The effect of Mitogen-Activated Protein Kinase Phosphatase-2 (MKP-2) over-expression via Infection with Adv.MKP-2 on Human Endothelial Cell Apoptosis and Vascular Smooth Muscle Cell Proliferation

A thesis presented by

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ABSTRACT

The mitogen-activated protein kinases (MAPKs) play key roles in the regulation of endothelial cell function and integrity through regulation of a number of cytosol and nuclear targets. These consist principally of the extracellular regulated kinases (ERKs) and isoforms of the p38 MAP kinases and the c-Jun N-terminal kinases (JNKs). A large body of evidence implicates JNK in endothelial cell apoptosis mediated by cytokines, hydrogen peroxide or high glucose concentration. Thus, novel approaches to limit JNK activation in endothelial cells would be useful. MAP kinase phosphatases are a family of dual-specific phosphatases (DUSPs) which negatively regulate the activity of the MAP kinases within different subcellular compartments. One such phosphatase, MKP-2, is a type one DUSP, nuclear located, induced in cells in response to a number of extracellular stimuli and selective for ERK and JNK *in vitro*. In this study an adenoviral version of MKP-2 was tested for its effects upon endothelial apoptosis and smooth muscle cell proliferation.

In human umbilical vein endothelial cells (HUVECs), apoptosis in response to TNF-α in combination with Adv.DNIKKβ was reversed by infection with an adenoviral MKP-2 (Adv.MKP-2) due to selective inhibition of JNK. Inhibition of apoptosis correlated with a reduction in the activation of nuclear pro-apoptotic proteins such as c-Jun, γ-H2AX and PARP-1. However surprisingly, the cleavage of caspase-3 and caspase-9, cytosolic enzymes and the release of mitochondrial cytochrome c was also inhibited by Adv.MKP-2 despite its strict nuclear location. In addition, Adv.MKP-2 over-expression was found to be far better in reversing cell death than pre-treatment with the JNK inhibitor, SP600125. These findings were also confirmed using other cellular stresses including Hydrogen peroxide (H_2O_2) or serum deprivation. In contrast, in human vascular smooth muscle cells, infection with MKP-2 adenovirus significantly inhibited cell proliferation which correlated with the abolition of growth factor-mediated JNK signalling. Taken together, these findings suggest a clear potential for MKP-2 to be used as a possible gene tool in some cardiovascular disorders, such as restenosis, which requires not only the inhibition of smooth muscle proliferation but endothelial cell survival.

PUBLICATIONS

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ABBREVIATIONS

Adv.	Adenovirus
Ang II	Angiotensin II
ANOVA	Analysis of variance
AP-1	Activating protein-1
APS	Ammonium persulphate
ASK	Apoptosis signal regulating kinase
ATP	Adenosine triphosphate
Bcl-2	B cell lymphoma
BH	Bcl-2 homology domain
BID	Bcl-2 interacting-Bid
BSA	Bovine serum albumin
CAD	Caspase activated DNase
CARD	Caspase associated receptor death domain
CAR	Coxackievirus-adenovirus receptor
Caspase	Cysteine dependent aspartate specific protease
COX	Cyclooxygenase
cDNA	Complementary DNA
c-FLIP	Cellular FLICE inhibitory protein
CHX	Cycloheximide
c-IAP-1	Cellular inhibitor of apoptosis
CRM-1	Chromosome region maintenence-1
DD	Death domain
DED	Death effector domain
DIABLO	Direct IAP-binding protein with low Pi
DISC	Death inducing signalling complex
DNIKKβ	Dominant negative inhibitory kappa B kinaseβ
DR	Death receptor
DTT	Dithiothreitol
DUSP	Dual-specificity phosphatase
EB	ERK Binding
ECL	Enhanced chemiluminescence

EDHF	Endothelium-derived hyperpolarising factor
EDRF	Endothelium-derived relaxing factor
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol-bis (β-aminoethyl ether) N,N,N,N' -tetra acetic acid
ERK	Extracellular-regulated kinase
ET-1	Endothelin-1
FADD	Fas-associated death domain
FBS	Foetal bovine serum
FCS	Foetal calf serum
FGF	Fibroblast growth factor
GPCR	G-protein coupled receptor
H_2O_2	Hydrogen peroxide
H2AX	Histone variant A2
HEK	Human embryonic kidney
HEPES	N-[12hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid]
HRP	Horseradish peroxidise
HSF	Heat shock factor
HSP	Heat shock protein
HUVECs	Human umbilical vein endothelial cells
IAP	Inhibitor of apoptosis protein
ICAD	Inhibitor of caspase activated DNase
ICAM-1	Intracellular adhesion molecule-1
IκB	Inhibitory kappa-B
IKK	Inhibitory kappa-B kinase
IL-1β	Interleukin-1 β
IP	Inositol phosphates
IP_3	Inositol 1,4,5,-triphosphate
ITR'S	Inverted terminal repeats
JIP	JNK interacting protein
JNK	c-Jun N-terminal kinase
kDa	kilo-Dalton
INF-γ	Interferon-gamma
LPS	lipopolysaccharide

MAPK	Mitogen-activated protein kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MEF	Mouse embryonic fibroblast
MEEK	MEK kinase
MEK	MAP kinase kinase
MEKK	MAP kinase kinase kinase
MKB	MAP kinase binding domain
MKP	Mitogen-activated protein kinase phosphatase
MMP	Matrix metalloproteinase
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
MSK	Mitogen and stress kinases
NES	Nuclear export signal
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor kappa-B
NIK	Nuclear factor kappa-B inducing kinase
NLK	Nemo-like kinase
NLS	Nuclear localisation sequence
NO	Nitric oxide
oxLDL	Oxidized low density lipoprotein
NOS	Nitric oxide synthase
PARP-1	Poly (ADP-ribose) polymerase enzyme
PBS	Phosphate buffered saline
PGE_2	Prostaglandin E-2
PGI_2	Prostacyclin
PI3K	Phosphatidyl inositol-3 kinase
PIKK	Phosphatidylinositol-3 kinase-related kinases
PKC	Protein kinase C
PLA_2	Phospholipase A-2
PMSF	Phenylmethylsulfonylfluoride
PS	Phosphatidyl-serine
ROS	Reactive oxygen species
RSK	Ribosomal S6 kinase

RTK	Receptor tyrosine kinase
PCR	Polymerase chain reaction
SAPK	Stress-activated protein kinase
SDS	Sodium dodecyl sulphate
siRNA	Small interfering RNA
SMAC	Second mitochondria derived activator of caspases
SODD	Silencer of death domain
STAT	Signal transducer and activator of transcription
TGFR	Transforming growth factor receptor
TEMED	N,N,N',N'-tetramethylenediamine
TF	Tissue factor
t-BID	Truncated –Bcl-2 interacting protein
THD	TNF homology domain
TNF-α	Tumour necrosis factor α
TNFR-1-2	TNF-receptor 1 & 2
TRAF2	TNF receptor associated factor 2
TRAILR1-2	TNF-related apoptosis inducing ligand receptor 1 &2
THR	Threonine
TYR	Tyrosine
TXA_2	Thromboxane A-2
UV	Ultraviolet
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VHR	VH-1-related phosphatase
XIAP	x-linked mammalian inhibitor of apoptosis protein

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GENERAL INTRODUCTION

1.1 Introduction

1.1.1 Physiological Functions of Endothelial Cells

The endothelium is a monolayer of endothelial cells (ECs) (approximately 1-6 x 10^{13} cells), which lines all blood vessels and the heart (Esper et al., 2006, Cines et al., 1998). The endothelium separates blood from the intima and media of arteries and veins, and from the interstitium of tissues throughout the body. Therefore, the strategic location of the endothelium between the wall of blood vessels and the blood stream assures that it is exposed constantly to a wide variety of extracellular stimuli that play a crucial role in regulating vascular homeostasis. These including the regulation of vascular tone and maintenance of blood flow as well as coagulation and regulation of permeability of blood vessels to leukocytes and inflammatory mediators (Behrendt and Ganz, 2002, Choy et al., 2001, Davignon and Ganz, 2004). By sensing mechanical stimuli, such as shear stress and pressure as well as hormonal and growth factor stimuli and other vasoactive mediators, endothelial cells synthesize and release a variety of substances into the blood stream or express constitutive or induced molecules on their surface. Among the substances released from endothelial cells which contribute to the regulation of blood pressure and flow are vasodilators such as nitric oxide (NO) (Furchgott et al., 1984), prostacyclin (PGI_2) (Wheeler-Jones, 2008, Vane, 1971), endothelium-derived relaxing factors (EDRFs) (Endemann and Schiffrin, 2004, Flammer and Luscher, 2010) and bradykinin (Drexler, 1998). These agents are well known to inhibit platelet aggregation, reduce thrombus formation, inhibit leukocyte adhesion, smooth muscle cell (SMC) proliferation and LDL oxidation (Constans and Conri, 2006). Such effects are partially controlled by inhibiting the release of contractile mediators including thromboxane A_2 (TxA_2), endothelin-1 (ET-1), angiotensin II (AngII) and reactive oxygen species (ROS) (Wheeler-Jones, 2008, Schiffrin, 2001). Other agents include; endothelium-derived hyperpolarizing factor (EDHF) (Feletou and Vanhoutte, 2009) and C-type natriuretic peptide, which also contributes to the regulation of vascular tone and maintenance of endothelial cell homeostasis (Schiffrin, 2001, Marasciulo et al., 2006). The endothelium also recruits circulating white blood cells and immunoglobulins into pathogen infected and inflamed tissue (Luscinskas and Gimbrone, 1996). In addition, endothelial cells contribute to angiogenesis and fluid balance (Endemann and Schiffrin, 2004). Unsurprisingly, when the

balance between vasodilatation and vasoconstriction is disturbed, endothelial dysfunction occurs, causing damage to the arterial wall, resulting in various cardiovascular diseases (See section 1.1.2).

1.1.2 Pathophysiology of Endothelial Dysfunction

In keeping with the importance of the endothelium in maintaining vascular homeostasis, dysfunction of the endothelium is implicated in the pathophysiology of several cardiovascular diseases. This can be mediated by inflammatory cytokines, oxidative stress, hypercholestremia, increased blood glucose, hypoxia, and alterations in blood flow (Drexler and Hornig, 1999, Alom-Ruiz et al., 2008, Widlansky et al., 2003). A number of key cellular events are initiated in response to endothelial cell dysfunction or activation. These include; a reduction in the release of vasodilators such as NO and PGI_2 (Endemann and Schiffrin, 2004) and an upregulation of adhesion molecules, such as ICAM-1, VCAM-1, E-selectin and P-selectin, which provide attachment for adhesion and migration of leukocytes through the intima (Haraldsen et al., 1996, Argenbright and Barton, 1992, Hess et al., 1996, Ley et al., 1995). Also, changes occur in the morphology of the plasma membrane and the cytoskeleton as well as expression of extra-cellular matrix, leading to endothelial permeability (Widlansky et al., 2003, Constans and Conri, 2006). Moreover, release of cytokines such as IL-1, TNF-α and INF-γ, chemokines, modified lipoproteins and growth factors (Blann et al., 2005), contribute to the inflammatory response following endothelial cell injury.

Endothelial dysfunction was first observed by Ludmer and colleagues as impaired vasodilatation to specific stimuli such as acetylcholine or bradykinin (Ludmer et al., 1986). This group observed a contradictory constriction in the arteries of patients with mild coronary artery disease (CAD) as well as in those with advanced CAD. This suggested that the damage of vascular endothelium was an important early and critical marker in the pathogenesis of atherosclerosis, contributing to plaque initiation and progression (Ribeiro et al., 2009, Rossig et al., 2001, Ross, 1999). Impairment of vasodilatation has been implicated in a number of other pathological diseases such as hypertension (Nitenberg, 2006), chronic heart failure (Miller et al., 2010, Reriani et al., 2010), diabetes (Vanhoutte et al., 2009, Urso et al., 2010, Nishio, 2010) and acute and chronic renal failure (Akcay et

al., 2009, Kohan, 2010), Furthermore, other disorders, such as dyslipidemia (Filip-Ciubotaru et al., 2009), obesity and hyperchromocyteinemia (Virdis et al., 2001, Raitakari et al., 2004), have all been associated with EC dysfunction.

Many clinical therapies have been used to restore the endothelial cell lining and improve function such as administration of acetyl salicylic acid (aspirin), which has been shown to reduce oxidative stress (Wu et al., 2002). Also, treatments with statins have been reported to improve endothelial cell function by increasing eNOS expression in human coronary artery endothelial cells (Dogra et al., 2002, Mehta et al., 2001). Recently, caveolin-1 has been shown to be involved in the regulation of eNOS turnover and consequently in NO release as result of shear stress (Frank et al., 2003), and may represent a novel future therapeutic target. Therefore, it is important to understand the mechanisms which regulate endothelial cell dysfunction and in particular apoptosis. Other agents including those which may prevent EC apoptosis are discussed extensively in section 1.3.

1.2 Signalling Pathways Involved in Endothelial Cell Function

The particular significance of the endothelium in mediating the effects of circulating factors is defined by the location of endothelial cells. This anatomic positioning assigns the cell a crucial role in the transduction of blood born extracellular signals to the inner vessel wall. Therefore regulation of intracellular signalling pathways represents a potential therapeutic avenue in a number of cardiovascular diseases outlined above. These signalling pathways include; mitogen-atctivated protein (MAP) kinases, nuclear factor-κB (NF-κB), phosphatidylinositol (PI)-3 kinase/Akt, sphingomyelinase and JAK/STAT. Of these, the MAP kinases represent a well recognised pathway involved in a wide range of endothelial cell functions including growth, migration, proliferation, differentiation, survival and apoptosis. Therefore, selectively limiting the activity of these kinases in EC may be a desirable strategy and as such is a focus a large number of studies in this area.

1.2.1 Mitogen Activated Protein Kinases (MAPKs) Family

In mammalian systems, three major groups of MAPKs have been well studied and are classified on the basis of sequence similarity, differential activation by agonists and substrate specificity. These are: the extracellular signal-regulated kinases including ERKs-1-4 and big MAP kinase (ERK5), c-Jun N-Terminal kinases (JNK-1/2/3) and isoforms of p38 MAP kinase (p38-α/β/γ/δ) (Gupta et al., 1996, Gupta et al., 1995). All these kinases contain the signature sequence-**TXY**-, where T and Y are threonine and tyrosine, and X is glutamate, proline or glycine, respectively, in ERK, JNK or p38 MAP kinase (Ahn et al., 1991, Payne et al., 1991, Robbins et al., 1993). Phosphorylation of both the threonine (Thr) and tyrosine (Tyr) within this signature sequence is essential for full MAP kinase activity. This activity may be achieved by triggering membrane receptors such as G-protein-coupled receptors (GPCRs), transforming growth factor β receptors (TGFR), receptor tyrosine kinases (RTKs) through the recruitments of specific adaptor/scaffold proteins or by cross-talk with other signalling pathways (Cullen and Lockyer, 2002). MAP kinase activity is initiated by phophorylation within their activation loop by a family of dual-specific MAP kinase kinases (MKKs or MEKs) which are in turn phosphorylated by MAPK kinase kinases (MKKK or MEKK), (Dhanasekaran and Johnson, 2007, Qi and Elion, 2005) (**Figure 1.1**).

Activation of the MKKKs is both stimuli and cell type-dependent but can also be via phosphorylation by other upstream kinases or interaction with small GTP binding proteins from the Rho or Ras family (Malumbres and Barbacid, 2003). The MKKs are the smallest group within the cascade and have specificity for their own MAPK substrate, however, the MKKKs are more varied in both their structure and substrate selectivity towards the MKKs. Activated MAPKs themselves are able to phosphorylate various substrate proteins including nuclear transcription factors such as ELK-1, c-Jun ATF2, and CREB (Derijard et al., 1994), and cytosolic proteins such as MAPKAP kinase-2/3 (Stokoe et al., 1992, McLaughlin et al., 1996) or PLA_2 (Kramer et al., 1995). This can be mediated through the formation of a signalling complex containing multiple kinases and scaffold proteins such as, kinase suppressor of Ras-1 (KAR) and MEK partner1 (MP1), JNK-interacting proteins (JIPs), β-Arrestin 2 (Morrison and Davis, 2003, Whitmarsh, 2006) and others.

Downstream transcription factors contribute to overlapping extents in the formation of homo/heterodimer complexes which bind to DNA (i.e c-Jun interacts with c-fos in the formation of the AP-1 complex), and regulate gene expression. This overlap is also reflected in kinase regulation of the transcription factors themselves. For example, early studies identified c-Jun as a substrate for both JNKs and ERKs (Derijard et al., 1994). Phosphorylation occurred at position Ser^{63} and Ser^{73}, following binding within JNKs within the N-terminus, whilst ERKs mediated phosphorylation within a C-terminal inhibitory site (Derijard et al., 1994). In the case of c-Jun and ATF2, JNKs binding within the N-terminus has been shown to be essential for phosphorylation (Gupta et al., 1996, Gupta et al., 1995, Livingstone et al., 1995), however, for Elk-1 initial binding by JNK within the N-terminus was not an essential (Gupta et al., 1996). Moreover, studies have demonstrated that the binding of JNK to Jun B (Gupta et al., 1996) and c-R (Hirano et al., 1996), was not followed by phosphorylation and these proteins therefore, functioned as molecular chaperones helping JNK to interact with and/or activate other nuclear targets (Paul et al., 1997).

Crosstalk between upstream components within each MAPK pathway further complicates the signalling process. A recent study showed that p38α MAP kinase antagonized the JNK-c-Jun pathway via inhibition of MEK7 (Hui et al., 2007). Also, JNK and p38 MAP

kinase pathways have been demonstrated to inhibit ERK1/2 activation and function in some cell lines (Zhang et al., 2001, Shen et al., 2003). More specifically, c-Jun transcriptional activity was found to be necessary for ERK inhibition, whilst JNK strongly inhibited ERK1/2 activation in primary cells (Shen et al., 2003, Jeffrey et al., 2006). This crosstalk between different MAPKs (Cuevas et al., 2007, Han and Sun, 2007, Schwacke and Voit, 2007) has been found to occur at both, mRNA production (Ambrosino et al., 2003) and protein phosphorylation (Dhillon et al., 2007). For example, the stability of all p38 MAP kinases was reduced by the p38α isoform (Ambrosino et al., 2003), while, JNK1 mediated activation of c-Jun was inhibited by JNK2 (Hochedlinger et al., 2002).

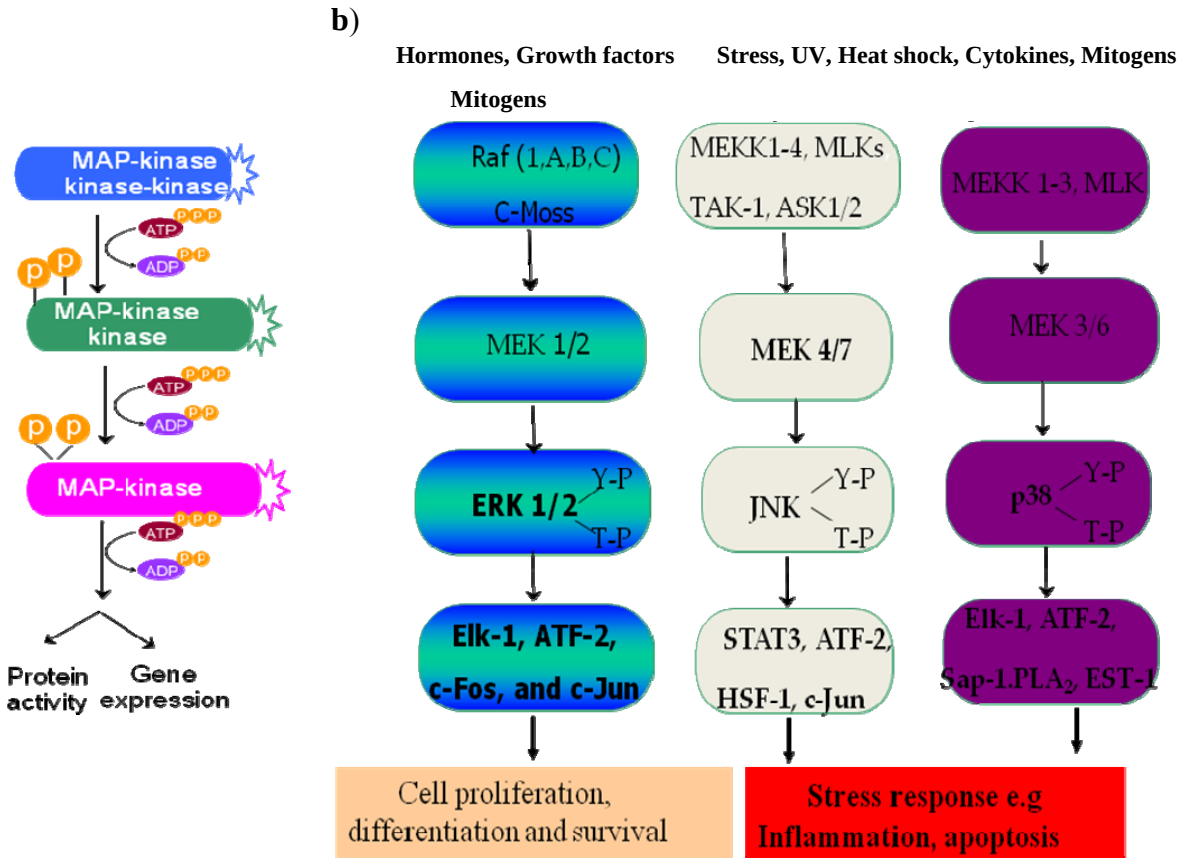


Figure 1.1: **The MAPK signalling transduction pathways. Schematic representation of the different MAPK cascades.** The mitogen activated protein kinase pathway is highly conserved and plays an important role in a variety of cellular processes from proliferation to apoptosis. **a**) Schematic diagram illustrates the components involved in the MAPK signalling paradigm. **b**) The upstream and downstream effectors of MAPK cascades.

1.2.1.1 Extracellular signal-regulated kinases (ERK's) Family.

ERK1 and ERK2 were the first mammalian MAP kinase isoforms discovered and to date, at least six isoforms have been identified (ERK1-6). The main ERK1/2 isoforms share 83% amino acid identity so they are often regarded as functionally redundant (Boutros et al., 2008). Nevertheless, some studies have reported that differences exist in relation to their respective substrate specificities (Seger and Krebs, 1995). The activation of ERK occurs upon dual phosphorylation of threonine^{183} and tyrosine^{185} residues within an **TEY** motif (where E is glutamate) on the subdomain VIII on its catalytic domain (Turjanski et al., 2007). Upon growth factor receptor activation, the small G-protein Ras becomes activated and contributes towards the phosphorylation of Raf (MEKK2). MEKK2 then phosphorylates MEK1/2, which then directly phosphorylates the dual site within ERK1/2. Once activated, ERK can then target cytosolic substrates (CD120a, calnexin), which has been shown to be significant in cytoskeletal rearrangement and alteration of cell morphology (Pullikuth and Catling, 2007). Under apropriate stimuli, ERK can also undergo translocation to the nucleus where it can target a number of nuclear substrates such as c-Jun, c-Fos, Elk-1, NF-AT, SRC-1, and STAT3 (Roux et al., 2004, Chen et al., 1996). ERK has been shown to translocate to the nucleus in various cell types (Chen et al., 1992, Gonzalez et al., 1993, Lenormand et al., 1993). Interestingly, however, the activators of ERK, MEK1 and 2, are restricted to the cytosol (Lenormand et al., 1993) due to the presence of a nuclear export sequence (NES) within the N-terminal tail (Fukuda et al., 1996). For nuclear translocation to occur, ERK must be released from MEK1/2, and this is concomitant with tyrosine phosphorylation (Adachi et al., 1999, Rubinfeld et al., 1999).

Mice lacking ERK1 or 2 have been used to distinguish between the functions of these isoforms. For example, some authors showed that ERK1-null mice have no overt phenotype, develop normally, and are fertile (Hommes et al., 2003), whilst others used behavioural tests to examine these mice and found that they had increased locomotor activity compared to their wild type littermates (Gerits et al., 2007, Aouadi et al., 2006). ERK2-null mice die *in utero* due to lack of mesoderm differentiation and lack of placental development (Gerits et al., 2007). This data suggests that whilst overall, ERK1/2 are equally expressed in many cells, they have different functions.

1.2.1.2 p38 MAP kinase Family.

Unlike ERK, p38 MAP kinase isoforms only share 60% amino acid identity with one another, which may explain their diverse functional capabilities and distribution. To date, four p38 MAP kinase splice variants have been identified, p38α and p38β are ubiquitously expressed whereas the presence of p38γ and p38δ is cell type dependant (Cuenda and Rousseau, 2007, Mayor et al., 2007). The p38γ isoform is expressed in skeletal muscle, whereas p38δ is expressed in the small intestine, pancreas, testis, and kidney (Hommes et al., 2003). Also, analysis of inflammatory cell lines indicates differences in p38 MAP kinase isoform expression in macrophages, T-cells and endothelial cells (Hale et al., 1999, Hu et al., 1999a). All isoforms of p38 MAP kinase are activated in response to stress factors such as UV light, osmotic shock, LPS, cytokines, as well as growth factors (Roberts and Der, 2007). These agents mediate their effect by activating MEKK4/ASK1 which directly activates MEK3 and MEK6 (Han and Sun, 2007), both of which are specific for p38 MAP kinase, as they do not activate either ERK or JNK. MEK6 but not MEK3 is able to activate the p38β isoform (Li et al., 1996), whilst both MKK3 and MKK6 can phosphorylate and activate the other three isoforms (Han and Sun, 2007). In some instances p38 MAP kinase activation has been shown to be MKK-independent, mediated through isoforms of a TGFβ-activating kinase-1 binding protein (Chen et al., 2005). JNK-interacting proteins (JIPs) can also bind to p38 MAP kinase and affect their subcellular localisation (Raman et al., 2007).

Activation of p38 MAP kinase requires the same dual phosphorylation (Thr^{183}, Tyr^{185}) but the motif within the activation loop of p38 MAP kinase contains glycine to give **T-G-Y** (Cuenda and Rousseau, 2007). Once phosphorylated, p38 MAP kinase can act upon cytosolic or nuclear substrates, including, cytosolic phospholipase A_2, heat shock proteins (HSP25/27), MAP kinase activated protein (MAPKAP-K2) (Rousseau et al., 2000, Rousseau et al., 2006), CREB, ATF1/2/6, p53, Elk-1, c-Fos and NF-κB (Kyriakis and Avruch, 2001, Raingeaud et al., 1996, Nick et al., 1999). Several studies show nuclear translocation of p38 MAP kinase in response to ischemia stress adaptation (Maulik et al., 1998), high glucose (Cheng and Feldman, 1998) and following hyperosmotic challenge (Raingeaud et al., 1996). Isoform specific knockout mice and pharmacological inhibitors

have been utilised to study the role of p38 MAP kinase signalling in a number of physiological and pathophysiological processes. These studies implicate p38 MAP kinase in rheumatoid arthritis, pulmonary diseases, CNS diseases and cancer.

1.2.1.3 c-Jun N-Terminal Kinases /Stress-Activated Protein Kinases (JNKs/SAPKs)

The c-Jun N-terminal kinases (JNKs) were first identified in the early 1990s, as a kinase activated *in vivo* in respone to cycloheximide treatment (Gupta et al., 1996). To date, three mammalian genes have been found to encode JNK, JNK1, 2, and 3, these are further divided into numerous splice variants. For JNK1 and 2 both α1 and α2 and β1 and β2 variants exist for both 46 and 54 isoforms, whereas JNK3 has only the α1 and α2 variants (p46) and (p54) (Waetzig and Herdegen, 2005, Johnson and Nakamura, 2007, Davis, 2000). There is very little data regarding the function of all 10 splice forms in one biological system and also functional information is generally lacking (Gupta et al., 1996, Yang et al., 1998b, Tsuiki et al., 2003). JNK1 and JNK2 are ubiquitously expressed whereas JNK3 is only observed in the brain, heart, and testis.

As JNK1,2 and 3 have been shown to differ in their ability to bind and phosphorylate diverse substrate proteins (Kallunki et al., 1994, Gupta et al., 1996, Bogoyevitch and Kobe, 2006) and display tissue specific distribution, particularly for JNK3, this has led to the idea that each isoform performs a different cellular role (Bogoyevitch and Kobe, 2006). JNKs are activated by many diverse stimuli including both chemical and physical stress as well as growth factors, UV irradiation, proinflammatory cytokines, serum deprivation, ischemia/reperfusion, hydrogen peroxide and cycloheximide (protein synthesis inhibitor) (Roberts and Der, 2007, Kyriakis and Avruch, 2001, Minden et al., 1994, Derijard et al., 1994, Bogoyevitch et al., 1996, Wadgaonkar et al., 2004, Robinson et al., 2001).

As with ERK and p38 MAP kinase, JNK requires the same dual phosphorylation (Thr^{183}, Tyr^{185}) within the activation loop to give **T-P-Y** (Bode and Dong, 2007, Kyriakis and Avruch, 2001). The immediate upstream activating kinases have been characterised, truncated and include MEK4, also known as JNKK1 and MEK7, also known as JNKK2 (Bode and Dong, 2007, Yamauchi et al., 1999). Several studies have shown that MEK4

specifically phosphorylates the (Tyr) residue, whereas MEK7 specifically phosphorylates the (Thr) residue (Meier et al., 1996). Moreover, MEK4 phosphorylates both JNK and p38 MAP kinase *in vitro* while MEK7 is specifically involved in the activation of JNK (Meier et al., 1996).

Similar to other MAP kinases, the JNK axis has a wide range of MAPKK kinase proteins that can cause JNK activation. These include MEKK-1,2,3, and 4, MLK 1,2,3, and 4, MUSK-1, Tp1-2, TAK-1, SPARK, MST and ASK-1-2 (Kyriakis and Avruch, 2001, Davis, 2000, Nishina et al., 2004, Dhanasekaran and Reddy, 2008, Raman et al., 2007). Experiments in knockout embryonic stem cells have shown that MEKK1 is essential in JNK activation by serum and proinflammatory stimuli (Yujiri et al., 1999, Xia et al., 2000) but not by UV irradiation, oxidative stress and anisomycin (Minamino et al., 1999), this suggests a possible role for MEKK1 in receptor activated signalling to JNK rather than in stress signals arising within the cytosol or nucleus. Other MAPKK kinases include the MLKs involved in transmitting signals from Rac/CDC42 to MKK4/7-JNK (Xu et al., 2001). Another kinase ASK1, links different apoptotic stimuli such as TNF-α, ROS, LPS and endoplasmic reticulum stress to JNK (Matsuzawa and Ichijo, 2001, Nagai et al., 2007). During TNF-α-mediated apoptotic signalling, it appears that ROS-dependent activation of ASK1 by TNF receptor-associated factor 2 (TRAF2) leads to stimulation of JNK and apoptosis (Matsuzawa and Ichijo, 2001, Nagai et al., 2007). Oxidative stress has also been shown to recruit both TRAF2 and TRAF6 to activate ASK1 and downstream JNK signalling (Fujino et al., 2007). Studies in mice lacking ASK1 has confirmed the requirement for this kinase in the sustained activation of JNK and apoptosis induced by TNF-α in embryonic fibroblasts (Tobiume et al., 2001).

In addition to phosphorylation of c-Jun at Ser^{36} and Ser^{37}, JNK is now recognised to phosphorylate several other transcription factors such as ATF2, Elk-1, p53 and c-Myc, Paxillin, MAP2 and γ-H2AX (Wada and Penninger, 2004, Chang and Karin, 2001, Weston and Davis, 2002, Manning and Davis, 2003, Nishina et al., 2003, Lu et al., 2006). Nuclear translocation of JNK is observed in respone to high glucose concentration (Cheng and Feldman, 1998), and in response to H_2O_2 (Robinson et al., 2001). It has also recently been discovered that JNK translocates to the mitochondria where it phosphorylates anti-

apoptotic proteins such as Bcl-2 and Bcl-xL (Wada and Penninger, 2004, Kharbanda et al., 2000, Nishina et al., 2003, Dhanasekaran and Reddy, 2008). Also, the arrestin proteins have recently been shown to interact with JNK to allow the re-localisation of JNKs from the nucleus to the cytosol (Song et al., 2006). In addition, other studies demonstrate that JNK interacts with JIPs to sequester JNK and regulate function (Raman et al., 2007). Whilst the exact physiological function of JNK within the cell is yet to be fully understood, progress has been made using isoform specific knockout mice and the JNK inhibitor, SP600125. These approaches have revealed roles in apoptosis, cell proliferation, tumour development (Liu and Lin, 2005, Lin and Dibling, 2002, Davis, 2000), cell migration (Ventura et al., 2006), neuronal death and diabetes (Huang et al., 2003, Bogoyevitch and Kobe, 2006). The involvement of JNK activation in endothelial cell apoptosis will be discussed in more detail in section 1.3.6.2.

1.3 Endothelial Cell Apoptosis

1.3.1 Apoptosis: historical overview and definition

Apoptotic cell death is an essential component of embryonic development and adult tissues by controlling tissues homeostasis, morphogenesis, remodelling of mature tissues and the removal of pathogens (Abud, 2004, Raff et al., 1993, Polunovsky et al., 1994). Inadequate or excessive apoptosis may lead to many pathological processes, including developmental defects, autoimmune diseases, neurodegenerative diseases or cancer. Lately, apoptosis has also generated interest in the pathophysiology of atherosclerosis as a feature of endothelial cell dysfunction (Kockx, 1998, Mallat and Tedgui, 2001, Bennett, 2002). Thus, understanding the exact signalling mechanism for apoptotic cell death regulation may allow therapeutic intervention strategies. The aim of this section is to focus on the mechanisms by which MAP kinases regulate HUVECs death in response to different stress stimuli. Underpining this is an understanding of the processes by which cellular apoptosis is regulated.

The first physiological cell death mechanism was identified in 1842 by Carl Vogt. This study suggested that cells die during the development of the nervous system of the toad and this cell death was important for embryogenesis and metamorphosis, in both insects

and mammals (Vogt,1842 taken from review by (Vaux and Korsmeyer, 1999). Later in 1965, programmed cell death was used to describe the controlled and complicated mechanism of cell-destruction happening during insect development (Lockshin and Williams, 1965). The term "apoptosis" was then coined in 1972 by Kerr and his colleagues (Kerr et al., 1972), who noticed that the morphological aspects of lymphocyte and liver cells dying in response to toxin or hormones, were similar to the developmental cell death described earlier by Glucksmann (Glucksmann, 1965). Apoptosis, derived from the Greek word, meaning ''falling off'', as of leaves from trees, was used to describe the novel cell death phenotype, morphologically different from accidental death known as ''necrosis''. The first study that defined apoptosis as a genetically regulated process came from studying development in *c-elegans* which showed that the ability of the human Bcl-2 gene to prevent programmed cell death in both mammalian cells and in nematode were similar (Vaux and Korsmeyer, 1999). This suggested that programmed cell death was a highly conserved process and was essential for the development and life of multicellular organisms.

A very large number of studies have now conclusively demonstrated the importance of apoptosis in diverse biological processes, including the formation of individualized digits in animals (Montero and Hurle, 2009), ablation of tadpole tails during amphibian metamorphosis, in development of the brain, deletion of the uterus and Wolffian duct during the formation of the male and female reproductive organs (Meier et al., 2000, Vaux and Korsmeyer, 1999). In the immune system, apoptosis has an essential role in the development, maintenance and regulation of the immune repertoire (Opferman and Korsmeyer, 2003). For example, during their development, lymphocytes undergo negative selection that results in the removal of non-functional and auto-reactive lymphocytes. In response to infection, the immune system rapidly activates and increases the number of antigen-specific lymphocytes through increased cell division and inhibition of apoptosis. Old, non-functional cells or those with severely damaged DNA that cannot be repaired, will also activate death signals to avoid formation of mutations (Vaux and Korsmeyer, 1999, Fadeel et al., 1999). Although apoptosis has long been recognized as a principal mechanism for the removal of redundant, autoreactive, or neoplastic cells, only recently

has a critical role of apoptosis been recognised in several cardiovascular diseases such as ischemic heart disease, heart failure and atherosclerosis (Pexieder, 1975).

1.3.2 Morphological classification of Apoptosis

In mammals, there are three different cell death morphologies that have been identified: apoptosis, necrosis and autophagy. As outlined above, apoptosis is a well-controlled process whereby cells activate pathways leading to their self-destruction in response to diverse stimuli, such as virus infection, serum or trophic factor withdrawal, DNA damage and signals from immune cells. Cells undergoing apoptosis are characterized by stereotypical morphological changes (Kroemer et al., 2005) including cytoplasm shrinkage and chromatin condensation, collapse of the mitochondrial membrane potential and loss of membrane phospholipid asymmetry. This is followed by plasma membrane blebbing, nuclear fragmentation and subsequent encapsulation of these fragments by apoptotic bodies, which contain cytosolic, nuclear and organelle material. These subcellular organelles remain integrated throughout the apoptotic process. Apoptotic bodies *in vivo* are recognized and engulfed by phagocyte cells such as macrophages and are therefore removed from the tissue to avoid an inflammatory reaction (Choy et al., 2001).

Phenotypic characteristics are the consequence of numerous cellular biochemical events, in particular the activation of endonuclease enzymes, which are responsible for internucleosomal cleavage of the DNA (Zamzami et al., 1997, Walker et al., 1994, Zheleznaya et al., 2009, Marcaida et al., 2009). In addition is the activation of a family of cysteine-dependent aspartate-specific proteases called caspases that mediate the degradation of specific cellular proteins. Caspase activation is considered to be one of the hallmarks of apoptosis and is therefore frequently used as a marker in cellular studies (Chowdhury et al., 2008, Demon et al., 2009, Logue and Martin, 2008, Pop and Salvesen, 2009).

Another biochemical hallmark of apoptosis is the externalization of phosphatidyl-serine (PS) plasma membrane phospholipids to the outer leaflet of the plasma membrane where it forms a recognition signal for phagocytes (Somersan and Bhardwaj, 2001, Venegas and Zhou, 2007, Schlegel and Williamson, 2007, Wu et al., 2006), Moreover, mitochondrial

membrane potential collapse and cytochrome c release is considered to be one of the central events in apoptosis (Green and Kroemer, 2004, Tait, 2008, Norberg et al., Cusimano et al., 2009, Robb et al., 2009, Brenner and Mak, 2009). Apoptosis is an energy-dependant process requiring ATP for optimal caspase activation.

Necrosis is characterized morphologically by an overall cytoplasmic swelling (oncosis) and decreased subcellular organelle and plasma membrane integrity. The release of cytosolic substances into the inter-cellular space causes inflammation in the affected tissue *in vivo*. Previously, necrosis had been considered to be a non-programmed, passive form of cell death initiated by cellular "accidents" such as toxic insult or physical injury. However, recently, evidence has accumulated pointing to a physiological role for necrosis suggesting that its activation may also be closely regulated (Golstein and Kroemer, 2007). For example, necrosis has been described during bone development and it also contributes in intestinal epithelial cell homeostasis (Barkla and Gibson, 1999, Roach and Clarke, 2000). Further studies have linked apoptosis to necrotic death (Chautan et al., 1999), suggesting that necrosis may in some circumstances, represent a default, programmed-cell death mechanism which is an alternative for failed apoptosis (Golstein and Kroemer, 2007, Edinger and Thompson, 2004).

Autophagy which means 'self-eating' (Levine and Kroemer, 2009) is morphologically characterised by the double membrane vesicles or autophagosomes that encapsulate cytosolic contents and organelles such as mitochondria (Levine and Klionsky, 2004). Combination of the autophagosome with the lysosome to form a new vesicle, results in the degradation of its contents by proteolysis. Electron microscopy shows these vesicles to be distinct from endosomes, lysosomes or apoptotic blebs (Levine and Klionsky, 2004). Autophagy was first identified as a survival strategy employed by yeast to survive nutrient deprivation, through providing amino acids and other essential substances to the cell (Reggiori and Klionsky, 2002). It is a highly conserved mechanism which plays a role in the turnover and elimination of long-lived proteins and organelles (Klionsky and Emr, 2000). Recent studies show that autophagy is essential in the early development of Drosophila, Caenorhabditis elegans and mice (Juhasz et al., 2003, Yue et al., 2003), but its particular role in this process remains to be elucidated. Knockout of autophagic genes in

mice and c. elegans increases cell death; supporting the idea that autophagy has a survival role during early development. However, autophagic vesicles are also observed in dying cells where the apoptotic pathway is repressed (Yu et al., 2004b, Shimizu et al., 2004). As outlined above for necrosis, autophagy may be an alternative pathway of cell death which could be unravelled when apoptosis is inhibited. It is not clear whether autophagy represents an appropriate mechanism of cell suicide, independent of caspases, or functions only as a survival strategy. Evidence suggests that even though these three types of cell death are morphologically very different, accumulated evidence indicates that they are strongly interrelated, dependent on the nature and strength of a particular stimulus and on cell type.

1.3.3 Apoptosis of Endothelial Cells (ECs)

An additional feature of endothelial cell dysfunction in response to prolonged injury is apoptosis and disruption of the endothelial lining (Rossig et al., 2001). Endothelial cell apoptosis can be triggered by cytokines (Yue et al., 1999, Karsan et al., 1996, Dimmeler et al., 1997b, Robaye et al., 1991, Van Antwerp et al., 1996), high glucose concentrations (Dimmeler et al., 1997a, Baumgartner-Parzer et al., 1995, Ho et al., 2000, Liu et al., 2007), oxidized low density lipoprotein (oxLDL) (Kamiyama et al., 2009, Chen et al., 2007), reactive oxygen species (Dimmeler et al., 1999, Sage et al., 2008), hydrogen peroxide (Hermann et al., 1997, Wang et al., 1999, Liu et al., 2007), serum deprivation (Harfouche and Hussain, 2006, Williams et al., 2006), angiotensin II (Li et al., 1999, Dimmeler et al., 1997c), adrenaline (Romeo et al., 2000), hypoxia/reoxgenation (Wang et al., 2005) and bacterial endotoxin lipopolysaccharide (LPS) (Bannerman and Goldblum, 2003, Munshi et al., 2002, Bernardini et al., 2009). Apoptosis results in increased vascular permeability through loss of EC number and gap formation (Choy et al., 2001). The loss of EC integrity also facilitates the migration and deposition of lipids, monocytes, smooth muscle proliferation into the intima, enhanced blood coagulation and plaque erosion which occurs since apoptotic cells are pro-coagulant (Dimmeler et al., 2002, Bombeli et al., 1997, Crisby et al., 1997) (**Figure 1.2)**. These factors contribute to acute coronary thrombosis (Dimmeler et al., 2002). In normal conditions, ECs are highly resistant to apoptosis (Bach et al., 1997, Polunovsky et al., 1994) and this may suggest that prolonged damage is necessary for death to occur. Increased apoptosis in endothelial cells is thus well

recognised as an important early marker of atherosclerosis and is associated with an increase in cardiovascular risk (Rossig et al., 2001, Kockx, 1998, Tricot et al., 2000).

Initially, the physiological consequence of EC apoptosis is increased SMC proliferation and monocyte accumulation (Ross, 1999, Crisby et al., 1997). The presence of the endothelium is important in the regulation of SMC growth, migration, proliferation and apoptosis. Denudation of the endothelial monolayer in aortic segments increases SMC migration into the lesion, and SMC proliferation occurs within the lesion, accompanied by increased intimal mass. Following re-endothelisation, the lesion can decrease intimal mass, suggesting that the presence of ECs is required to properly regulate SMC number in the vascular wall (Choy et al., 2001). This may occur through EC's initiation of SMC death, since increased re-endothelisation is correlated with a decrease in SMC number. This may suggest that an increase in the frequency of both cell death and cell proliferation is important in plaque formation. Other evidence for the involvement of endothelial cell death in atherosclerosis is provided by the observation that endothelial cells in lesion-prone regions, where atherosclerosis preferentially develops, are characterized by increased cell turn-over rates. Since cell turnover depends on cell death and regeneration, increased cell death leads to increased turnover (Caplan and Schwartz, 1973, Choy et al., 2001). This also confirms that endothelial cell apoptosis is one of the main players in the development of atherosclerotic plaques (Dimmeler et al., 1998).

The link between endothelial cell apoptosis and the development of atherosclerotic plaques is further supported by studies assessing protein expression. Human umbilical vein endothelial cells (HUVECs) incubated with serum from congestive heart failure patients has been shown to have reduced eNOS production and higher levels of apoptosis (Rossig et al., 2000, Agnoletti et al., 1999). Apoptosis was also associated with impaired eNOS dependent vasodilatation in response to acetylcholine which was age dependent (Asai et al., 2000). Increased expression of pro-apoptotic factors such as Bax and Fas have also been observed in endothelial cells in atherosclerotic lesions in allograft vasculopathy (Dong et al., 1999, Dong et al., 1996). *In vitro* evidence has shown that the expression of A20, an anti-apoptotic protein, decreases with increasing passage number and this sensitizes EC to growth factor withdrawal induced apoptosis (Varani et al., 1995). Levels

of CD44, a receptor for LDL which is linked to human ECs apoptosis is increased (Chi et al., 2003), whereas disruption of CD44 in mice results in a decrease in atherosclerosis (Cuff et al., 2001) and increased endothelial lipase is associated with reduced high density lipoprotein (HDL).

These findings and those outlined above strongly support the concept that endothelial dysfunction plays a key role in plaque formation and disease progression in patients. Therefore, a number of clinical approaches have been used to restore endothelial cell integrity by altering the expression and function of cellular proteins. For example, administration of L-arginine and tetrahydrobipetrin mimetics increase the synthesis of NO and protect ECs from apoptosis (Hyndman et al., 2002). In addition, adenoviral expression of VEGF and other related endothelial cell growth factors or specific endothelial enzymes such as eNOS (Tsurumi et al., 1997, Lake-Bruse et al., 1999) and superoxide dismutase (Chu *et al.*, 2003) have also been tested with positive effects upon endothelial integrity *in vitro* and *in vivo*. In injured carotid artery, treatment with adenoviral eNOS increases endothelial regeneration and provides a potential therapeutic strategy for the prevention of restenosis after vascular injury (Cooney et al., 2007). Moreover, treatment with erythropoietin in patients with chronic renal failure has been shown to increase endothelial progenitor cell mobilisation by upregulating eNOS synthesis. MAP kinases themselves have also been identified as a targeted approach to prevent endothelial cell apoptosis. Therefore, the role of MAP kinase in endothelial cell apoptosis will be discussed in detail in section 1.3.6.

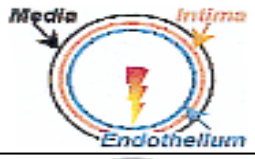
Stage	Histopathological event	Scheme	Apoptosis
1.	*Injury* of the endothelium		Endothelial activation by neurohormonal stimuli, cytokines, and ROS induced endothelial cell apoptosis and causes endothelial dysfunction
2.	*Proliferation* Formation of the neointima		Endothelial susceptibility of proliferating smooth muscle cells to undergo apoptosis
3.	*Plaque Development* Inflammatory cells Lipid enrichment		Proinflammatory cell-cell signalling by apoptotic VSMC via MCP-1 leads to macrophage migration
4.	Plaque *Destabilization* Foam cells		Apoptosis of VSMC contributes to thinning of the fibrous cap, destruction of the extracellular matrix, and increased plaque vulnerability
5.	Plaque *Rupture/Erosion*		Endothelial cell apoptosis leads to plaque erosion, endothelial and smooth muscle cell apoptosis might be involved in triggering plaque rupture
6.	*Lumen Thrombosis*		Procoagulant activity of apoptotic vascular cells and shed membrane particles enhances thrombus formation

Figure 1.2: Histopathological events and apoptosis in the vascular wall during atherosclerotic lesion development and plaque rupture. MCP-1 monocyte chemoattractant protein-1; ROS reactive oxygen species; VSMC vascular smooth muscle cell. Adapted from (Rossig et al., 2001).

1.3.4 Cellular Events Mediating Apoptosis

Apoptosis can be mediated by a wide variety of physiological, pathogenic or cytotoxic stimuli (section 1.3.5). In mammalian cells, induction of apoptosis occurs through two distinct pathways; extrinsic and intrinsic **(Figure 1.3)**. The extrinsic pathway integrates extracellular signals through the binding of external ligands to death receptors located at the plasma membrane (e.g. Tumour Necrosis Factor-1 (TNFR-1), Fas and TRAILs). The intrinsic pathway is activated under conditions of intracellular stress (such as DNA damage, deprivation of survival signals and oxidative stress) and involves the use of organelles to propagate death signals. Over a number of years, mitochondria have been identified as central executors of intrinsic apoptosis following a wide variety of death signals. Both extrinsic and intrinsic pathways lead to the activation of the cysteine-dependant aspartate-specific proteases, called caspases, which are responsible for the morphological features of apoptosis, see section 1.3.6.3

1.3.4.1 Death Receptor extrinsic pathway

The death receptors (DRs) belong to the TNF receptor superfamily and are involved in the transduction of either survival or apoptotic signals. DRs are transmembrane proteins composed of cysteine-rich extracellular domains and a characteristic cytoplasmic region composed of around 80 residues called the death domain (DD) essential for transmitting the death signal. To date, in humans there have been 8 members of the gene family identified: TNF receptor 1, TNF receptor 2, CD40, Fas receptor (also known as DR2, CD95 and APO-1) and nerve growth factor receptor (NGFR), TNF-Related Apoptosis-Inducing Ligand receptors 1 and 2 (TRAILR1, TRAILR2), DR6 and ectodysplasin A receptor (EDAR) (Peter et al., 1999, Madge and Pober, 2001, Gaeta et al., 2000, Slowik et al., 1993). Two types of DRs can be identified depending on their distinct signalling complexes. The Fas receptor and TRAILR1/2 activation leads to the formation of the Death Inducing Signalling Complex (DISC) and results in the induction of apoptosis (Peter and Krammer, 2003, Peter and Krammer, 1998). However, TNFR-1, DR3, DR6 and EDAR recruit a different set of molecules and transduce, in addition to apoptosis, other cellular responses including proliferation, differentiation and survival (Lavrik et al., 2005). The binding of TNF-α to the TNFR-1 can initiate the formation of the DISC but also the assembly of two other signalling platforms that result in the activation of two transcription

factors, nuclear factor κB (NF-κB) and c-Jun. These transcription factors are essential for the expression of genes involved in diverse biological processes such as cell growth, death, development, oncogenesis, inflammation and immune responses. TNF-α, FasL and TRAIL contain a conserved TNF homology domain (THD) which is important for binding the DR. These receptors are required to oligomerise prior to ligand binding in order to function (Chan et al., 2000); Their cytoplasmic domains form platforms for the binding of one of two classes of signalling molecules; the TRAFs or the ‘ death domain’ (DD) containing proteins (Locksley et al., 2001).

In unstimulated receptors the cytoplasmic domain is associated with a cytoplasmic DD–containing protein known as silencer of death domain (SODD). The DD of SODD is thought to bind to the DD of TNFR-1 thereby preventing signalling, however, following ligand binding, DRs promote apoptosis by the formation of the multiprotein death-inducing signalling complex (DISC), which contains Fas-associated DD-containing protein (FADD) and procaspase-8 (**Figure 1.3)**. The receptors recruit FADD via its DD, while Fas and TRAIL1/2 associate directly with FADD, TNFR-1 requires TNFR associated death domain (TRADD) as an intermediate adaptor for FADD recruitment. Procaspase-8 (also known as FLICE) binds through N-terminal death effector domains (DEDs) to an N-terminal DED in FADD. Autocatalytic activation of procaspase-8 between p10 and p18 domains leads to its release from the DISC and subsequent activation of the downstream effector caspases, such as caspase-3 (Kischkel et al., 1995, Medema et al., 1997), and the degradation of nuclear substrates such as poly (ADP-ribose) polymerase enzymes (PARP) and caspase-activated DNase (Type I cells) which are responsible for morphological features of apoptosis (Enari et al., 1998, Lazebnik et al., 1994). Alternatively, active caspase-8 cleaves another protein, Bid (Bcl-2 interacting protein), within its N-terminus, which causes it to translocate into the mitochondria, as truncated-Bid (tBid) and mediates cytochrome c release (Li et al., 1998, Luo et al., 1998). This process is augmented by the release of a second protein, termed SMAC (second mitochondria-derived activator of caspases) or Diablo (direct IAP-binding protein with low Pi), which enhances the ability of caspase-9 to activate caspase-3 by binding to and sequestering inhibitor of apoptosis protein (IAP) (Chai et al., 2000, Stennicke and Salvesen, 2000). The activation of caspase-8 in the intrinsic pathway is delayed and

requires a mitochondrial step regulated by Bcl-2 and Bcl-xL (Scaffidi et al., 1999, Madge and Pober, 2001, Scaffidi et al., 1998, Luo et al., 1998). Bcl-xL can block the activity of caspase-8 at the mitochondrial membrane by indirectly sequestering the enzyme at the outer surface (Stegh et al., 2002). These data suggest that the signal transmission of the two pathways for caspase activation can sometimes be inter-connected.

In addition to the role of DRs in mediating apoptosis, DR stimulation can also activate anti-apoptotic NF-κB regulators such as cFLIP (cellular FLICE inhibitory protein), cellular inhibitor of apoptosis 1(c-IAP-1), c-IAP-2, x-linked mammalian inhibitor of apoptosis protein (XIAP) and survivin that function to inhibit induction of apoptosis by the DISC (Wang et al., 1998). Basically they inhibit apoptosis by interfering with caspase-8 activation, directly binding and inhibiting effector caspases, or by preventing permeability transition, depolarization of mitochondria and cytochrome c release (Karin and Lin, 2002, Kucharczak et al., 2003, Micheau et al., 2001). If NF-κB signals are absent, these anti-apoptotic proteins are not upregulated and DISC formation results in cell death.

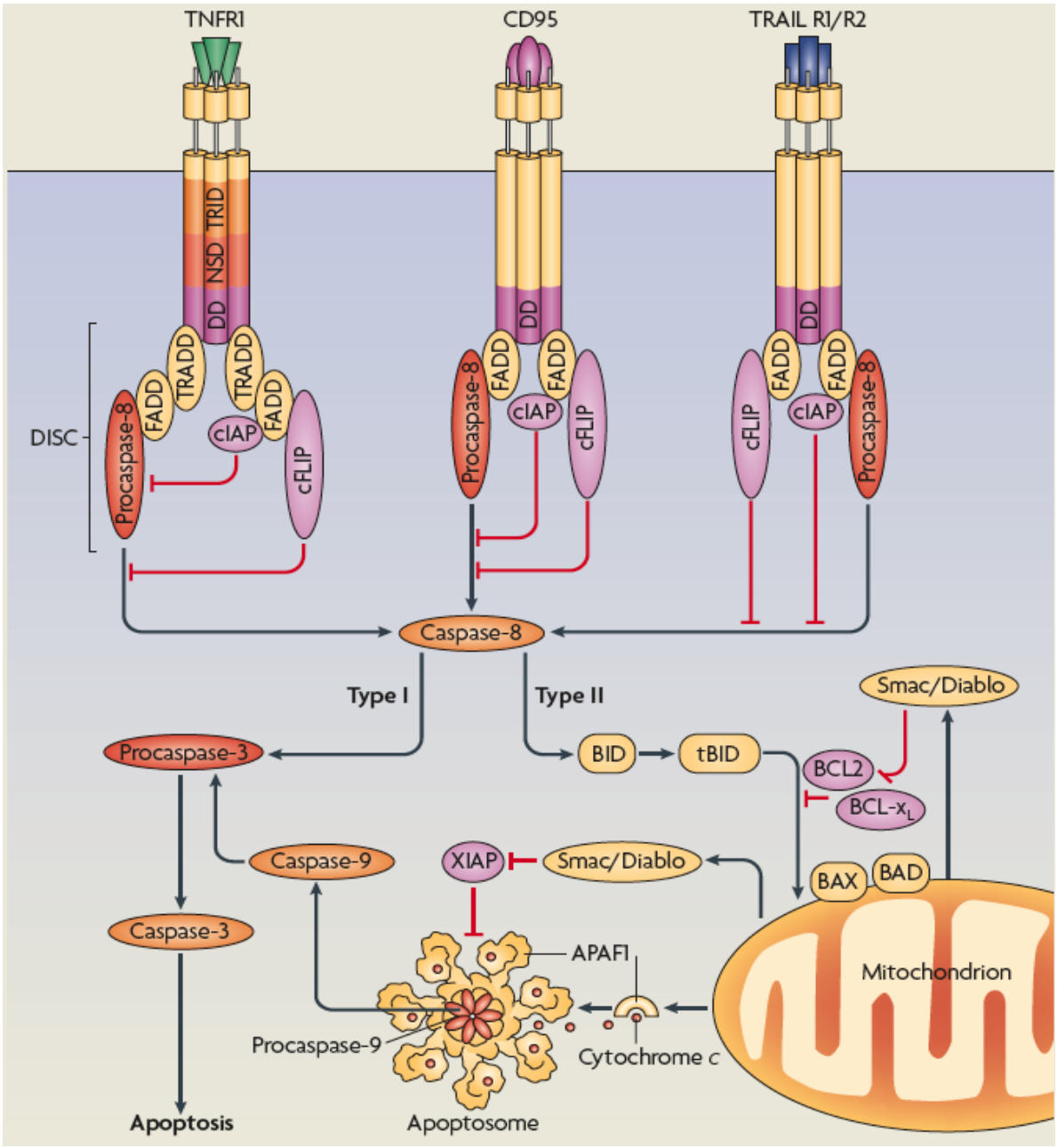


Figure 1.3: The death receptor and mitochondrial pathways of apoptosis.
Detailed description within the text. Adapted from (Schutze et al., 2008).

1.3.4.2 The Mitochondrial intrinsic pathway

Mitochondria are intracellular double-membrane organelles that possess an intermembrane space surrounded by the outer membrane and a matrix surrounded by the inner membrane. For over a century mitochondria have been recognised as providing energy in the form of ATP. However, in the early 90's the mitochondria was uncovered as an essential organelle in apoptotic signalling pathways through two key discoveries; firstly that the apoptotic inhibitor Bcl-2 protein was present on the outer mitochondrial membrane and secondly, that the mitochondrial fraction from xenopus laevis oocytes *in vitro* was essential for apoptosis (Hockenbery et al., 1990, Newmeyer et al., 1994). This evidence was followed by the finding that cytosolic extracts from apoptotic cells were able to mediate DNA fragmentation in a caspase-dependent and Bcl-2 inhibitable manner (Martin et al., 1995).

Exposure to intracellular stresses such as DNA damage, ROS, and treatment with chemotherapeutic drugs affect the integrity of the mitochondria. If these stresses are sufficient, permeablisation of the mitochondrial membrane occurs. Following this, cytochrome c is released from the mitochondrial intermembrane space into the cytosol (Choy et al., 2001). Here, cytochrome c binds with apoptotic protease activating factor-1 (APAF-1), procaspase-9 and ATP to form a pro-apoptotic complex termed the apoptosome. Active caspase-9 is released from this complex and can activate downstream effector caspases such as caspase-3 and 6 (Zou et al., 1999, Chinnaiyan, 1999). The apoptosome is regulated by the B cell lymphoma 2 (Bcl-2) family of protein. There are over 20 different Bcl-2 proteins, each containing a conserved Bcl-2 homology (BH) domain, which have either pro- or anti-apoptotic properties. Anti-apoptotic family members include Bcl-2 proper, Bcl-X_L, Bcl-w, A1 and Mcl1, while the pro-apoptotic members include the multi-domain Bax family and the BH3-only proteins (Cory and Adams, 2002). Anti-apoptotic Bcl-2 proteins are linked to intracellular membranes, including those of the mitochondria, nucleus and endoplasmic reticulum (ER). Bcl-2 is an integral membrane protein (Janiak et al., 1994), while Bcl-w and Bcl-X_L only associate with the membrane in response to apoptotic signals (Cory and Adams, 2002). An early study in *c. elegans* has shown that in normal cells, Ced-9, a Bcl-2 homologue, sequesters Ced-4 (the Apaf-1 homologue), blocking binding to the mitochondrial membrane and preventing it from activating the caspase Ced-3 (Spector et al., 1997). However, in

apoptotic cells, Ced-4 is displaced from Ced-9 by the BH3- only protein egl-1 and Ced-4 assumes a perinuclear localisation (Chen et al., 2000). A similar analogous mechanism occurs in mammalian cells; for the apoptosis activator egl-1 protein are the pro-apoptotic Bcl-2 family members, for the apoptosis inhibitor Ced-9 protein is Apaf-1 (apoptotic protease-activating factor-1) and for the apoptosis executor Ced-3 protein, the caspases (Lamkanfi et al., 2002).

An additional component of the pathway involves the Bax family of pro-apoptotic Bcl2 proteins, including the ubiquitously expressed Bak and Bax. Mutation of Bak or Bax alone has little effect on apoptosis; however, mutation of both proteins clearly inhibits apoptosis (Lindsten et al., 2000). During apoptosis, Bax which is normally present as a monomer in the cytosol becomes inserted in the outer mitochondrial membrane where it oligomerises (Nechushtan et al., 2001, Antonsson et al., 2001). In quiescent cells, Bak presents in the mitochondrial membrane as an oligomer, but during apoptosis it alters conformation (Griffiths et al., 1999). These Bax and Bak oligomers affect mitochondrial membrane permeability and initiate cytochrome c release (Pastorino et al., 1998, Gross et al., 1998), by forming pores (Antonsson et al., 2001, Saito et al., 2000) or by enlarging existing mitochondrial permeability transition pores (Tsujimoto and Shimizu, 2000). Bax and Bak also bind and inactivate anti-apoptotic proteins such as Bcl-2 (Chittenden et al., 1995). Furthermore, the BH3 domain of Bid is also able to bind and activate Bax and Bak (Wang et al., 1996), enhancing oligmerisation, subsequent pore formation in the mitochondrial membrane and the release of cytochrome c into the cytosol (Korsmeyer et al., 2000).

1.3.4.3 Activation of caspases in apoptosis

Apoptotic cell death is caused mainly by cleavage of critical cellular proteins by a family of structurally related intracellular proteases known as active cysteinyl, aspartyl-directed proteases (caspases). Caspases belong to a conserved family of proteases that use a cysteine residue as a catalytic nucleophile to cleave their substrates directly after a spartic acid residue. The first member of the family discovered in humans in 1992, as a regulator of inflammation, was caspase-1, named interleukin-1β converting enzyme (ICE) (Thornberry et al., 1992). Since then the role of the caspase family of cysteine proteases in apoptosis has been extensively examined. Yuan and Horvitz generated *c. elegans* mutants

in order to identify genes which triggered the execution of the cell (Yuan and Horvitz, 1992). They cloned *ced-3,* the first cell death gene, and they found that it encoded a cysteine protease (CED3) with similarity to mammalian ICE (Yuan et al., 1993). To date, 14 caspases have been characterized in humans, 10 in mouse and 4 in *c. elegans* (Lamkanfi et al., 2002), however the precise identities of their substrates are unclear. In normal cells, caspases are present as inactive precursors, called procaspases or zymogens, that require activation allosterically or by proteolytic cleavage (Saikumar et al., 2007). During apoptosis, caspases undergo a hierarchical cascade of activation from the initiator to the effector caspases. Active caspases have highly conserved, dimeric structures resulting from the association of two identical catalytic subunits. Each catalytic subunit contains one active site and is composed of one large and one small active domain.

Human caspases are classified depending on their function, structure and substrate specificity. As outlined above, caspase-8 and 9 are activated by both the extrinsic death receptor pathway and the intrinsic mitochondrial pathway. These caspases are called 'initiator' caspases because once activated, they cleave and activate a downstream caspase cascade. The cascade culminates in activation of 'effector' caspase-3, 6 and -7; which represent a point of connection between the death receptor and mitochondrial pathways. In contrast to the initiator caspases that are monomeric as zymogen, the effector caspases are held in the cytosol as in-active dimers. Within the caspase family, caspase-3 has been demonstrated to be a key mediator of apoptosis in mammalian cells and is synthesized as a latent proenzyme composed of 227 amino acids (Kuida et al., 1996). Translocation of caspase-3 fragments is dependent upon prior cleavage of procaspase-3 in the cytosol and association with recognised substrates (Kamada et al., 2005). Recent studies have uncovered a possible link between cytosolic caspase activation and apoptotic events within the nucleus (Sakahira et al., 1998, Enari et al., 1998).

Effector caspases such as caspase-3 cleave a number of important nuclear substrates including poly(ADP-ribose) polymerase-1 (Mehta et al., 2007), inhibitor of caspase-activated DNase (ICAD) (Sakahira et al., 1998) and Cyclin E (Mazumder et al., 2002). In addition however, caspases cleave numerous proteins which promote intracellular integrity or mediate survival pathways such as IκB (Barkett et al., 1997), NF-κB (Kang et al., 2001)

and anti-apoptotic Bcl-2 family members, Bcl-2 and Bcl-X_L (Cheng et al., 1997, Clem et al., 1998, Chowdhury et al., 2008). In addition, Bid cleavage by caspase-8 enhances its apoptotic properties, and this result in the apoptotic signal transduction from cytosol to the mitochondria (Li et al., 1998). Taken together, these data clearly demonstrate that caspases promote apoptosis through cleavage of multiple substrates.

1.3.4.4 The role of JNK in Cellular Apoptosis

Whilst the MAP kinases ERK and p38 have been implicated in the regulation of apoptosis under some conditions (Agell et al., 2002, Murphy and Blenis, 2006, Boutros et al., 2008), in relation to both the intrinsic and extrinsic pathways JNK has a predominant role. It has been shown utilising JNK isoform specific knockout mice, that JNK-1 mediates cytochrome-c mediated fibroblast death (Tournier et al., 2000b) and T-cell apoptosis, (Sabapathy *et al.*, 1999) whilst JNK3 plays a role in neuronal excitotoxicity (Yang et al., 1997). Recently, it has been demonstrated that disruption of JNK2/JNK3 genes in mice caused an increased small blood vessel contraction in arteries of mice compared to wild type and single knockout mice (Laukeviciene et al., 2006). Interestingly, JNK1/JNK3 and JNK2/JNK3 double knockout mice developed normally, while, JNK1/JNK2 double knockout mice die, this possibly suggests that JNK1 and 2 are able to compensate for the loss of each other.

Several recent studies have demonstrated that JNK plays a link between the extrinsic and intrinsic apoptotic pathways (Elmore, 2007, Papa et al., 2004, Bouillet and Strasser, 2002, Danial and Korsmeyer, 2004, Deng et al., 2003, Verhagen et al., 2000). Studies in embryonic stem cells from JNK1/2 knockout mice have demonstrated a role for JNK in activation of effector caspases, including caspase-3, in response to UV-C (Tournier et al., 2000a). This was confirmed in another study using γ-ray stimulation (Chen et al., 1996), the duration of JNK signalling in ECs was found to be crucial in mediating apoptotic signals. This observation was supported by other studies which demonstrated that sustained activation of JNK for more than 1 hr was associated with apoptosis, whereas shorter durations were linked to anti-apoptotic activities (Chen and Tan, 2000, Ventura et al., 2006, Yu et al., 2004a, Maundrell et al., 1997, Fuchs et al., 1998b).

The mechanism of JNK-dependent apoptosis is unclear but is unlikely to involve a single-specific event (**Figure 1.4**). On exposure to genotoxic stress such as ionising radiation or cisplatin, JNK can translocate to the mitochondria and phosphorylates numerous targets such as Bcl-$X_{L,}$ Bax and 14-3-3σ, a cytoplasmic anchor of Bax. These proteins are involved in release of cytochrome-c which represents a crucial mitochondrial event in initiating apoptosis (Cadalbert et al., 2005, Kharbanda et al., 2000, Kim et al., 2006, Yamamoto et al., 1999, Dhanasekaran and Reddy, 2008). Moreover, β-adrenergic-mediated apoptosis of cultured adult cardiomyocytes was observed to be inhibited by over-expression of dominant-negative JNK through a mitochondria dependent effect (Remondino et al., 2003, Aoki et al., 2002). These findings suggest that JNK has an essential role in balancing both the pro- and anti- apoptotic proteins located in the mitochondria.

The exact mechanism by which JNK translocates to the mitochondria and activates cytochrome c release has not been fully elucidated. There are several targets that have also been identified. Studies have been shown that JNK mediates Bid phosphorylation which resulted in Bax activation (Bossy-Wetzel and Green, 1999, Kuwana et al., 2002, Madesh et al., 2002). JNK has also been shown to induce caspase-8 independent cleavage of Bid during TNF-α mediated apoptosis of HeLa cells, and the resultant 21 kDa fragment of Bid (jBid) translocates to the mitochondria and selectively promotes the release of the pro-apoptotic protein Smac/DIABLO (Deng et al., 2003). Whilst the exact mechanism by which JNK triggers the cleavage of Bid needs to be identified, it is more likely that activated JNK mediates the release of cytochrome c from mitochondria through a similar pathway involving the pro-apoptotic proteins Bid and Bax. It is quite possible that a similar mechanism causes the release of Smac/ DIABLO during stress mediated apoptosis of multiple myeloma cells (Chauhan et al., 2003). The ability of JNK to induce Bid cleavage and therefore promote the release of Smac/DIABLO associated with another apoptotic signalling mechanism**,** suggests that JNK may provide a critical link between the extrinsic pathway and the mitochondrial events.

Moreover, JNK has also been demonstrated to modulate the activities of other mitochondrial pro-apoptotic BH3-only subgroup Bcl-2 family proteins such as Bim and

Bmf (Lei and Davis, 2003). During UV-induced apoptosis of HEK293T cells, phosphorylation of Bim and Bmf by JNK promotes their release from dynein and myosin V motor complexes. The phosphorylated Bim and Bmf can then activate Bax and/or Bak to mediate apoptosis (Letai et al., 2002, Lei et al., 2002, Marani et al., 2002). However, phosphorylated Bim can also bind and reverse the anti-apoptotic activities of Bcl-2 and Bcl X_L, thereby promoting apoptosis (Puthalakath et al., 1999, Puthalakath and Strasser, 2002). It has also been demonstrated that JNK phosphorylates the Ser^{128} of BAD (another BH3-only pro-apoptotic member of Bcl2 family) and promotes the apoptotic effect of BAD in the primary granule neurons of the rat cerebellum (Donovan et al., 2002). The apoptotic effect of BAD involves the inhibition of the pro-survival Bcl-2 proteins (Gross et al., 1999). JNK phosphorylates BAD at Ser^{128} and inhibits its interaction with 14-3-3 proteins so that BAD can antagonize the anti-apoptotic Bcl-2 proteins to promote apoptosis (Wang et al., 2007b). Moreover, JNK has been shown to activate the pro-apoptotic role of BAD by phosphorylating 14-3-3z proteins at Ser^{184} (Tsuruta et al., 2004), this phosphorylation led to dissociation of BAD from this protein. Furthermore, JNK also directly regulates the anti-apoptotic Bcl-2 through phosphorylation. For example, it has been demonstrated that JNK phosphorylates Bcl-2 at Ser^{70} in response to microtubule-damaging agent, paclitaxel, in breast cancer cells to suppress its anti-apoptotic activity (Srivastava et al., 1999, Yamamoto et al., 1999).

Numerous studies also support JNK regulation of nuclear events as a mechanism to initiate apoptosis. In some cell types, JNK translocates to the nucleus where it phosphorylates and transactivates c-Jun (Davis, 2000; Chang and Karin, 2001). Phosphorylation of c-Jun leads to the formation of activator protein 1 (AP-1), which is involved in the transcription of a wide variety of genes, some of them encoding known pro-apoptotic factors (Dhanasekaran and Johnson, 2007, Raman et al., 2007, Turjanski et al., 2007). It has been noted that the JNK/AP-1 pathway is involved in the increased expression of pro-apoptotic genes such as TNF-α, Fas-L and Bak (Fan and Chambers, 2001), suggesting that the nuclear activation of c-Jun is required for its apoptotic activity.

In rat heart tissue nuclear accumulation of JNK1 occurs following ischemia, then during reperfusion JNK becomes activated by phosphorylation by nuclear SEK1 (Bak and

Ingwall, 1994, Mizukami et al., 1997), a finding confirmed by another study in ischemic mouse liver (Webster et al., 1994). Recent studies show clustering of cell surface receptors in response to UV irradiation or osmotic stress, and this consequently induces the activation and nuclear entry of JNK (Cavigelli et al., 1995, Rosette and Karin, 1996). However, it has been demonstrated that γ-radiation activates a nuclear-located JNK1 without nuclear translocation (Chen et al., 1996b, Robinson et al., 2001). Kharbanda et al. (1995) have shown that JNK1 is activated by c-AbI tyrosine kinase activation within both the nucleus and the cytosol in response to DNA-damaging agents (Kharbanda et al., 1995). Taken together, these studies suggest that JNK is activated within the nucleus or within the cytosol and is then translocated this compartment.

Resolving the nuclear action of JNK has centred on the regulation of c-Jun. A mutant version of c-Jun was able to prevent apoptosis induced by ceramide, a major product of TNF-α activation (Verheij et al., 1996). This was supported by another study showing that MEFs expressing mutant c-Jun were resistant to UV-induced apoptosis (Behrens et al., 1999). In contrast, other work has questioned the role of JNK and c-Jun in apoptosis as c-Jun phosphorylation status did not correlate with cell death (Hochedlinger et al., 2002). Such differences may be JNK isoform related; JNK1 was found to be the main JNK isozyme responsible for the phosphorylation of c-Jun and subsequently apoptosis, whilst JNK2 negatively regulated c-Jun phosphorylation (Sabapathy and Wagner, 2004). Consistent with this observation, JNK1 inhibition was shown to protect cardiac myocytes from ischemia-induced apoptosis, whereas JNK2 inhibition had no effect (Hreniuk et al., 2001).

Therefore, these studies provide sufficient evidence to suggest that the nuclear activities of JNK are necessary for it is apoptotic activity. However, if both nuclear and mitochondrial JNK dependent events are involved in mediating apoptosis they may take place either sequentially, simultaneously or exclusively. Recent studies have implicated the involvement of multiple phases of JNK activation in mediating apoptosis in response to stress stimuli (Krilleke et al., 2003, Ham et al., 2003), and the role of the different JNKs (JNK1 and JNK2) in apoptosis (Eminel et al., 2004). In this study, JNK2 has been shown to translocate to the mitochondria in PC12 cells in response to 6-hydroxy dopamine. An

initial phase was associated with nuclear JNK activation and a second phase resulted from dissociation of p21WAF/CIP1, mitochondrial translocation of JNK and subsequent cytochrome c release (Chauhan et al., 2003, Vivo et al., 2003). Cadalbert et al. (2005) suggested interdependence between the two phases of JNK activation; the mitochondrial phase is dependent upon the earlier nuclear phase. This hypothesis was supported by the finding that cytochrome c release from mitochondria is mediated by JNK activation following DNA damage, an event initiated within the nucleus (Tournier et al., 2000a).

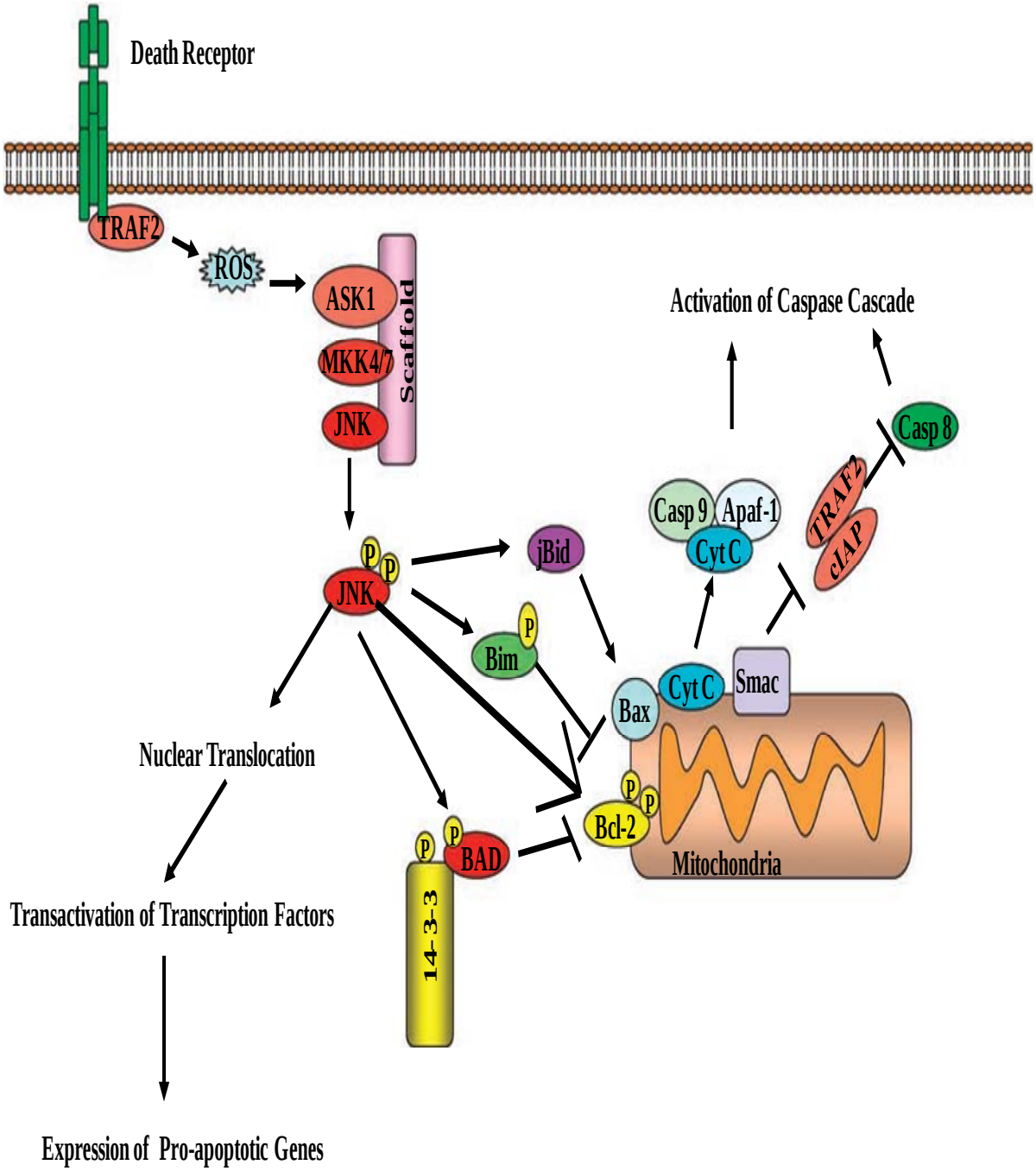


Figure 1.4: Nucleus and Mitochondria-Targeted Signalling by JNK.
Detailed description within the text (Dhanasekaran and Reddy, 2008).

1.3.4.5 Role of MAP kinase in Endothelial Cell Apoptosis

Whilst several studies have examined MAP kinase signalling pathways and their structures, localisations and substrates, more remains to be identified about the biological function of this pathway in endothelial cell apoptosis (EC). Nevertheless, in general, whilst ERK is associated with cell survival (Mavria et al., 2006, Pintus et al., 2003, Yu and Sato, 1999), evidence suggests that both p38 MAP kinase and JNK are strongly associated with endothelial cell apoptosis (Nakagami et al., 2001, Wadgaonkar et al., 2004). Indeed, a large body of evidence implicates JNK in the regulation of EC death (Sabapathy et al., 1999, Yang et al., 2009, Harfouche and Hussain, 2006). However again, the mechanism of JNK-dependent apoptosis in unclear but is unlikely to involve a single mechanism and in some instances both survival and death-enhancing outcomes have been demonstrated.

1.3.4.6 JNK mediated Endothelial Cell Apoptosis

A role for JNK in mediating apoptosis in response to a number of stimuli stressful to the endothelium is well established. These include: infection with streptococcus pneumoniae R6x (N'Guessan *et al.*, 2005), high glucose (Ho et al., 2000), hypoxia / reoxygenation (Dougherty et al., 2004, Kunz et al., 2001), H_2O_2 (Wang et al., 1999), homocysteine (Dong et al., 2005), ischemia/reperfusion (Yang et al., 2009), LPS (Hull et al., 2002) and serum deprivation. In contrast, activation of JNK by TNF-α in HUVECs occurs without significant induction of death (Wajant et al., 2003, Karsan et al., 1996, Hull et al., 2002, Kamata et al., 2005). This is because TNF-α causes only a transient increase in JNK activity, which is not normally a signal sufficient for apoptosis (Franzoso et al., 2003). Thus, to trigger apoptosis, JNK must instead signal chronically under conditions of NF-κB inhibition which prevents induction of JNK suppressor (Kamata et al., 2005, Sakon et al., 2003). Recently, several studies have demonstrated that in HUVECs, stimulation with TNF-α in conjunction with cycloheximide (CHX), a protein synthesis inhibitor, or actinomycin D (ActD), results in sustained JNK activation and apoptosis (Madge and Pober, 2001, Wadgaonkar et al., 2004, Deng et al., 2003, Stehlik et al., 1998).

Other pro-apoptotic agents include members of the reactive oxygen species (ROS) family, such as H_2O_2, O_2 and HO radicals, generated mainly from mitochondria by several enzymatic pathways (Sakon et al., 2003, Nakano et al., 2006). Several studies have shown

that TNF-α increased reactive oxygen species (ROS), however this again seems to be under conditions of NF-κB inhibition. For example, TNF-α induced ROS accumulation in NF-κB deficient cells, which resulted in sustained JNK activation (Nakano et al., 2006, Kamata et al., 2005, Sakon et al., 2003). Under these conditions expression of several antioxidant genes was reduced and led to inhibition of mitogen-activated protein kinase phosphatase (MKP) activity through oxidation of a highly reactive cysteine residue in their catalytic site (Kamata and Hirata, 1999). Stimulation with H_2O_2 in HUVECs for over 12 hr has been shown to mediate SEK1/JNK phosphorylation and subsequent caspase-3 activation and this lead to apoptosis (Murakami et al., 2005). In the same study, apoptosis was significantly reversed by adenoviral expression of DN-JNK and SEK1 (Murakami et al., 2005), this suggested a role for JNK in mediating caspase-3 dependent apoptosis in ECs. These findings were supported by other studies which demonstrated that caspase-3 and its nuclear substrate PARP-1 were cleaved in HUVECs over-expressing c-Jun. Apoptosis in this instance was significantly attenuated by zDEVD.fmk, an irreversible cell-permeable inhibitor of caspase-3 (Wang et al., 1999). Other work has demonstrated that the c-Jun/AP-1 complex activation was required for H_2O_2-induced apoptosis (Ishikawa et al., 1997, Shono et al., 1996). Furthermore, adenoviral mediated over-expression of dominant negative mutants of c-Jun such as TAM67, TAM67, both increased EC resistance to H_2O_2 induced apoptosis (Wang et al., 1999). Additional studies also link H_2O_2 to cell death via glutathione reductase-dependent glutathione redox-cycling (Hermann et al., 1997, Hojo et al., 2002, Cai, 2005), Src-dependent activation of EGFR (Chen et al., 2001a), Fas up- regulation (Suhara et al., 1998), enhanced intracellular ion uptake (Kotamraju et al., 2003), and induction of mitochondrial DNA damage (Ballinger et al., 2000).

Apoptosis of ECs can also be induced by serum deprivation. For example, it has been demonstrated that HUVECs starved with 0.1% foetal bovine serum (FBS) for 24 hr elicited a significant increase in JNK phosphorylation followed by apoptosis, outcomes reversed by adenoviral over-expression of dominant negative JNK. Furthermore, it has been shown in other studies that serum starvation for 24 hr was able to induce JNK activation and subsequently caspase-3 and 9 activation, thus creating a positive feedback

loop that culminated in the progression of apoptosis (Harfouche et al., 2003, Cardone et al., 1997). Recently, it has been demonstrated that serum starvation of HUVECs mediated release of cytochrome c to the cytosol (Williams et al., 2006). High glucose (33 mmol/l) has also been shown to induce HUVECs apoptosis by sequential activation of ROS-mediated JNK and caspase-3 cleavage (Ho et al., 2000). Furthermore, stimulation of microvessel endothelial cell (HMEC) with LPS has been demonstrated to lead to caspase-3 cleavage through JNK activation in presence of CHX (Karahashi et al., 2009).

Most recently, a study utilising JNK-1 deficient mice, showed that JNK-1 is associated with endothelial death *in vivo* at a site in the vessel prone to plaque development (Chaudhury et al., 2010). In coronary artery segments isolated from rat cardiac allografts, it has been shown that around 50% of EC stained positively to Bax (Dong et al., 1999). These studies are some of the very few which link JNK to apoptosis *in vivo*.

1.4 Control of MAP Kinases by Protein Phosphatases

As with any signalling transduction process, as well as being able to induce and sustain a signal or response, there has to be a means of switching the signal off once it has served its purpose. Protein phosphatases dephosphorylate and inactivate protein kinases, thus limiting the amplitude and duration of cellular activation. During development, protein phosphatases function as key regulators in the balance between cell proliferation, differentiation and apoptosis. Protein phosphatases can be divided into two subfamilies depending on substrate specificity. Firstly, serine/threonine-specific phosphatases (such as PP2A, PP2C) and tyrosine-specific phosphatases (such as SREP, HePTP and PTP-SL). These phosphatases have been reviewed elsewhere (Tonks, 2006, Mumby and Walter, 1993, Stoker, 2005, Berndt, 1999, Shi, 2009) and will not been disscused further. An additional sub family of protein phosphatases are dual specific phophatases (DUSPs), also known as the MAP kinases phosphatases (MKPs). These are reviewed below.

1.4.1 Dual specificity MAP Kinase Phosphatases (MKPs) Family

In mammalian systems to date, around 10 MKP family members have now been identified (Keyse, 2008, Kondoh and Nishida, 2007, Patterson et al., 2009, Soulsby and Bennett, 2009). However, this list is expanding with the identity of homologous MKP isoforms

within subgroups. MKPs specifically target and dephosphorylate both the phospho-threonine and the phospho-tyrosine residues within the TXY activation motif of MAP kinase enzymes, thus abolishing MAP kinase activity. Early studies demonstrated that MKPs could be induced by a number of agents such as growth factors (Misra-Press et al., 1995), oncogenes (Fu et al., 2000) and hormones (Zhang and Roberson, 2006, Brondello et al., 1997). Many of these agents activated MAP kinases, and this lead to the idea that the MAP kinases themselves promoted a negative feedback affect through the induction of the MKPs.

All MKPs have a highly conserved catalytic domain that contains the consensus PTPase motif. This domain is located within the C-terminal portion of all MKPs and shares sequence similarity with the prototypic VH-1 DUSP encoded by vaccinia virus, $DX_{26}(V/L)_X(V/I)H_{CX}AG(I/V)SRS_XT(I/V)_{XX}AY(L/I)M$. The D (Asp), C (Cys) and R (Arg) residues have been reported to be essential for catalysis activity (Denu and Dixon, 1998, Patterson et al., 2009), whilst the N-terminal region, containing the MAP kinase binding domain (MKP), is believed to be involved in substrate binding and specificity. All MKPs also have two short regions of homology with the Cdc25 phosphatase (also termed CH_2 domain) (Keyse and Ginsburg, 1993).

Whilst the DSP domains share strong homology with each other they do not discriminate between MAP kinase members. In contrast, the N-terminal MAP kinase binding domain (MKB) has a major role in regulating enzymatic specificity as a docking site for each MAP kinase (Muda et al., 1998, Tanoue et al., 1999). The MKB domain contains a cluster of positively charged amino acids which play a role in determining binding specificity of MKPs towards MAP kinases (Tanoue et al., 2000, Tanoue et al., 2001a). A cluster of hydrophobic amino acids and another cluster of positively charged amino acids are required for the specific binding of MKPs with MAP kinases (Tanoue et al., 2002), and it has been reported that several MKPs are catalytically activated by binding to their MKB domain (Camps et al., 1998b, Chen et al., 2001b, Dowd et al., 1998, Zhang et al., 2005) (**Figure 1.5**). This suggests that this binding changes the domain structure leading to increased catalytic activity.

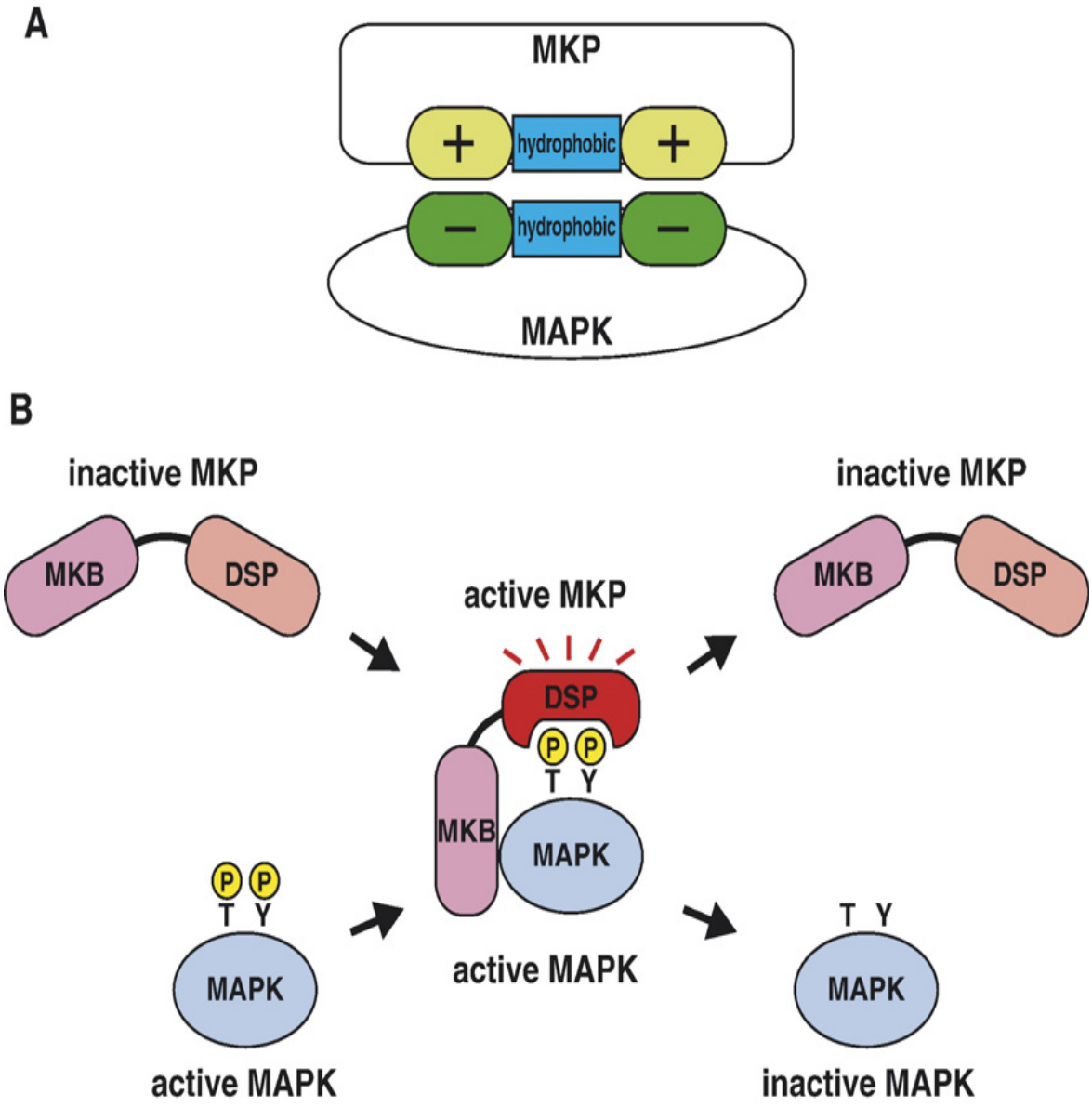


Figure 1.5: Interaction of MKPs with MAPK. **A**) Activation of MKPs by MAPK; the dual-specificity phosphatase (DSP) domain in the MKPs is inactive without its substrate. **B**) Binding of activated MAPK to the MKB domain induces conformational changes in the DUSP domain which causes the increase of its catalytic activity. Adapted from (Kondoh and Nishida, 2007)

A number of characteristics based on sequence similarity, protein structure, substrate specificity, subcellular localization and patterns of transcriptional regulation allow MKPs to be divided into three main groups (see reviews by (Patterson et al., 2009) (**Figure 1.6**). Group one MKPs are approximately 300-400 amino acids in length, localised mainly in the nucleus due to the presence of nuclear targeting sequences, and are transcriptionally regulated by a large number of stimuli that activate MAP kinase. Therefore, these MKPs have been shown to play a crucial role in the feedback control of MAP kinase signalling in the nucleus. MKPs encoded by highly inducible genes include: DUSP-1/MKP-1, DUSP2/PAC-1, DUSP4/MKP-2 and DUSP5/Hvh-3. They exhibit broad substrate specificity and can inhibit ERK, p38 MAP kinase and JNK. The second group of MKPs consists of DUSP6/MKP-3, DUSP-7/MKP-X and DUSP9/MKP-4. All three proteins are cytoplasmic enzymes and exhibit a degree of selectivity towards ERK1/2. These MKPs have a nuclear export sequence (NES) and are distributed principally in the cytosol, and MKPs in this group show restricted tissue distribution. The final group of MKPs comprises DUSP8/hVH-5, DUSP10/MKP-5, M3/6 and DUSP16/MKP-7. Whilst they are too large to enter the nucleus by passive diffusion, these proteins are found in both the cytosol and the nucleus. They selectively dephosphorylate the stress-activated MAP kinase, p38 MAP kinase and JNK, whilst showing little or no activity towards ERK1/2.

Although the biochemical and structural properties of MKPs have been studied extensively over the past few years, the physiological roles of these MKPs in mammalian cells and tissues have not been fully elucidated. This is due to the large number of MKPs with overlapping substrate specificities. In the following section the current knowledge regarding the function of the major MKP family members will be discussed. The properties of these and other isoforms not discussed are summarised in **Table 1.1**

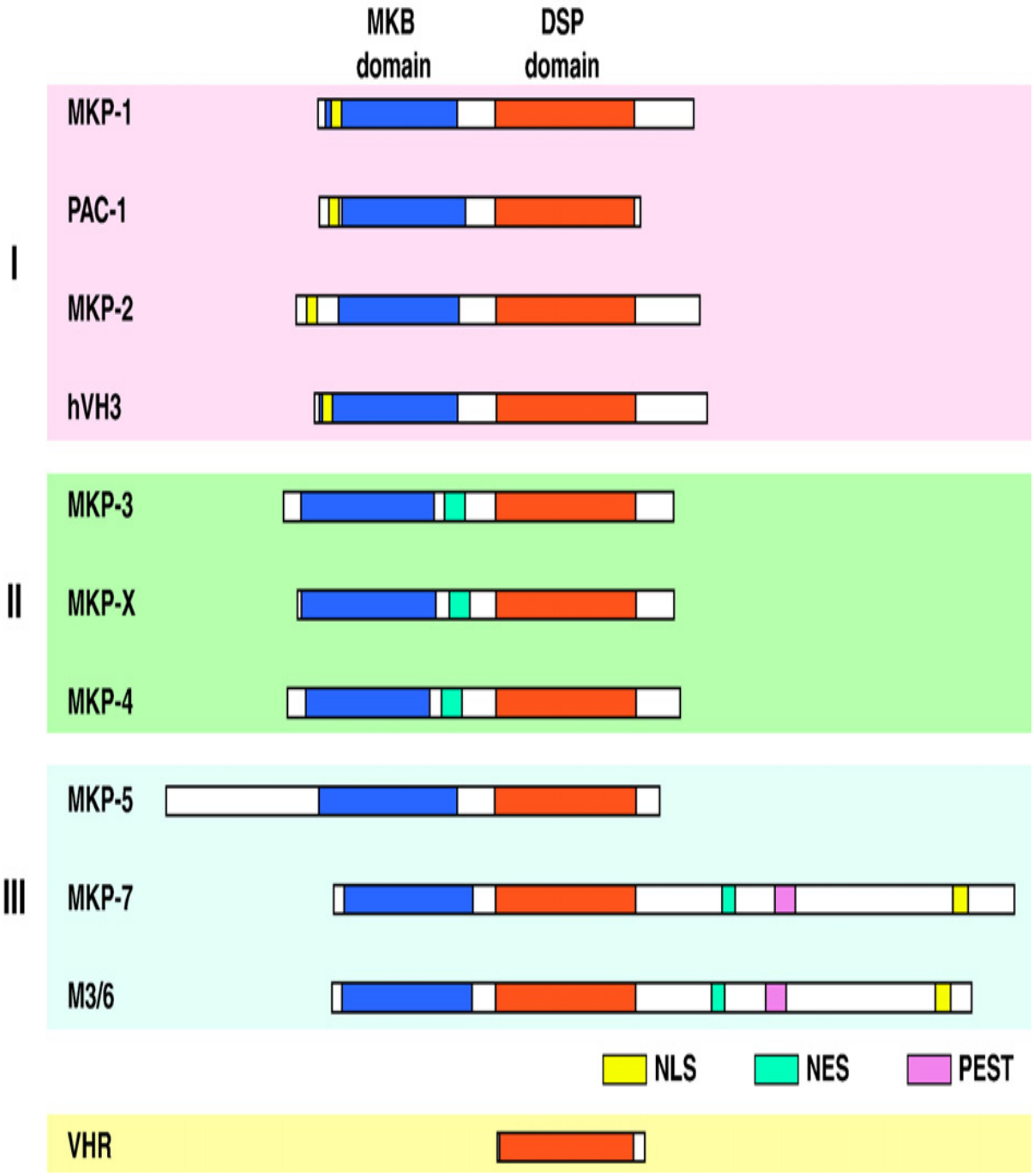


Figure 1.6: Classification and domain structure of the MKP family
Domain structures of the three subgroups of MKPs are shown. VHR is an atypical "MKP". In addition to the MAPK binding (MKB) domain and dual-specificity phosphatase (DUSP) domain, nuclear localization signal (NLS), nuclear export signal (NES), and PEST sequences are indicated. Adapted (Kondoh and Nishida, 2007).

Table 1.1: The MAP kinase Phosphatases

Gene	MKP	Trivial names	Subcellular Localisation	Substrate Specificity	Tissue distribution	References
DUSP-1	**MKP-1**	CL-100, hVH1, erp, 3CH134	Nuclear	p38~JNK~ERK	Heart, skeletal muscle, pancrease, placenta, liver, testes, stomach, lung and brain	(Farooq and Zhou, 2004; Keyse and Emslie, 1992; Noguchi *et al.*, 1993).
DUSP2	**PAC-1**	N.D	Nuclear	ERK~p38	lymphoid cells (spleen and thymus)	(Gerondakis *et al.*, 1994; Rohan *et al.*, 1993; Ward *et al.*, 1994).
DUSP4	**MKP-2**	hVH-2, Typ1, Sty8	Nuclear	JNK~ERK1 > p38	Placenta, skeletal muscle, spleen, kidney and brain, heart, liver, testis, and pancreas.	(Chu *et al.*, 1996; Guan *et al.*, 1995; Misra-Press *et al.*, 1995).
DUSP5	**N.D**	hVH3, B23	Nuclear	ERK	Placenta, liver, heart, lung, brain, kidney, pancreas, skeletal muscle	(Ishibashi *et al.*, 1994; Martell *et al.*, 1994).
DUSP6	**MKP-3**	rVH-6, Pyst1	Cytoplasmic	ERK	Placenta, skeletal muscle, spleen, kidney, and brain, heart, liver, testis, and pancreas.	(Groom *et al.*, 1996; Muda *et al.*, 1996a; Muda *et al.*, 1996b).
DUSP7	**MKP-X**	Pyst2, B59	Cytoplasmic	ERK	Heart, brain, placenta, lung, skeletal muscle, kidney and pancreas	(Smith *et a*l., 1997, Shin *et al.*, 1997)
DUSP9	**MKP4**	Pyst3	Cytoplasmic	ERK > p38	Placenta, kidney, embryonic liver	(Muda *et al.*, 1997, Smith *et al.*, 1999)
DUSP10	**MKP-5**	N.D	Cytoplasmic/ Nuclear	JNK~p38	Heart, lung, skeletal muscle, liver, and kidney> brain, spleen and testis.	(Tanoue *et a*l., 1999, Theodosiou *et al.*, 1999)
DUSP16	**MKP-7**	MKP-M	Cytoplasmic/ Nuclear	JNK~p38	N.D	(Masuda *et al.*, 2001)
DUSP14	**MKP-6**	MKP-L	N.D	ERK~JNK	Human trachea, placenta, liver, heart and thyroid.	(Marti *et al.*, 2001)
N.D	**MKP-8**	N.D	Cytoplasmic/ Nuclear?	p38	Brain, heart, skeletal muscle	(Sanchez-Prieto *et al.*, 2000; Vasudevan *et al.*, 2005).

* N.D : not determined

1.4.1.1 Type 1 Nuclear MKPs

1.4.1.1.1 MAP Kinase Phosphatase-1 (MKP-1)

MKP-1, also referred to CL100, was the earliest discovered MKP. The cDNA for this gene was initially cloned because of its inducibility by serum stimulation and peroxide-induced stress in human fibroblasts, and was found to be homologous to Vaccinia virus H1 phosphatase (Lau and Nathans, 1985). The gene locus of MKP-1 is present on human chromosome 5q34, an area of chromosome 5 correlated to malignancies. This region is deleted in leukaemias and translocations is linked to lymphoma (Emslie et al., 1994). MKP-1 was identified as an immediate nuclear early gene product that is induced rapidly after exposure to growth factors, hormones, heat shock, cytokines, DNA damage and oxidative stress (Charles et al., 1992, Keyse and Emslie, 1992, Noguchi et al., 1993, Wang et al., 2008, Teng et al., 2007). Nuclear translocation is mediated by its N-terminal LXXLL motif (Wu et al., 2005).

Initial studies *in vitro* demonstrated selectivity in the dephosphorylation of the MAPK family with the following order of affinity; p38 MAP kinase > JNK > ERK1/2 (Franklin and Kraft, 1997, Camps et al., 2000, Farooq and Zhou, 2004) and within these ERK2 > ERK1 (Slack et al., 2001). MKP-1 was also later found to dephosphorylate ERK5 (Sarkozi et al., 2007). Studies *in vivo* utilising MKP-1 knockout mice have, by and large, confirmed such a specificity order (Brondello et al., 1999, Hutter et al., 2000, Wu and Bennett, 2005). Moreover, ERK1/2 activity is not affected in the MKP-1 deficient fibroblasts (Franklin and Kraft, 1997, Chu et al., 1996, Dorfman et al., 1996). However, despite its apparent selectivity *in vivo*, it has been shown that the catalytic activity of MKP-1 is enhanced by its binding to all three major MAP kinases through the MKB domain (Slack et al., 2001, Hutter et al., 2000).

MKP-1 expression is high in liver, placenta, lung and pancreas, with lower levels detected in heart and skeletal muscle (Kwak et al., 1994). In a variety of different cell systems studies have shown that MKP-1 expression is mediated predominantly by ERK activation (Brondello et al., 1997, Franklin and Kraft, 1997, Beltman et al., 1996). In addition, it has been reported that phophorylation of MKP-1 by ERK reduces proteosomal mediated

degradation of MKP-1 enhancing the half life of the protein (Brondello et al., 1999). This suggestes that MKP-1 is involved in a negative feedback system to prevent over activation of the MAP kinases (Bokemeyer et al., 2000). This hypothesis is supported by a recent study which reports that p38 MAP kinase activation also stabilizes MKP-1 by MAPKAP kinase 2 (Hu et al., 2007).

The role of MKP-1 has been studied in several differing cell types and situations, in particular in relation to apoptosis and proliferation. Initially MKP-1 deficient mice were found to be normal and fertile, and studies using MEFs isolated from the knockout mice revealed no changes in either ERK1/2 activation or the growth of the cells in response to serum (Dorfman et al., 1996). However, recent studies provide evidence for an essential role in the regulation of p38 MAP kinase and JNK. MEFs derived from mice lacking MKP-1 show hyperactivation of p38 MAP kinase and JNK in response to serum, osmotic stress and anisomycin and increased sensitivity to stress-induced apoptosis (Wu and Bennett, 2005). In tumour cells the chemotherapeutic drug cisplatin was found to induce apoptosis but the tans-isomer of the same drug, transplatin, was ineffective. This was discovered to be due to the ability of transplatin to induce MKP-1 mRNA production; inactivating JNK and preventing apoptosis (Sanchez-Perez et al., 2000). In addition, over expression of MKP-1 increased breast cancer cell (MCF-7) resistance to H_2O_2-induced death by JNK activation (Zhou et al., 2006), and inhibition of MKP-1 expression also potentiated apoptosis (mediated by TNF-α) through JNK activation in rat mesangial cells (Guo et al., 1998). In the same cell type, another group has shown that H_2O_2 was able to induce MKP-1 which in turn inhibited JNK activation and apoptosis (Xu et al., 2004). Furthermore, it has been shown that MKP-1 expression inhibited PMA and UV irradiation induced JNK activation and apoptosis in human leukemic cells (U937). Taken together these studies demonstrate that MKP-1 exerts an anti-apoptotic effect by the down-regulation of JNK activity.

MKP-1 also has effects on various other systems, including immune and cardiovascular. It has been demonstrated that MKP-1 induction in response to VEGF and thrombin leads to EC migration (Kinney et al., 2008). In relation to inflammation, Macrophages from mice lacking MKP-1 show increased JNK and p38 MAP kinase activities in response to LPS,

and the cells are highly sensitive to endotoxic shock (Zhao et al., 2006, Zhao et al., 2005, Hammer et al., 2006). In vascular smooth muscle cells, MKP1 has been shown to be a positive regulator of hypertrophic genetic responses due to its ability to inhibit activation of MAP kinases (Duff et al., 1993, Duff et al., 1995). Recently, MKP-1 has been implicated in preventing atherosclerotic plaque development by suppressing pro-inflammatory activation (Chaudhury et al., 2010, Zakkar et al., 2008, Chaudhury et al., 2009). Bueno et al.(2001) have shown that transgenic mice constitutively expressing MKP-1 in the heart displayed decreased normal developmental hypertrophy, enhanced ventricular dilation and resistance to hypertrophic response by isoproterenol or pressure overload (Bueno et al., 2001). This suggests that MKP-1 reduces the cardiac hypertrophic response *in vitro* and *in vivo*. Moreover, it has been shown that inhibition of p38 MAP kinase in MKP-1 gene-targeted mice inhibits cardiac injury and cell death by ischemic-perfusion (Kaiser et al., 2004), however, the role of MKP-1 in atherosclerosis and other cardiovascular diseases has not yet been studied well.

1.4.1.1.2 MAP kinase Phosphatase-2 (MKP-2)

MKP-2 (also known as hVH-2) is an inducible nuclear protein of 394 amino acids (M.W approx. 43 kDa)(Chen et al., 1996a). MKP-2 was cloned concurrently from rat and human (Guan and Butch, 1995, Misra-Press et al., 1995), although recently a novel variant of MKP-2 with a shortened N-terminus has been identified (Cadalbert et al., 2010). MKP-2 has a similar gene structure organisation to MKP-1. The intron and exon size and splicing patterns of the two genes are almost the same, suggesting that they are both derived from the same ancestral gene (Zhang et al., 2001a). However, MKP-2 is distinct from MKP-1 in tissue distribution, which may indicate different physiological functions for each phosphatase. Guan et al. (1995) observed that MKP-2 is expressed in most rat tissues including spleen, testes, brain, heart and lung (Guan and Butch, 1995). Similarly, Chu et al. (1996) found that MKP-2 is expressed in most human tissues, with highest amounts in prostate, testes, stomach and pancreas (Chu et al., 1996). Misra-Press et al. (1995) also found additional expression in human placenta and pancreas, but undetectable amounts in the lung (Misra-Press et al., 1995). Nuclear localisation of MKP-2 is determined by the presence of two putative nuclear localisation sequences (NLS), NLS2 is present within the

MKB domain and NLS1 within the DUSP domain; the disruption of both being required to alter the cellular localisation of MKP-2 (Sloss et al., 2005).

Several studies have shown that MKP-2 is induced by growth factors, gonadotrophin-releasing hormone (GnRH), retinoic acid, the oncogenic gene v-Jun (a constitutively active form of c-Jun), the tumour suppressor p53, E2F-1,UV-light, and oxidative stress (Zhang et al., 2001b, Palm-Leis et al., 2004, Fu et al., 2000, Cadalbert et al., 2005, Eljaschewitsch et al., 2006, Wang et al., 2007a, Teng et al., 2007). In some of these studies expression is mediated by ERK1/2 (Brondello et al., 1997), suggesting that as with MKP-1, MKP-2 regulates MAP kinase activity by a negative feedback mechanism. Furthermore, MKP-2 stability is enhanced in response to cellular senescence by blocking its degradation (Torres et al., 2003).

Regulations of MKP-2 catalytic activity and substrate specificity have been examined in a manner similar to MKP-1. Early *in vitro* studies demonstrated that MKP-2 specifically dephosphorylates ERK1/2 and JNK *in vitro* (Chu et al., 1996). This was consistent with studies that demonstrated that the catalytic activity of MKP-2 could be enhanced considerably by interaction with ERK1/2 and JNK (Chen et al., 2001b). MKP-2 was also found to interact with p38 MAP kinase but had no effect on its catalytic activity (Chen et al., 2001b). The interaction of ERK1/2 and p38 MAP kinase with MKP-2 involved three arginine residues within a basic motif in the NH_2 terminus of MKP-2 (MKP). However, for JNK this interaction was not properly defined. Although JNK activated MKP-2 *in vitro* and MKP-2 efficiently inactivates JNK *in vivo*, this interaction was unaffected by mutation of the MKP-2 basic motif (Chen et al., 2001b). This suggested that JNK was also likely to interact with an additional site on MKP-2. This finding is supported by Slack et al. (2001) who reported that a basic motif on MKP-1, which is highly similar to that on MKP-2, did not contribute in the interaction between JNK and MKP-1. Interestingly, mutation of an LXL motif within a cytosolic MAP kinase phosphatase, hVH5/M3/6, blocks JNK mediated phosphorylation of hVH5 and inhibits the phosphatase activity of hVH5 toward JNK (Slack et al., 2001, Johnson et al., 2000).

Although the substrate specificity of MKP-2 *in vitro* is well established the specificity *in vivo* has not been clearly established. This is due to the lack of a MKP-2 deletion mouse model. In cellular studies, it has been shown that MKP-2 dephosphorylates JNK, but not ERK1/2 in UV-C or cisplatin stimulated HEK293 cells (Cadalbert et al., 2005). In these cells JNK translocates to the nucleus whilst ERK1/2 remains in the cytosol. Another study reported that suppressing MKP-2 by introducing siRNA MKP-2 into HEK 293 cells led to sustained JNK activation in response to H_2O_2 treatment (Teng et al., 2007). In addition, in EAhy926 cells, over-expression of MKP-2 had little effect on PMA-stimulated ERK phosphorylation despite its translocation to the nucleus, however, in the same cells nuclear activation of JNK was completely abolished (Robinson et al., 2001). This suggests the possibility that MKP-2 is far less active against ERK than against JNK *in vivo* under normal conditions of stimulation. This contrasts with another study that showed efficient ERK1/2 and JNK dephosphorylation in MKP-2 transfected NIH3T3 and Hela cells (Chu et al., 1996). Recently, the crystal structure of the catalytic domain of human MKP-2 (MKP-2C) has been identified; Twenty-four subunits of MKP-2C form a hollow, spherical complex in the crystal. All catalytically active sites are exposed to the solvent, whilst N-terminal residues comprising the MKB moieties would be buried in the sphere according to the direction of N-terminus of the catalytic domain (Jeong et al., 2009). However, these findings have not shed any additional light on the regulation of JNK by MKP-2.

MKP-2 is thought to play a role in a number of pathological processes, most prominently in cancer. Genetic studies show over-expression of MKP-2 correlates with pancreatic tumours metastasis (Yip-Schneider et al., 2001), multiple endocrine neoplasia (MEN), familial medulatory thyroid carcinoma (FMTC), papillary thyroid carcinoma (Hasegawa et al., 2008), and hepatomas survival (Yokoyama et al., 1997). In these studies the mRNA levels of MKP-2 was strongly expressed, suggesting a role for MKP-2 as a negative regulator in cancer proliferation. In contrast, other screening studies have implicated MKP-2 as tumour suppressor/cancer susceptibility gene (Cloos et al., 2006), linked to gene locus 1q8 which is frequently mutated in breast and prostate cancers (Armes et al., 2004).

In cell studies, MKP-2 has been shown to play important regulatory roles in various physiological and pathological processes, including senescence (Tresini et al., 2007).

MKP-2 expression is reported to have a dual function, inhibiting both human fibroblast cell proliferation and apoptosis. Hasegawa et al. (2008) showed that inhibition of MKP-2 attenuated the *in vitro* and *in vivo* proliferation of MKK-f cells, which was established from a mammary tumour developed in a RET-MEN2A transgenic mouse. This was mediated by the suppression of cyclin B1 expression, leading to misregulation of the cell cycle (Hasegawa et al., 2008). Furthermore, it has been reported that MKP-2 plays an important protective role in HEK293 cells following genotoxic stress including UV-C or cisplatin (Cadalbert et al., 2005). This study shows that MKP-2 is able to dephosphorylate and inactivate nuclear JNK and therefore reverse genotoxic triggered apoptosis. In addition, it has been reported that the basic motif of MKP-2 is essential to effectively protect Hela cells from cisplatin-induced apoptosis.

Taken together, these findings indicate that MKP-2 is able to play a role in many aspects of cellular function, in particular proliferation and apoptosis. However, more studies are clearly required to define the role of MKP-2 in processes such as immune function, inflammation and cardiovascular function.

1.4.1.1.3 PAC-1

PAC-1 was initially identified in human T cells stimulated by mitogenic agents phythohemagglutinin and phorbol 12-myristate 13-acetate (PMA) (Rohan et al., 1993). It is a 314 amino acid, nuclear, dual specificity phosphatase that is expressed mainly in T-cells and B-cells (Grumont et al., 1996). PAC-1 is regarded as one of the most regulated transcripts in activated immune effector cells (Jeffrey et al., 2006). Activation of the ERK1/2 pathway mediates PAC-1 expression (Rohan et al., 1993), whilst PAC-1 specifically dephosphorylates ERK1/2 and p38 MAP kinase *in vitro* with little activity towards JNK (Chu et al., 1996, Ward et al., 1994). Notably, whilst the interaction of the PAC-1 MKB domain with ERK1/2 enhances PAC-1 catalytic activity, its binding with p38 MAP kinase does not (Zhang et al., 2005). Surprisingly, in PAC-1 deficient macrophages stimulation with LPS leads to decreased ERK activity and an increased in JNK. This suggests that a loss of PAC-1 *in vivo* may decrease ERK1/2 by increasing JNK activity.

Studies have shown that PAC-1 regulates aspects of immune cell proliferation and differentiation. PAC-1 mRNA and protein show strong upregulation in activated human primary leukocytes, dendritic cells, macrophages and Th1 and Th2 cells following induction with LPS, PMA, cross-linking of CD3 or IgE cell surface receptors. Macrophages and mast cells from Pac-$1^{-/-}$ mice show impaired induction of IL-6, COX-2 and TNF-α gene during LPS stimulation (Jeffrey et al., 2006, Pulido and Hooft van Huijsduijnen, 2008). A recent study has found that transcription of PAC-1 is activated by p53 through a palindromic site in the PAC-1 promoter during apoptosis. PAC-1 over-expression increases sensitivity to apoptosis and suppresses tumour formation, suggesting that PAC-1 is required for p53-triggered apoptosis (Yin et al., 2003).

1.4.1.1.4 Type II Cytosolic MKPs

1.4.1.1.5 MAP Kinase Phosphatase-3 (MKP-3)

MKP-3 (DUSP6) was cloned from two sources; human brain cDNA by using an EST closely linked to the human MKP-1 (Groom et al., 1996), and from a rat brain cDNA library using RT-PCR (Muda et al., 1996a). Identification of a second partial cDNA clone (MKP-X) encoding an additional phosphatase, 76% identical to MKP-3, indicated the existence of a different subfamily of structurally homologous MAP kinase phosphatase genes. MKP-3 is cytosolic due to a single leucine rich NES and has activity towards ERK1/2 and ERK5 but not for p38 MAP kinase and JNK in mammalian cells (Mourey et al., 1996, Muda et al., 1996b, Kamakura et al., 1999, Groom et al., 1996). To date, MKP-3 is the only MKP that is found to dephosphorylate ERK5 (Kondoh and Nishida, 2007). As with the nuclear phosphatases, ERK1/2 binding to the MKB domain of MKP-3 increases its phosphatase activity (Muda et al., 1998, Camps et al., 1998b), however, ERK 2 binding to MKP-3 is not dependent on ERK phosphorylation as both wild type MKP-3 and its catalytically inactive form bind consistently to ERK2 (Farooq et al., 2001). Deletion of the KIM sequence from MKP3 results in a 135-fold reduction in ERK2 binding (Karlsson et al., 2004).

The mechanisms regulating MKP-3 expression and function are cell type dependent. Whilst MKP-3 has been shown to be constitutively expressed in some cell types such as skin fibroblasts (Groom et al., 1996), its induction is also mediated by growth factors

including nerve growth factors and basic fibroblast growth factor (Groom et al., 1996, Camps et al., 1998a, Reffas and Schlegel, 2000). This suggests that unlike MKP-1 and 2, MKP-3 is not stress inducible and not encoded by an immediate early gene. In P19 cells and MM14 muscle cells, MKP-3 mRNA is strongly induced at several stages of differentiation (Mourey et al., 1996, Reffas and Schlegel, 2000). In *Drosophila*, MKP-3 is critically involved in cell differentiation, development and gene expression controlled by ERK (Kim et al., 2004). In the developing mouse embryo, MKP-3 mRNA is observed in the presegmental paraxial mesoderm, limb bud and branchial arch mesenchyme and mid-brain/hindbrain isthmus (Dickinson et al., 2002a). As most of these have been reported as sites of FGF/FGFR signalling, this suggests that MKP-3 contributes to the regulation of FGF mediated ERK signalling during early development. Moreover, expression of MKP-3 was also found to be regulated by a maternal β-catenin and retinoic acid signalling (Echevarria et al., 2005, Smith et al., 2005).Whilst inhibition of MKP-3 expression induces apoptosis in the mesenchyme (Kawakami et al., 2003), mice lacking MKP-3 showed an excess perinatal mortality and developmental defects with skeletal dwarfism, effects likely due to enhanced FGFR signalling (Li et al., 2007).

1.4.1.1.6 MAP kinase-4 (MKP-4)

MKP-4 is homologous to other family members, including MKP-X (61% amino acid identity) and MKP-3 (57% amino acid identity) and contains two N-terminal Cdc25 homology domains as well as an extended active site motif characteristic of this gene family. MKP-4 displays a distinctive tissue distribution, among a large range of cell and tissue types mRNA was detected only in placenta, kidney, and embryonic liver, migrating muscles and insulin-response tissues (Dickinson et al., 2002b, Xu et al., 2003). MKP-4 is clearly a cytosolic protein with additional punctuates nuclear staining observed in a subset of cells (Muda et al., 1997). The MKP-4 gene locus was found to be Xq28 which is located on the long arm of chromosome X. This region of chromosome X has not been shown to be associated with any cell growth abnormalities (Muda et al., 1997).

Only MKP-4 inactivates ERK both *in vitro* and *in vivo*. This enzymatic selectivity, together with a distinct subcellular localization and highly restricted pattern of tissue expression, suggests a specific regulatory role for MKP-4. However, it has been shown

that MKP-4 reduces p38 MAP kinase activity and inhibits arsenite-mediated glucose uptake in adipocytes (Bazuine et al., 2004). This finding suggests that MKP-4 regulates insulin signalling negatively by p38 MAP kinase dephosphorylation. MKP-4 has been identified as candidate gene for the regulation of the stress responses involved in insulin resistance (Xu et al., 2003). In addition, gene targeting experiments in mice have recently reported that MKP-4 plays an important role in placental development and function (Christie et al., 2005).

1.4.1.2 Nuclear and Cytoplasmic MKPs

1.4.1.2.1 MAP kinase Phosphatase-5 (MKP-5)

MKP-5/DUSP10 was cloned by virtue of its binding to p38 MAP kinase in yeast two-hybrid screening of a human liver cDNA library (Tanoue et al., 1999). Specific *in vitro* substrates are p38 MAP kinase and JNK, with little activity towards ERK. The cDNA encodes a protein 482 amino acids in length with all the hallmarks of a MKP family member. However, there is an additional 150 amino acid N-terminal MAPK-binding domain, which is absent from other MKPs, that binds to p38MAP kinase and is essential for efficient p38 MAP kinase dephosphorylation (Tanoue et al., 2000, Theodosiou et al., 1999). Unlike other MKPs, which show strong catalytic activation upon substrate binding, MKP-5 activity is enhanced less than 2-fold in the presence of either p38 MAP kinase or JNK. The MKP-5 gene is well-conserved among mammals (Masuda et al., 2000) and humans and is localised to human chromosome 1q32 (Theodosiou et al., 1999), which is known to be missing in some renal carcinomas and breast tumours (Steiner et al., 1996, Benitez et al., 1997). Like other MKPs, its expression is highly regulated; it is induced by TNF-α, anisomyscin and osmotic stress, but not UV radiation or phorbol ester and there is some evidence of induction requiring p38 MAP kinase activation but not JNK.

A recent study has reported that vitamin D also enhances MKP-5 expression, resulting in inactivation of p38 MAP kinase (Tanoue et al., 1999, Nonn et al., 2006). However, another recent study (Zhang et al., 2004) suggests JNK as the key substrate for MKP-5 following the observation that MKP-5 is the closest mammalian homologue for *pluckered*, the main JNK phosphatase in *Drosophila*. As with MKP-1, MKP-5 knockout mice show no change in phenotype, and both TH1 and TH2 cells that lack MKP-5, have increased JNK activity

whilst p38 MAP kinase remains unchanged (Zhang et al., 2004). This finding again indicates that MKP-5 is specific for JNK *in vivo*. MKP-5 is located in the cytoplasm and the nucleus due to NLS and NES sequences and its subcellular localisation does not change even after stimulation (Theodosiou et al., 1999, Dickinson and Keyse, 2006).

1.4.1.2.2 MAP kinase Phosphatase-7 (MKP-7)

MKP-7/DUSP16 is the biggest molecule in the MKP family, it has a unique long C-terminal region that contains both NLS and NES. Like MKP-5, MKP-7 is localised mainly in the cytosol but shuttles to and from the nucleus by the effect of NLS and NES sequences (Masuda et al., 2001, Tanoue et al., 2001b). This changing subcellular distribution suggests that MKP-7 may function as chaperone protein. Expression of MKP-7 is regulated by JNK activation and MKP-7 binds to and dephosphorylates both JNK and p38 MAP kinase but not ERK1/2 (Masuda et al., 2001, Tanoue et al., 2001b). Whilst MKP-7 is able to bind to JNK2 and JNK3 (but not JNK1) and p38α and p38β (but not p38γ and p38δ) (Tanoue et al., 2001b), inactive mutants of MKP-7 caused increased JNK signalling suggesting MKP-7 is selective for JNK *in vivo* (Masuda et al., 2001). It has been reported that the half life of MKP-7 is short due to presence of a PEST, a poly peptide sequence enriched in proline glutamic acid, serine and threonine, located within the c-terminus (Matsuguchi et al., 2001) . It has been reported that MKP-7 binds to ERK1/2 through its C-terminal region, which is phosphorylated by ERK1/2 in response to several extracellular stimuli (Katagiri et al., 2005). This phosphorylation of MKP-7 by ERK1/2 inhibits the proteasomal degradation and increases the stability of MKP-7. Thus, while MKP-7 dephosporylates JNK and p38 MAP kinase but not ERK1/2, it is likely that the ERK1/2 pathway indirectly suppresses JNK or p38 MAP kinase by MKP-7 stabilization. MKP-7 also binds to JNK-interacting protein-1 (JIP-1) and β-arrestin 2, a scaffold protein for the JNK, through its C-terminal region (Willoughby et al., 2003, Willoughby and Collins, 2005).

1.4.1.3 Other MKPs

1.4.1.3.1 MAP Phosphatase -6 (MKP-6)

MKP-6/DUSP14 is not a typical MKP and is composed of a DSP domain alone. It was identified in a yeast-two-hybrid screen as a binding partner of the cytoplasmic tail of the

CD28 T cell co-stimulatory receptor (Marti et al., 2001). Like VHR (DUSP3), MKP-6 can dephosphorylate all three MAP kinases *in vitro* (Marti et al., 2001). It is expressed ubiquitously, but notably levels are higher in cell types such as human trachea, placenta, liver, heart and thyroid. Signalling from the CD28 receptor to the nucleus has been reported to involve the MAP kinase cascades, dominant negative MKP-6 enhances both ERK and JNK activation in response to CD28 activation. This finding suggests a specific effect for MKP-6 in ERK and JNK inactivation (Marti et al., 2001). However, the reason for this interaction between MKP-6 and CD28 receptor remains unclear but it is possible that CD28 works as anchor protein to keep MKP-6 in close proximity to sites of MAP kinase activation.

1.4.1.3.2 MAP kinase phosphatase-8 (MKP-8)

MKP-8/DUSP26 is also a truncated DUSP, approximately 20 kDa in size. MKP-8 localizes to the nucleus and has substrate specificity for p38 MAP kinase, and to a lesser extent ERK1/2 (Vasudevan et al., 2005). It was shown that MKP-8 binds to heat shock transcription factor 4 in a complex with ERK1/2, controlling transcription factor DNA binding (Hu and Mivechi, 2006). MKP-8 is highly expressed in childhood cancers such as retinoblastoma, neuroepithelioma, and neuroblastoma and has limited expression in normal tissues (Vasudevan et al., 2005). The gene locus of MKP-8 is within 8p11-12 on the long arm of chromosome X. This region is amplified in breast, urinary bladder, lung and ovary cancers. Yu and co-workers have presented evidence the MKP-8 is a key gene in this chromosomal region, and that over-expressed MKP-8 maintains p38 MAP kinase in an inactive state and prevents caspase-3-mediated apoptosis (Yu et al., 2007). Blocking MKP-8 expression using siRNA reduces tumour cell proliferation.

1.5 Adenoviruses Approaches to Treating Cardiovascular Diseases

1.5.1 Structure of Adenoviruses:

Adenoviruses (Ads) are non-enveloped double stranded DNA viruses under the family Adenoviridae that contain a linear genome of between 26 and 44 kilobases (kb), and they have inverted terminal repeats (ITR's) at the end of their genome (around 100-140bp in length) (Sharma et al., 2009). In 1953, Rowe et al. isolated a virus that caused primary cell culture degeneration, these cells were derived from adenoid tissues (Rowe et al., 1953).

Later, in 1956, Hilleman et al. isolated virus from respiratory secretions and showed that it induced cytopathatic effects in human cell cultures (Hilleman and Werner, 1954). The viruses isolated by the two distinct groups were later reported to be linked, and in 1956 these agents were termed adenoviruses (Enders et al., 1956). Since their discovery adenoviruses have been isolated from every class of vertebrates including fish, birds, amphibians and reptiles (Davison et al., 2003).

There are 51 human Ad serotypes; classified on the basis of their resistance to neutralisation by animal antisera from other know adenovirus serotypes. Type specific neutralisation results mainly from binding of the antibody to the hexon protein and the terminal knob of the fibre protein (Russell, 2009). Many of the human serotypes are associated with respiratory, gastrointestinal and ocular disease. Recently studies showed that species C adenoviruses persisted and could cause latent infections in approximately 80% of individuals investigated (Garnett et al., 2002). Of the human adenoviruses serotypes 2 and 5 are the most extensively studied, and they belong to same species (Bergelson et al., 1997, Tomko et al., 1997). Adenoviruses are non-enveloped icosahedral particles and have a diameter of 80- 120nm (Sharma et al., 2009). Each virion consists of a protein shell surrounding a DNA containing core. The protein shell (capsid) is composed of 252 subunits (capsomeres), of this 240 are hexons and 12 are pentons. Each penton contains a base, which forms part of the surface of the capsid, and a projecting fibre, whose length varies among the different serotypes. The capsid is comprised of seven known polypeptides (types II, III, IIIa, IV, VI, VIII and IX) (Russell, 2009), which are essential for the structure of the virus. The pentons (composed of a penton base and a fibre) are responsible for the attachment and internalisation of the adenovirus into the host cell. The N terminus of the fibre is attached to the penton base, whilst the C-terminus folds into a “knob”. The “knob” is required for binding of the virus to the host cell, but it is the penton base interacting with α integrins on the cell surface that triggers virus internalisation and membrane permeabilisation (Russell, 2009). An arginine-glycineasparartic acid (RGD) motif which is present within the penton base of many adenovirus serotypes serves as a recognition site for these intergrins (Leopold and Crystal, 2007).

1.5.2 Expression of CAR Receptors

The best known adenovirus receptor is the coxackievirus-adenovirus receptor (CAR), a type 1 trans-membrane glycoprotein, although this receptor is not used by all serotypes. It is present at intracellular junctions, such as the cardiac intercalated disc and the tight junctions of epithelial cells; however, its tissue distribution in human cell types is not well defined. CAR mRNA presents in a range of organs such as the heart, brain, pancreas, intestine, lung, liver and kidney (Zhang and Bergelson, 2005). Expression of CAR has been observed in HUVECs (Vincent et al., 2004) and ovarian cancer tissues (Kim et al., 2002). Upon binding to the receptor, interaction between the penton base and integrins causes the virus to be internalised in a clathrin-coated vesicle and transported to endosomes (Wickham et al., 1993, Sharma et al., 2009). Here the acidic pH of the endosome results in partial disassembly of the capsid and escape into the cytoplasm. From here the virion is transported to the nucleus via microtubules where the viral DNA associates with the nuclear matrix, via the terminal protein and transcription of the viral genome is initiated (Zhang and Arcos, 2005) (**Figure 1.7**)**.**

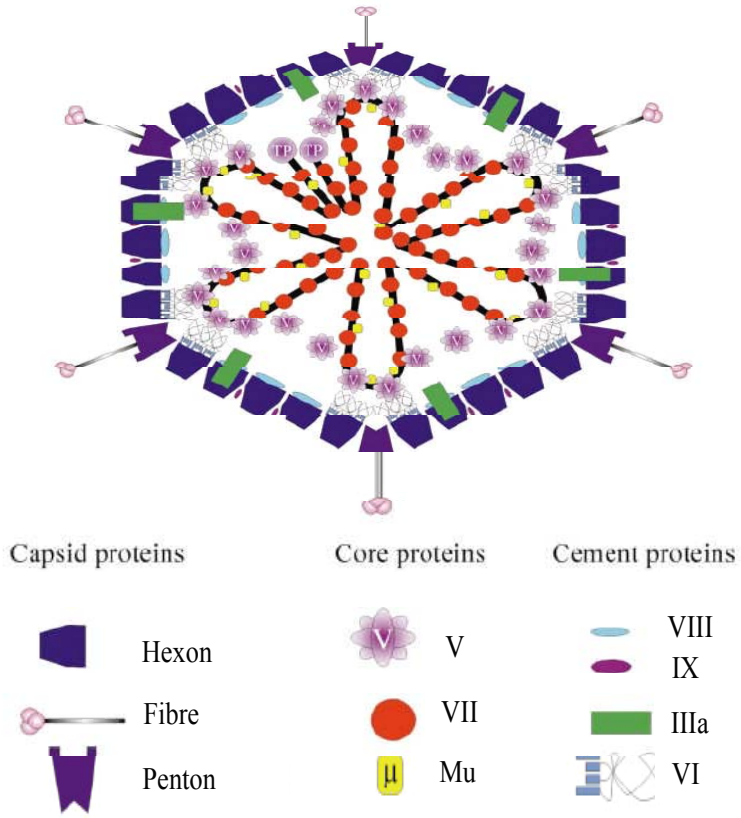


Figure 1.7: Structure of adenovirus. A schematic depiction of the structure based on cryo-electron microscopy and crystallography. (Russell, 2009)

1.5.3 Cardiovascular studies utilising Adenoviruses:

The properties of adenoviruses, in particular its high delivery efficiency and ability to target specific cells or tissues, has prompted its use in gene therapy for a number of cardiovascular diseases. These studies include a number of animal models of atherosclerosis, heart failure, hypertension, restenosis, graft failure and ischemic damage. Delivery of an adenoviral vector encoding endothelial NO synthase (eNOS) to the blood vessel wall, using phosphorylcholine (PC)-coated stents, showed an a significant acceleration of re-endothelialization in the eNOS-stented blood vessels as early as 14 days after surgery and persisted for up to 28 days in comparison with Adv.βgal-treated control vessels (Sharif et al., 2008). This correlated with the reduction in formation of neointima in the eNOS-stented vessels of hypercholesterolemic animals, as observed by histomorphometric analysis and quantitative coronary analysis (Sharif et al., 2008). Stent-based eNOS gene delivery could result in a reduction of in-stent-restenosis (ISR) whilst promoting endothelial regeneration, and this study was supported by previous works demonstrating that eNOS and prostacyclin synthase genes, which when delivered to the rat carotid artery after balloon injury, resulted in accelerated re-endothelialization (Cooney et al., 2007, Numaguchi et al., 1999), altered vascular reactivity (Kullo et al., 1997, Sato et al., 2000) and reduced neointimal formation (Cooney et al., 2007). Moreover, intravenous injection of mice with recombinant adenovirus encoding human apolipoprotein (apo) A1 resulted in over-expression of apo A1 and, consequently, increased HDL cholesterol to levels known to be protective in humans (Herz and Gerard, 1993).

Viral approaches to regulate kinase activity have also been utilised; infection with mutant IκB kinase 2 inhibited the response of endothelial cells to inflammatory stimuli (Oitzinger et al., 2001). In relation to apoptosis, adenovirus-mediated over-expression of human catalase (Ad-Cat) attenuated oxLDL mediated apoptosis in human arterial EC (HAEC), caused by inhibition of JNK phosphorylation and increased ERK activation (Lin et al., 2004). Consistent with this study, adenovirus mediated transfer of the human catalase cDNA was found to protect HUVECs from H_2O_2 triggered apoptosis (Erzurum et al., 1993). In addition, adenoviral expression of VEGF and other related endothelial cell growth factors or specific endothelial enzyme such as superoxide dismutase have also been

tested with positive effects upon endothelial integrity *in vitro* and *in vivo* (Modarai et al., 2008, Chu et al., 2003).

Delivery to the smooth muscle layer also profoundly influences proliferation and migration within the intima. Adenoviral mediated over-expression of tissue inhibitor of metalloproteinase-3 (Adv.TIMP-3) blocked smooth muscle cell proliferation and migration in human saphenous vein, an effect which was maintained following grafting (George et al., 2000). Wild-type TIMP-3 gene transfer inhibited neointima formation in human saphenous vein by modulating smooth muscle cell migration and inducting apoptosis (George et al., 2001). MAP kinases have also been identified as a target to prevent smooth muscle remodelling, gene transfer of ERK oligonucleotides or DN mutants of JNK and ERK have been found to significantly reduce intimal formation (Izumi et al., 2001, Liu et al., 2002). Also, infection with adenovirus containing dominant negative c-Jun gene in rat vascular smooth muscle cells (VSMC) has been shown to inhibit VSMC proliferation and neointimal hyperplasia (Yasumoto et al., 2001). Adenoviruses have gained status as gene delivery vectors for therapeutic and prophylactic applications. Gene therapy aims to tackle the ability of the genome in a clinical relevant setting, with a focus on diseases with unfulfilled clinical need. However, natural tropism of Ads usually does not always equal the therapeutic requirement. Numerous studies are developing strategies to ablate the native tropism of Ad vectors and introduce novel tropism towards target cells. Recently, cell-based delivery of Ad vector is emerging as a novel delivery approach in which cells infected with Ad *in vitro* carry the Ad vector to the target tissue (Power et al., 2007). This non-receptor mediated Ad transduction system prevents vector neutralisation by anti-Ad antibodies and elicits the desired therapeutic effect (Stevenson et al., 2007).

This work indicates that adenoviruses are relatively promiscuous in their ability to infect a wide range of species and tissues with efficient selective effects on the gene of questions. Studies using pharmacological inhibition are also promising, but they are compromised due to lack of selectivity and the transient nature of the inhibition (Koyama et al., 1998). Thus, adenoviral delivery may be of some advantage.

1.6 AIMS

MAP kinases often play opposing roles in the regulation of endothelial cell function. Whilst ERK and to a lesser extent p38 MAP kinase, are linked to endothelial survival, there is a growing evidence that JNK is linked to apoptosis in response to a number of stimuli stressful to the endothelium. Studies have been confirmed that JNK plays a crucial role in regulating endothelial cell apoptosis in response to a number of pro-apoptotic agents, and potentially influences the genesis of a number of diseases such as atherosclerosis, where dysfunction of endothelium is a feature. Therefore, strategies to inhibit JNK are essential in developing therapeutics targets in vascular disease. The MAP kinase phosphatases (MKPs) are a family of dual specific phosphates, which negatively regulate the magnitude and duration of ERK, p38 MAP kinases and JNK activation within different cellular compartment. There are at least 10 isoforms classified in terms of substrate specificity, subcellular location and mechanisms of regulation. Whilst these phosphatases have been studied extensively in cancer and immune function, much less attention has been directed at other disease conditions. Their functions within the cardiovascular system are largely unknown, however, if these phosphatases could be utilised to inhibit JNK specifically this maybe a useful clinical approach.

The aim of the present study was to investigate the effect of over-expressing one such DUSP, MKP-2, in regulating JNK induced apoptosis in HUVECs in response to TNF-α, in combination with an adenoviral construct encoding dominant negative IKKβ (Adv.DNIKKβ), H_2O_2 or serum deprivation. This utilised over-expression of MKP-2 as an experimental tool to study the underlying effect on extrinsic and intrinsic apoptotic pathways. The possible cross-talk between MKP-2 over-expression and the NF-κB pathway was also examined. Using human vascular smooth muscle cells, as another cellular model, the effect of MKP-2 over-expression on cell proliferation and apoptosis respectively was examined.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 General Reagents

All materials and reagents used were of the commercial grade possible and were obtained from Sigma Aldrich Chemical Company Ltd. (Pool, Dorset, UK) unless otherwise stated.

GE Healthcare Ltd (Buckinghamshire, UK)

Amersham™ Hybond™ -ECL nitrocellulose membrane

Roche diagnostics GmbH.

Dithiothreitol (DTT)

Lonza (Slough, UK).

Endothelial Cell Basal Medium-2 (EBM-2),

Endothelial Growth Media (EGM^{TM}-2) SingleQuots

Invitrogen (Paisley. U.K)

F-12 Nutrient mixture (Ham) 1X (21765)

Waymouth's MB752/1 (31220)

Bio-Rad Laboratories (Hertfordshire, UK).

Bio-Rad AG® 1-X8 Resin, pre-stained SDS-Page molecular weight markers.

Calbiochem (Nottingham, U.K).

SP600125 (JNK inhibitor)

Santa Cruz Biotechnology Inc. (CA, USA).

Recombinant human TNF-α

Boehringer Mannheim (East Sussex, UK).

Bovine serum albumin (BSA, Fraction V)

Corning Costar (Buckinghamshire, UK).

Nitrocellulose membranes

Corning B.V (Buckinghamshire, UK).

All tissue culture flasks, plates, dishes and graduated pipettes

Whatmann (Kent, UK).

Nitrocellulose Membranes, 3MM blotting paper

Invitrogen (Paisley. U.K).

Foetal calf serum (FCS), L-glutamine, Geneticin (G418), Medium 199 with Earls salts (M199), Minimal Essential Medium (x10), Non-essential amino acids, Opti-MEM® I Reduced Serum media, Penicillin/Streptomycin, Sodium Bicarbonate.

2.1.2 Reagents for Apoptosis

PE Annexin V Apoptosis Detection Kit 1(559763)(BD Pharmingen^{TM} ,Oxford, UK)
Mito Tracker Red CMXRos (M-7512) (Eugene, Oregon, USA)
Hydrogen Peroxide (H_2O_2) (Sigma-Aldrich, Poole, UK)

2.1.3 Adenoviruses

Clontech Laboratories, Inc. (Mountain View, CA, USA).
Adeno-X virus purification kit
Vector Biolabs (Philadelphia, USA)
Adv.MKP-2
NF-κB-Luciferase human adenovirus type 5 (Ad.NF-κB-Luc)
The following adenovirus were kindly provided by
Dr D. Goeddel (Tularik Inc., CA, USA)
Adv.DNIKKβ

2.1.4 Microscopy

Merck-Calbiochem (Nottingham, UK)
Mowiol
Molecular Devices Corp. (Downingtown, PA, USA)
MetaMorph Imaging Series software (version 7.0 or 7.6.4)
Nikon Instruments (New York, USA)
Nikon TE-300 Epifluorescence microscope
Nikon Eclipse TE2000-E inverted Epifluorescence microscope
40 x or 100 x oil-immersion Plan Fluor Objective Lens, NA=1.3
VWR International Ltd (Leicestershire, UK)
No. 0, (0.09-0.13 mm thick), circular glass 13 or 22 mm diameter coverslips.
0.8-1.0mm thick glass microscopy slides

2.1.5 Antibodies

Santa Cruz Biotechnology Inc. (CA, USA).

Mouse monoclonal anti-p-ERK (E-4)

Rabbit polyclonal anti-IKBα (C-21)

Rabbit polyclonal anti-MKP-2 (S-18)

Rabbit polyclonal anti-p38 (N-20)

Rabbit polyclonal anti-p-c-Jun $Ser^{63/73}$ (R)

Rabbit polyclonal anti-JNK-1 (FL)

Rabbit polyclonal anti-ERK-1 (K-23)

Rabbit polyclonal IKKα/β (H-470)

Rabbit polyclonal NF-κB p65 (C-20)

Invitrogen (Paisley, UK).

Rabbit polyclonal anti-p-JNK1 & 2 (44- 682G)

Rabbit polyclonal anti-p-p38 (44684-G)

Cell Signalling Technology, Inc. (New England Biolabs, UK)

Rabbit polyclonal p-p65 (Ser^{536})

Rabbit polyclonal cleaved Caspase-3 (Asp^{175})

Rabbit polyclonal cleaved Caspase-9 (Asp^{315})

Rabbit polyclonal PARP-1 (Asp^{214})

BD Pharminogen™ (BD Biosciences, UK)

Mouce monoclonal anti-cytochrome c (55-64-33)

Upstate Biotechnology Inc (Lake Placid, NY, USA).

H2AX (Ser^{139})

Jackson ImmunoResearch Laboratories Inc (PA, USA)

Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (111-035-144)

Horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG

Texas Red dye-conjugated AffiniPure donkey anti-goat IgG (705-076-147)

Fluorescein (FITC) conjugated AffiniPure donkey anti-Mouse IgG (715-095-130)

Fluorescein (FITC) conjugated AffiniPure donkey anti-Rabbit IgG (711-095-132)

2.1.6 Radiochemicals

PerkinElmer life science (Cambridge, UK)

γ [^{32}P]-ATP (3000 Ci/mmol^{-1})

2.2 CELL CULTURE

All cell culture work was performed in a class II cell culture hood under aseptic conditions. All the cells were grown in 75cm^2 flasks unless otherwise stated.

2.2.1 Human Umbilical Vein Endothelial Cells (HUVECs).

Cryopreserved Primary HUVECs (≥ 500,000 cells/vial) were purchased from Cascade Biologics. HUVECs were maintained in Endothelial Basal Media (EBM-2) supplemented with EGM-2 Single Quots (2% Foetal bovine serum, 0.2 ml Hydrocortisone, 2 ml hFGF-B, 0.5 ml VEGF, 0.5 ml R^3-Insulin like Growth Factor-1, 0.5 ml hEGF, 0.5 ml GA 1000, 0.5 ml Ascorbic Acid, 0.5 ml Heparin). (Concentrations not disclosed by the company). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2. Medium was changed every 2 days thereafter until cells became confluent.

2.2.2 Subculturing HUVECs

Cells were subcultured upon reaching approximately 90% confluency. The medium was removed and the cells washed twice with a sterile solution of 0.5% (w/v) trypsin, 0.2% (w/v) EDTA in phosphate buffered saline (PBS). The solution was then removed and the flask placed in an incubator at 37°C, 5% CO_2 and 95% air, for 2-5 min until cells began to change shape, indicating that they had begun to detach from the flask. The flask was then gently tapped, to encourage the cells to completely detach from the surface and then washed in EBM-2. Cells were then diluted three times with additional EBM-2 and transferred to fresh flasks (75 cm^2) or seeded into plates (12 or 6 wells). Cells were maintained at 37°C in an incubator and the media replaced every 2 days with full medium until they reached 90% confluency and ready for stimulation. Cells were stimulated with different reagents in full media.

2.2.3 Human Embryonic Kidney (HEK293)

Low passage human embryonic kidney (HEK) 293 cells were maintained in Minimal Essential (MEM) with Eagle's salts (GIBCO®, Invitrogen Ltd) supplemented with 10% (v/v) foetal calf serum (FCS), penicillin (250 units/ml), streptomycin (100 µg/ml), L-glutamine (27 mg/ml), 1x (v/v) non-essential amino acids and 0.375% (v/v) sodium

bicarbonate (all GIBCO®, Invitrogen Ltd). Cells were than incubated at 37°C in a humidified atmosphere with 5% CO_2 with media replaced every second day. At 90% confluence, the HEK 293 cells were passaged using 1x sodium sodium citrate (1x SSC) solution (8.78g NaCl and 4.41g sodium acetate) dissolved in 100 ml of distilled water then pH adjusted to 7.0. Cells were used for experimentation between passage 30 and 40.

2.2.4 Subculturing HVSMCs

HVSMCs were subcultured as described for HUVECs and the cells then washed in F-12 and waymouth's (50:50) media and transferred to fresh flasks (75 cm^2) or seeded into plates (12 or 6 wells). Cells were then maintained at 37°C in an incubator and the media replaced every 4-5 days until they reach 90% confluency and were ready for stimulation. Cells were stimulated with different reagents in 0.1% serum media.

2.3 WESTERN-BLOT ANALYSIS

2.3.1 Preparation of Whole Cell Extracts

Cells were exposed to appropriate agonists or vehicle for the relevant period of time and they were then placed on ice to stop the reaction. Cells were immediately washed twice with ice cold PBS before 200µl of pre-heated Laemmli's sample buffer (63 mM Tris-HCl (pH6.8), 2 mM $Na_4P_2O_7$, 5 mM EDTA, 10% (v/v) glycerol, 2% (w/v) SDS, 50 mM DTT, 0.007% (w/v) bromophenol blue) was added. The cells were then scraped and the chromosomal DNA sheared by repeatedly pushing through a 21 guage needle. The cells were then transferred to Eppendorf tubes and boiled for 4 min, to allow the proteins in the samples to be denatured, before storing at -20 °C until use.

2.3.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Gel kit apparatus was first cleaned in 70% ethanol before assembly, then distilled water added to check the glass plates were flush and not leaking. Resolving gels were prepared containing an appropriate amount of (7.5% (w/v), 9% (w/v), 10% (w/v), 11% (w/v) acrylamide: (N, N'-methylenebis-acrylamide (30:0.8), 0.375 M Tris (pH8.8), 0.1% (w/v) SDS and 10% (w/v) ammonium persulfate (APS)). Polymerisation was initiated by the addition of 0.05% (v/v) N, N, N', N'- tetramethylethylenediamine (TEMED). The

solution was poured between two glass plates assembled in a vertical slab configuration according to the manufacturer's instruction (Bio-Rad) and overlaid with 200 µl 0.1% (w/v) SDS. Following gel polymerisation the layer of 0.1% SDS (w/v) was removed and a stacking gel containing (10% (v/v) acrylamide: N, -methylenebis-acrylamide (30:0.8) in 125 mM Tris (pH6.8), 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate and 0.05% (v/v) TEMED) was poured directly on top of the resolving gel, and a teflon comb was immediately inserted into the stacking gel solution. After polymerisation was complete the comb was removed and the polyacrylamide gels were assembled in a Bio-Rad Mini-PROTEAN II™ electrophoresis tank, with both reservoirs filled with electrophoresis buffer (25 mM Tris, 129 mM glycine, 0.1% (w/v) SDS). Aliquots of samples (20-30 µg per lane) were then loaded into the wells using a microsyringe. A prestained SDS-PAGE molecular weight marker of known molecular weights was run concurrently in order to identify the polypeptide of interest. Samples were electrophoresed at a constant voltage of 120 V, until the bromophenol dye had reached the bottom of the gel.

2.3.3 Electrophoretic Transfer of Proteins to Nitrocellulose Membrane

The proteins separated by SDS-PAGE were transferred to nitrocellulose membranes by electrophoretic blotting following a standard protocol (Towbin et al., 1979). The gel was pressed firmly against a nitrocellulose sheet and assembled in a transfer cassette sandwiched between two pieces of Whatman 3 MM paper and two sponge pads. The cassette was immersed in transfer buffer (25 M Tris, 19 mM glycine, 20% (v/v) methanol) in a Bio-Rad Mini Trans-Blot ™ tank and a constant current of 300 mA was applied for 2 hr, whilst the tank was cooled by inclusion of an ice reservoir. The presence of SDS in the resolving gel confers a negative charge on the proteins so the cassette was oriented with the nitrocellulose towards the anode.

2.3.4 Immunological Detection of Protein

Following transfer of the proteins from the gel to the nitrocellulose membrane, the membrane was removed and any remaining protein blocked by incubation in a solution of 2% (w/v) BSA in NaTT buffer (150 mM NaCl, 20 mM Tris (pH 7.4), 0.2% (v/v) Tween-20) for 2 hr with gentle agitation on a platform shaker. The blocking buffer was then

removed and membranes incubated overnight with antiserum specific to the target protein appropriately diluted in NaTT buffer containing 0.2% (w/v) BSA. On the following day membranes were washed in NaTT every 15 min for 90 min with gentle agitation. The membranes were than incubated for a further 2 hr at room temperature with secondary horseradish peroxidase-conjugated IgG directed against the first immunoglobulin diluted to approximately 1:10000 in NaTT buffer containing 0.2% (w/v) BSA. After six additional washes in NaTT as described before, immunoreactive protein bands were detected by incubation in enhanced chemiluminescene (ECL) reagent for 2 min with agitation. The membranes were blotted onto a paper towel to remove any excess liquid. The blots were then mounted onto an exposure cassette and covered with cling film, then exposed to X-ray film (Kodak Ls X-OMAT) for the required time under darkroom conditions and developed using on X-OMAT machine (KODAK M35-M X-OMAT processor).

2.3.5 Nitrocellulose membrane stripping and reprobing

Nitrocellulose membranes processed by Western blotting were reprobed for the subsequent detection of other cellulose bound proteins. This involved stripping the membrane of any previous antibody using a stripping buffer (0.05 M Tis-HCl, 2% SDS, and 0.1 M of β-meracptoethanol). The membrane was incubated in 15 ml of stripping buffer for 60 minutes at 70^{o}C in an incubator/shaker (Stuart Science Equipment). The stripping buffer was discarded in a fume hood sink and the membrane washed three times with NATT buffer at 15 min intervals to remove residual stripping buffer. After the final NATT wash, membranes were then incubated overnight with primary antibody prepared in 0.2% BSA (w/v) in NATT buffer. At this stage the blots were ready for the immunological detection protocol (section 2.3.4).

2.4 PREPARATION OF NUCLEAR EXTRACTS

HUVECs were grown on 10 cm culture plates and exposed to vehicle or agonist for the appropriate time. After washing twice with 1 ml of ice cold PBS, cells were removed by scraping into 0.5 ml PBS and harvested by centrifugation at 13000 rpm for 1 min. Nuclear extracts were prepared as previously described (Schreiber et al., 1989). The

pellet was resuspended in 400 µl buffer 1 (10 mM Hepes pH 7.9, containing 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1mM DTT, 0.5 mM PMSF, 10 $\mu g ml^{-1}$ aprotinin, 10 $\mu g ml^{-1}$ leupeptin, 10 $\mu g ml^{-1}$ pepstatin) and incubated on ice for 15-30 min. 25 µl of 10% (w/v) NP-40 prepared in buffer 1 was added and the tubes vortexed at full speed for 10 sec. Insoluble material was then pelleted by centrifugation at 13000 rpm for 1 min and supernatants were removed and the pelleted material resuspended in 20 µl of buffer 2 (20 mM Hepes, (pH 7.9), 25% (v/v) glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1mM DTT, 10 $\mu g ml^{-1}$ aprotinin, 10 $\mu g ml^{-1}$ leupeptin, 10 $\mu g ml^{-1}$ pepstatin) and agitated at $4^{o}C$ for 15 min. Extracts were then sonicated on ice in a bath-type sonicator for 2 x 30 sec. Nuclear proteins were then recovered by centrifugation and the soluble nuclear extract collected and stored at $-80^{o}C$ until use.

2.4.1 Determination of Protein Concentrations in the Nuclear Extracts

Quantification of protein concentration was determined using the Bio-Rad™ protein assay kit based on the Bradford method. For each assay performed, a standard curve was prepared using dilutions of BSA (5-20 $\mu g ml^{-1}$) as a protein standard prepared in the appropriate buffer. Appropriate dilutions of the standards and samples were made in H_2O to a volume of 160 µl, to which 40 µl of dye reagent was added. Samples were pipetted into a 96 well micro-titre plate and left for 15 min at room temperature for colour to develop. Colour development was quantified at 595nm by a Dynex microtitre plate reader. The protein concentration of each sample was calculated from the standard curve.

2.5 IN VITRO KINASE ASSAY

2.5.1 Solid Phase c-Jun N-Terminal Kinase (JNK) Activity Assay

c-Jun N-terminal kinase activity was assessed in affinity precipitates of c-Jun bound to a recombinant truncated N-terminus of c-Jun ($c\text{-}Jun_{5\text{-}89}$) substrate immobilized on glutathione (GSH)-sepharose beads (Dai et al., 1995). Cells were stimulated for the required time and then the reaction was terminated by rapid aspiration and the addition of ice-cold phosphate-buffered saline (PBS). The cells were solubilized in JNK solubilization buffer containing (20 mM HEPES buffer (pH 7.7), 50 mM NaCl, 0.1 mM

EDTA, 0.1 mM Na_3VO_4, 0.1 mM PMSF, 10 $mgmL^{-1}$ aprotinin, 10 $mgmL^{-1}$leupeptin and 1% (w/v) Triton X-100). The cells were removed by scraping and placed in eppendorf tubes, vortexed briefly and left on ice for 30 min. The samples were centrifuged at 13,000 rpm at 4°C to remove cell debris and the lysates are mixed with GST-c-Jun beads overnight at 4°C. The beads were then pelleted by centrifugation at 13,000 rpm for 1 min and washed once in solubilization buffer and once in kinase buffer, (25 mM HEPES (pH 7.6), 20 mM $MgCl_2$) on the day; 5mM β-glyecrophosphate, 0.1 mM Na_3VO_4, 2 mM DTT was added. The beads were resuspended in 25 µl of kinase buffer and the reaction started by addition of 5 µl of 150 µm ATP and 1 µCi [γ^{32}P]-ATP to a total volume of 30 µl. The samples were agitated at 30°C for 30 min, the reactions were terminated by the addition of 10 µl of 4x Laemmli sample buffer. The samples were then boiled for 5 min and resolved by 11% (w/v) SDS-PAGE (as described in section 2.3.2). Gels were fixed for 30 min in a solution containing 20% methanol, 10% acetic acid and 70% H_2O, before being sandwiched between cellophane sheets and dried for approximately 90 min at 80°C by Hoefer Eazy-BreezeTM gel dryer Scientific Instruments, UAS). The incorporation of [γ^{32}P] into the substrate was detected by autoradiography. Kodak X-OMAT LS X-ray film exposed to the dried gels for 16 hours at -20°C in a spring loaded metal cassette and developed by a KODAK M35-M X-OMAT processor.

2.6 LUCIFERASE REPORTER ACTIVITY ASSAY

Luciferase encoding Adenovirus NF-κB was purchased from (Vector Biolabs Inc). HUVECs were grown to confluency in 6-well plates then infected with Adv.NF-κB-Luc for 40 hr prior to stimulation with TNF-α at the indicated time points. Stimulation was terminated by aspiration of medium, followed by washing once with cold PBS. Cells were scraped from the plates and transfered to 1.5 ml Eppendorf tubes and then spun at 13,000 rpm for 2 min. Then 100 µl of lysis buffer (25 mM Tris phosphate (pH 7.8), 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X, 15% (v/v) glycerol) containing 1 mM ATP, 1% (v/v) BSA and 0.2 mM luciferin substrate was added into each cuvette. The relative light units were measured using a luminometer (F12 luminometer, Berthold detection system).

2.7 FLOW CYTOMETRY ASSAY OF APOPTOSIS (FACS)

HUVECs or HVSMCs were grown in 6 well culture plates until they reached 50-60% confluence. Cells were infected for 40 hr then stimulated for a further 24 hr prior to analysis. Non-adherent cells were collected from the wells and placed in 15 ml tubes. The adherent cells were trypsinized and collected with adherent cells then centrifuged at 1000 rpm for 2 min. The pellet was then resuspended in 500 μL of 1x annexin binding buffer (10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl and 2.5 mM $CaCl_2$). Phycoerythyrin–Annexin V and 7-AAD (BD Bioscience, UK) were added to the cells according to the manufacturer's instructions, and the samples were read in a FACS scan flow cytometer using FACS Diva software (FACS scan, Becton Dickinson, Oxford, UK). The data were analysed using FACS Diva (Becton Dickinson) and RCS Express (De Novo Software, Thornhill, Canada) software. A total of 10,000 events were measured per sample. Gating was determined using PE–Annexin V FL-2 and 7–AAD FL-3 standards attached to beads (Becton Dickinson, Oxford, UK) and preliminary experiments were conducted using paraformaldehyde and serum deprivation to define apoptotic and necrotic populations as outlined by the manufacturer's instructions.

2.8 PREPARATION OF RECOMBINANT ADENOVIRUSES

2.8.1 Crude Adenoviral Lysates

Crude lysates of wild type mitogen activated protein kinase phosphatase-2 (Adv. MKP-2), β-galactosidase (Lac*Z*) and dominant negative inhibitory kappa β kinase (Adv.DN-IKKβ) viruses were generated in HEK 293 cells, by infecting a 75cm^2 flask with 0.75 μl of original stock virus. Flasks were incubated at 37ºC, 5% CO_2, 95% air for 5-7 days until the cytopathic effect had occurred and the cells had started to detach from the flask. Cells were then removed from the flask and subjected to centrifugation (1500 rpm for 5 min). The supernatant was removed and pellet was washed twice with PBS and centrifuged at 1500 rpm for 5 min. The collected pellet was then resuspended in HE buffer (10 mM HEPES pH 7.5, 1mM EDTA), frozen in liquid nitrogen and thawed in a 37ºC water bath. This was repeated a further two times. After the third cycle, cells were centrifuged at

1500 rpm for 5 min to pellet the debris and the supernatant, which constitute the crude adenoviral lysates, was collected in sterile tube and stored at -80ºC until required.

2.8.2 Generation and Purification of High-Titre Stocks of Adenovirus.

The high-titre stocks of adenovirus were generated by large scale amplification of the crude adenoviral lysates described in section 2.8.1. Twenty T175 flasks of HEK 293 cells were grown to approximately 60-70% confluency. The medium was then changed to 2% FCS medium (19 ml) before receiving 1ml of diluted virus media (100 µl crude adenoviral lysates added to 20 ml of HEK 293 medium). These flasks were incubated at 37ºC, 5% CO_2 until the cytopathic effect had occurred and cells had detached from the flask. The cells were then removed together with medium from the flasks and pelleted by centrifugation in a 50 ml centrifuge tube at 1500 rpm for 5 min. The supernatant was collected in a sterile centrifuge tube (around 100 ml of total supernatants) and stored at 4ºC until use. Cells were resuspended in 20 ml of supernatant and frozen in liquid nitrogen and thawed in a 37ºC water bath, this was repeated a further two times. Subsequently, cells were mixed by vortexing after each thaw. Following the third cycle, cells were pelleted by centrifugation at 1500 rpm for 5 min. Supernatant was collected in a sterile tube while the pellet was discarded. A BD Adeno-X virus purification kit from Clontech Laboratories was used for the adenovirus purification. The supernatant was subjected to filtering and then incubation with benzonase (25 units/µl) (50 mM Tris-HCl pH 8.0, 20 mM NaCl, 2 mM $MgCl_2$, 50% glycerol) at 37ºC for exactly 30 min. Meanwhile, 1x dilution buffer and 1x wash buffer were prepared by diluting the provided 5x buffers with sterile H_2O, according to the manufacturer's instructions. The benzonase treated filtrate was mixed with an equal volume of 1x dilution buffer and passed through BD Adeno-X syringe-Filter followed by washing with 1x washing buffer. Adenovirus was later eluted from the mega filter using the elution buffer and the elutate collected in sterile Eppendorf tubes and aliquots then stored at -80ºC until titration.

2.8.3 Titration of Adenovirus by End-Point Dilution

Titration of the end point dilution method was used to quantify virus production (Nicklin and Baker, 1999). Serial dilutions of the adenovirus were applied to a 96 well plate of HEK 293 cells that reached approximately 70-80% confluency **(Figure 2.1)**. The plate

was incubated at 37ºC, 5% CO_2 overnight after which the medium containing adenovirus was replaced with fresh medium. The plate was then incubated for 5-7 days at 37º C, 5% CO_2 until the cytopathic effect of the virus had reached a steady state. Wells containing plaques were counted and the titre of adenovirus stock in terms of plaque-forming units (pfu) was calculated as in the example shown below **(Figure 2.2)**.

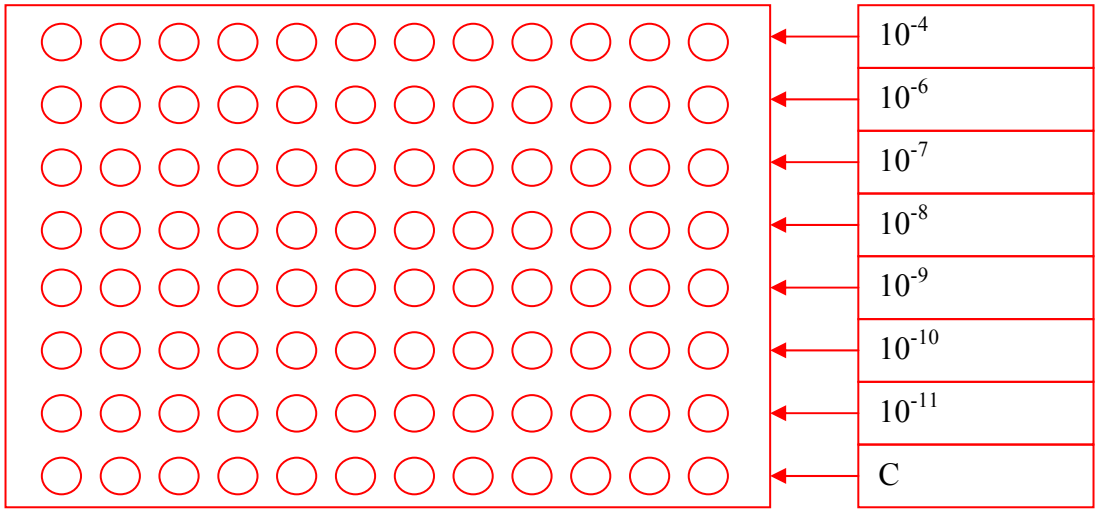


Figure 2.1: Layout of 96-well plate used for characterisation and titration of adenovirus. The numbers represent adenoviral dilutions. Cells are subcultured into all wells and adenovirus is added to the middle 10 wells on each row at the appropriate dilutions.

Number of wells containing plaques:

10^{-4}= 10/10= 100%

10^{-6}= 10/10 = 100%

10^{-7}= 10/10 = 100%

10^{-8}= 7/10 = 70%

10^{-9}= 2/10 = 20%

10^{-10}= 0

10^{-11}= 0

The proportionate distance =

$$\frac{\%\text{ wells positive above 50\%-50}}{\%\text{ wells positive above 50\% - \%wells positive below 50\%}}$$

$$= \frac{70-50}{70-20} = 0.67$$

Log ID_{50} = dilution factor at % wells positive above 50% + (proportionate x -1)

= -8 + (0.67x -1)

= -8.67

ID_{50} = $10^{-8.67}$

TCID (Tissue culture infectivity dose) $_{50}$/100 µl = $\frac{1}{10^{-8.67}} = 10^{8.67}$

$TCID_{50}$/1 ml = $10^{8.67}$ x dilution factor

= $10^{8.67}$ x10

= $10^{9.67}$

= 4.68 x10^{9}

1 TCID50 = **0.7 plaque forming units (9 pfu**), therefore final titre of adenovirus is

4.68 x 10^9 x 0.7= 2.21 x 10^9 pfu/ml

= 3.27 x 10^6 pfu/ µl

Figure 2.2: Example of calculation to establish titre of adenovirus.

2.8.4 Infecting HUVECs with Adenovirus

To establish the appropriate volume of adenovirus to add to cells in order to give an appropriate multiplicity of infection (MOI), cells were grown to approximately 50-60% confluency in either 6 or 12-well plates. The cell number was determined using a haemocytometer. An appropriate MOI of adenovirus (50-600 pfu/cell) was added to the cells and incubated for 40 hr in normal growth medium before stimulation. Later cells were exposed to vehicle control, TNF-α, H_2O_2 or serum deprivation for the indicated times.

2.8.5 Infecting HVSMCs with Adenovirus

Using a similar approach as for HUVECs, HVSMCs were incubated with adenovirus encoding MKP-2 gene (300 pfu/cell) or LacZ gene (300 pfu/cell) for 24 hr in normal growth medium and then cells were serum starved in 0.1% FCS for further 24 hr before stimulation with 10% FCS for the indicated times.

2.9 PROLIFERATION ASSAY

Confluent HVSMCs were detached with trypsin-EDTA, seeded on coverslips in 12 well plates (5,000 cells/well) in 10% FCS-(F12 + waymouth's medium) and allowed to attach for 24 hours. Cell were starved in 0.1% serum free media for 24 hr and then stimulated further for 24, 48, 72 hr with 10% FCS. Cultures were washed with PBS, and stained with Hematoxylin. The number of cells was determined by counting the number of cells in 10 random fields per each coverslip.

2.10 INDIRECT IMMUNOFLUORESCENCE MICROSCOPY

HUVECs or HVSMCs were grown to approximately 70-80% confluence in 12 or 6 well plates that contained glass coverslips and then stimulated as appropriate, coverslips were washed in ice cold PBS twice prior to fixation with 4% paraformaldehyde at room temperature for 10 min. After fixation, cells were washed twice with ice cold PBS and permeabilised with 0.3% Triton X-100. Slides were then washed twice in PBS and incubated with 1% BSA diluted in PBS (w:v) for 1 hr to prevent non-specific binding. After blocking, the coverslips were placed cell side down onto 30 µl of primary antibody

(1:100 for polyclonal antibody in PBS) overnight in the dark at 4ºC in a humidified chamber to minimise evaporation. The cells were washed three times in PBS and the coverslips placed cell side down onto FITC or Texas Red conjugated secondary antibody (1:200 dilution in PBS) for 1 hr at room temperature in the dark. The coverslips were then washed a further 2 times in PBS followed by incubation at room temperature in the dark for 5 min with 1 ml of PBS containing 100 ng/ml of DAPI. The coverslips were then washed with PBS twice and the coverslips were placed cell side down on to glass microscope slides containing 15 µl Mowiol. The microscope slides were then stored in the dark at 4ºC overnight to allow the coverslips to dry. Cells were visualised using a Nikon TE300-E upright epifluorescence microscope and imaged at x100 or x40 magnification using an oil-immersion Plan Fluor objective lens. Images were collected using a cool digital Cool Snap-HQ CCD camera (Roper Scientific, Photometrics, Tucson, AZ). MetaMorph Imaging Series 7.0 (Molecular Devices Corp., Downington, PA, USA) was used for control of image acquisition, processing and modification of all image data. The background average statistical correction editing function in MetaMorph was used to produce background corrected images. This was achieved by determining the average background level of fluorescence from regions of interest drawn adjacent to cells expressing fluorescence.

2.11 SCANNING DENSITOMETRY

Western blots were scanned on an Epson perfection 1640SU scanner using Adobe photoshop 5.0.2 software. The captured images were then normalised to a control and quantified using Scion Image (Scion Corp., Maryland, USA).

2.12 DATA ANALYSIS

All data shown were expressed as mean ± s.e.m and were representative of at least three separate experiments. The statistical significance of differences between mean values from control and treated groups were determined by either one-tailed Student's Unpaired t-test or a two tailed one-way analysis of variance (ANOVA) with Dunnet's post test ($P<0.05$ was considered significant).

CHAPTER 3

THE EFFECT OF MKP-2 OVER-EXPRESSION ON MAP KINASE-INDUCED APOPTOTIC PROTEIN EXPRESSION IN HUVECS: A POSSIBLE INTERACTION WITH THE NF-κB PATHWAY

3.1. Introduction

As outlined above in section 1.1.1, the position of endothelium assures its simultaneous and constant exposure to a wide range of stimuli, many of which have the potential to induce apoptosis. EC apoptosis is a well recognised feature of atherosclerosis, myocardial infarction, congestive heart failure and diabetes. A variety of stimuli can mediate pro-apoptotic protein expression such as TNF-α, H_2O_2 or serum deprivation (Wadgaonkar et al., 2004, Wang et al., 1999, Williams et al., 2006). These events are controlled by a variety of receptors and intrinsic signal transduction pathways.

Among the signalling pathways involved in regulating EC apoptosis is the MAP kinase cascade including; ERK, JNK and p38 MAP kinases (discussed in section 1.3.6.1). The MAP kinase family has been reported to play a major role in the control of many critical cellular processes including apoptosis, differentiation and proliferation. They exert their effects on the cell by the phosphorylation of a multitude of cellular proteins such as transcription factors, which subsequently change levels of gene expression. Of these kinases, JNK is believed to play a key role in EC apoptosis. These kinases are in turn controlled by reversible dual phosphorylation within their conserved threonine and tyrosine residues in their tri-peptide activation motif (Tyr-X-Thr) by specific MAP kinase phosphatses, MKPs. To date, 10 members of this family have been identified and their substrate specificities, subcellular distribution and regulation by extracellular stimuli were defined (Keyse, 2008, Kondoh and Nishida, 2007, Soulsby and Bennett, 2009).

MKP-2, one of the earliest MKPs to be identified, is expressed in a wide range of tissues as an immediate early gene in response to different stimuli and is localised within the nucleus (Chu et al., 1996, Misra-Press et al., 1995, Robinson et al., 2001). Originally, MKP-2 was found to display selectivity for JNK and ERK over p38 MAP kinase *in vitro*. Given this selectivity it maybe possible that over-expression of MKP-2 (Adv.MKP-2) may limit JNK activity and thus prevent apoptosis. Moreover, MKP-2 is known to play important regulatory roles in various physiological and pathological processes (section 1.4.1.1.2).

The aim of this chapter was firstly to characterize the TNF-α signalling pathways involved in pro-apoptotic protein expression in HUVECs. Secondly, an adenoviral encoding MKP-2 gene was utilised as a potential tool to reduce endothelial apoptosis by its possible inhibition of JNK phosphorylation and subsequent regulation of nuclear targets. Thirdly, the possible interaction between MKP-2 and the NF-κB pathway was investigated. In addition, Adenovirus encoding MKP-2 gene was also used to regulate apoptosis mediated by other stress agents, including H_2O_2 and serum deprivation.

3.2. CHARACTERISATION OF TNF-α SIGNALLING PATHWAYS IN HUVECS

3.2.1. TNF-α mediated activation of c-Jun N-terminal kinase (JNK) and c-Jun in HUVECs

The effect of TNF-α treatment on MAP kinase phosphorylation in HUVECs was investigated. Cells were stimulated with 10 ng/ml TNF-α over a period of 90 min. In HUVECs JNK phosphorylation was transient, reaching a maximum phosphorylation between 5 and 15 min, approximately 40 fold of basal values (fold stim. at 15 min = 43.72 ± 8.65, n=3) (Figure 3.1 **a**). The response rapidly declined thereafter and fell towards basal levels by 90 min. Cellular expression of JNK, as assessed by total JNK blotting, was not changed suggesting that the increase in density of the phosphorylated JNK bands was not due to any change in protein expression.

As a result of JNK activation the down stream transcription factor c-Jun, which is known to be a direct target for JNK was also found to be activated (Figure 3.2 **b**). An increase in c-Jun phosphorylation was observed after 15 min of TNF-α stimulation, approximately 3.5 fold of basal values and reached maximal phosphorylation by 30 min (fold stim. at 30 min = 4.95 ± 0.88, n=3). The response rapidly declined towards basal levels by between 60 and 90 min.

3.2.2. TNF-α mediated activation of ERK and p38 MAP kinase in HUVECs

Figure 3.2 shows p38 MAP kinase (panel **a**) and ERK (panel **b**) stimulated by TNF-α over a 90 min period. As with JNK, phosphorylation of p38 MAP kinase was transient;

peaking at 15 min (fold stim. at 15 min = 9.28 ± 2.89, n=3) and subsequently returning towards basal levels by 60 min. In contrast, phosphorylation of ERK was rather weak and give a smaller response which reached a maximum after 15 min (fold stim. at 15 min = 3.15 ± 0.90, n=3). This response rapidly dropped off and returned to basal levels by 60 min. Total p38 MAP kinase and ERK were unchanged, indicating equal protein loading.

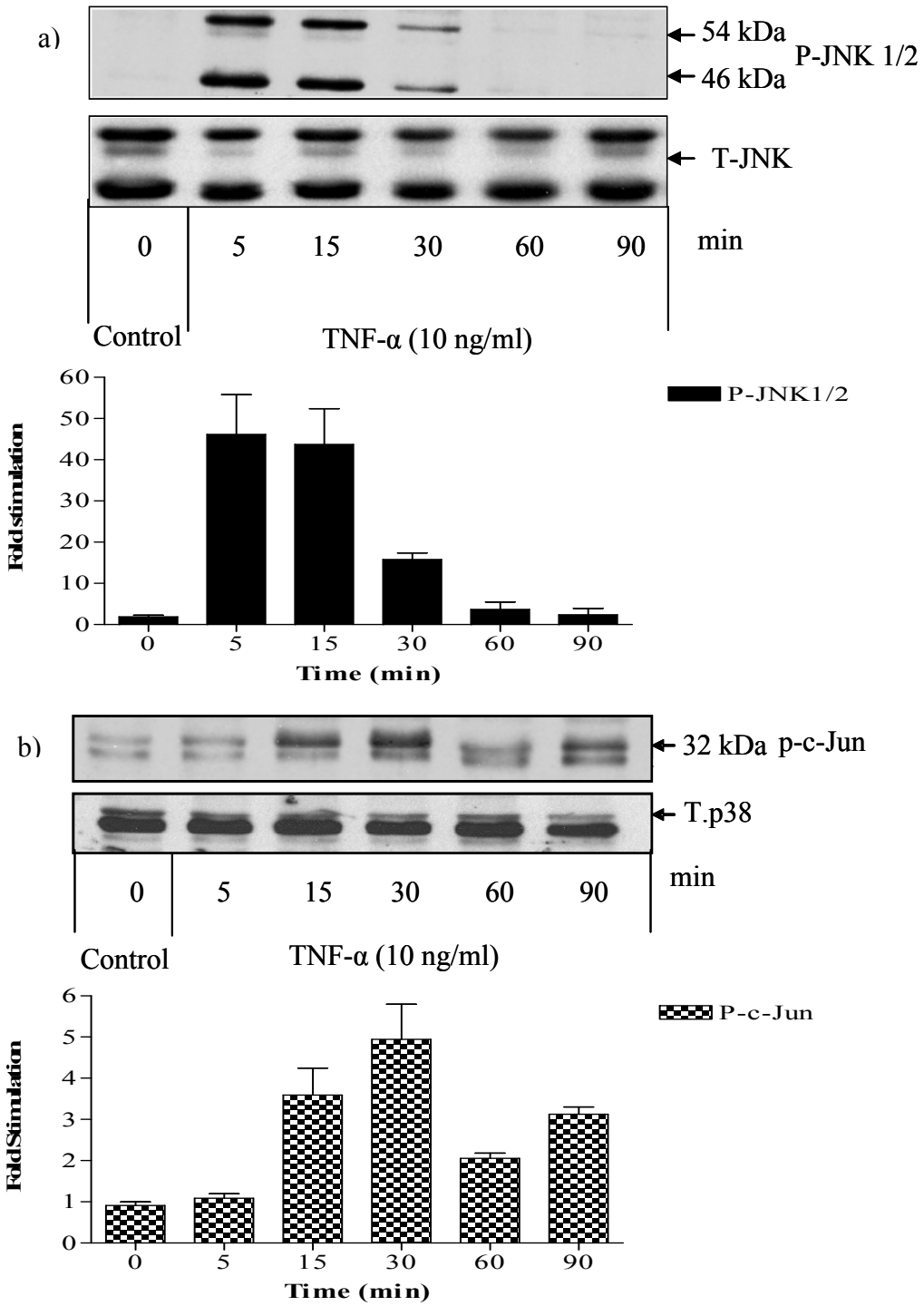


Figure 3.1: Time course of TNF-α-mediated JNK and c-Jun phosphorylation in HUVECs.

Cells were stimulated with TNF-α (10 ng/ml) as indicated. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for **a**) p-JNK (46/54 kDa) and T-JNK **b**) p-c-Jun (32 kDa) and T.p38 as outlined in section 2.3. The results are representative of 4 independent experiments. Blots were quantified for fold stimulation by scanning densitometry; each value represents the mean ± s.e.m.

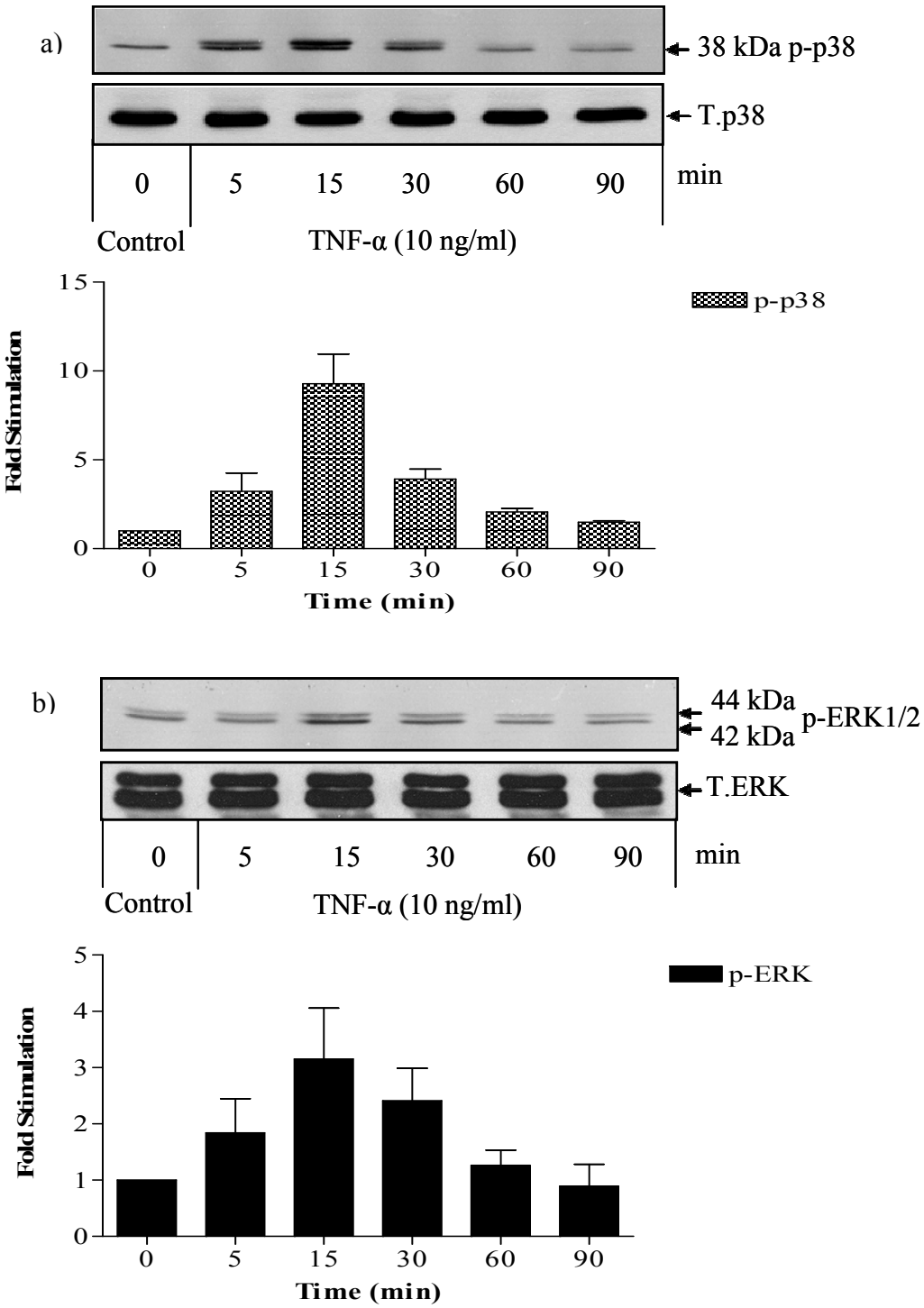


Figure 3.2: Time course of TNF-α-mediated p38 MAP kinase and ERK phosphorylation. HUVECs were stimulated with TNF-α (10 ng/ml) as indicated. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for **a**) p-p38 (38 kDa), T.p38 and **b**) p-ERK1/2 (42/44 kDa) and T.ERK as outlined in section 2.3. The results are representative of 3 independent experiments. Blots were quantified for fold stimulation by scanning densitometry; each value represents the mean ± s.e.m.

3.3. The effect of Adv.DNIKKβ on TNF-α induced IκBα loss in HUVECs

It has been suggested that IKK enzymes play an essential role in NF-κB activation (Perkins, 2007). Thus, in order to confirm the role of dominant negative IKKβ (Adv.DNIKKβ) in regulating endothelial cell apoptosis, an adenoviral construct encoding dominant negative IKKβ (Adv.IKKβ) was generated, purified in the lab (section 2.6) and employed as an experimental tool in this study. This construct has an alanine residue in place of lysine-44 within the kinase domain, therefore, rendering it catalytically inactive (Regnier et al., 1997).

3.3.1. Kinetics of TNF-α induced IκBα degradation in HUVECs

The effect of TNF-α on IκBα degradation is shown in Figure 3.3. TNF-α (10 ng/ml) stimulated a time-dependent loss in cellular IκBα which was maximum within 15 min post stimulation, less than 9% of control values (% control expression = 8.82 ± 5.97). Levels then gradually returned towards basal values by 90 min. Cellular expression of protein, as assessed by p-65 NF-κB blotting was unchanged.

Figure 3.4 shows the effect of Adv.DNIKKβ on TNF-α stimulated IκBα loss. Infecting cells with 300 pfu/ml of Adv.DNIKKβ resulted in a small but significant increase in basal expression of IκBα ($P<0.05$). When cells were exposed to TNF-α (10 ng/ml) for 15 min a distinct loss of IκBα expression was demonstrated (% inhibition = 77.75 ± 11.71, n=3). This response was not altered in the presence of LacZ (% inhibition = 80.56 ± 9.41, n=3), however, the extent of degradation was significantly decreased in the presence of Adv.DNIKKβ. Infection with 100 pfu/ml of Adv.DNIKKβ significantly reversed the inhibition of IκBα degradation mediated by TNF-α ($P<0.5$, Panel a). A similar effect was noticed at both 200 and 300 pfu/ml of Adv.DNIKKβ ($P<0.5$, $P<0.001$ respectively, panel b). Total p65 was unchanged, indicating equal protein loading.

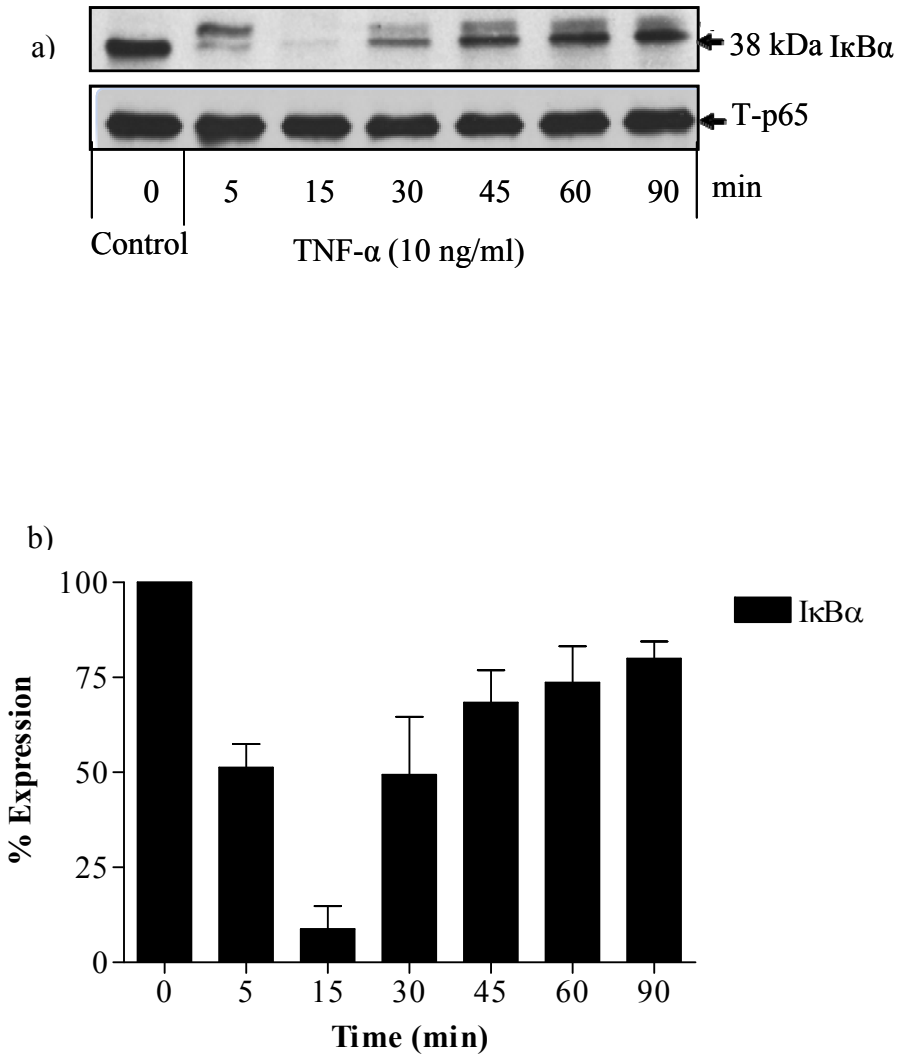


Figure 3.3: TNF-α-mediated IκBα loss in HUVECs. Cells were stimulated with TNF-α (10 ng/ml) for the times indicated. Whole cell lysates were prepared, separated by SDS PAGE and assessed for **a**) IκBα (38 kDa) and T-p65 as outlined in section 2.3. Blots were quantified for **b**) % expression by scanning densitometry; each value represents the mean ± s.e.m. The results are representative of 3 independent experiments.

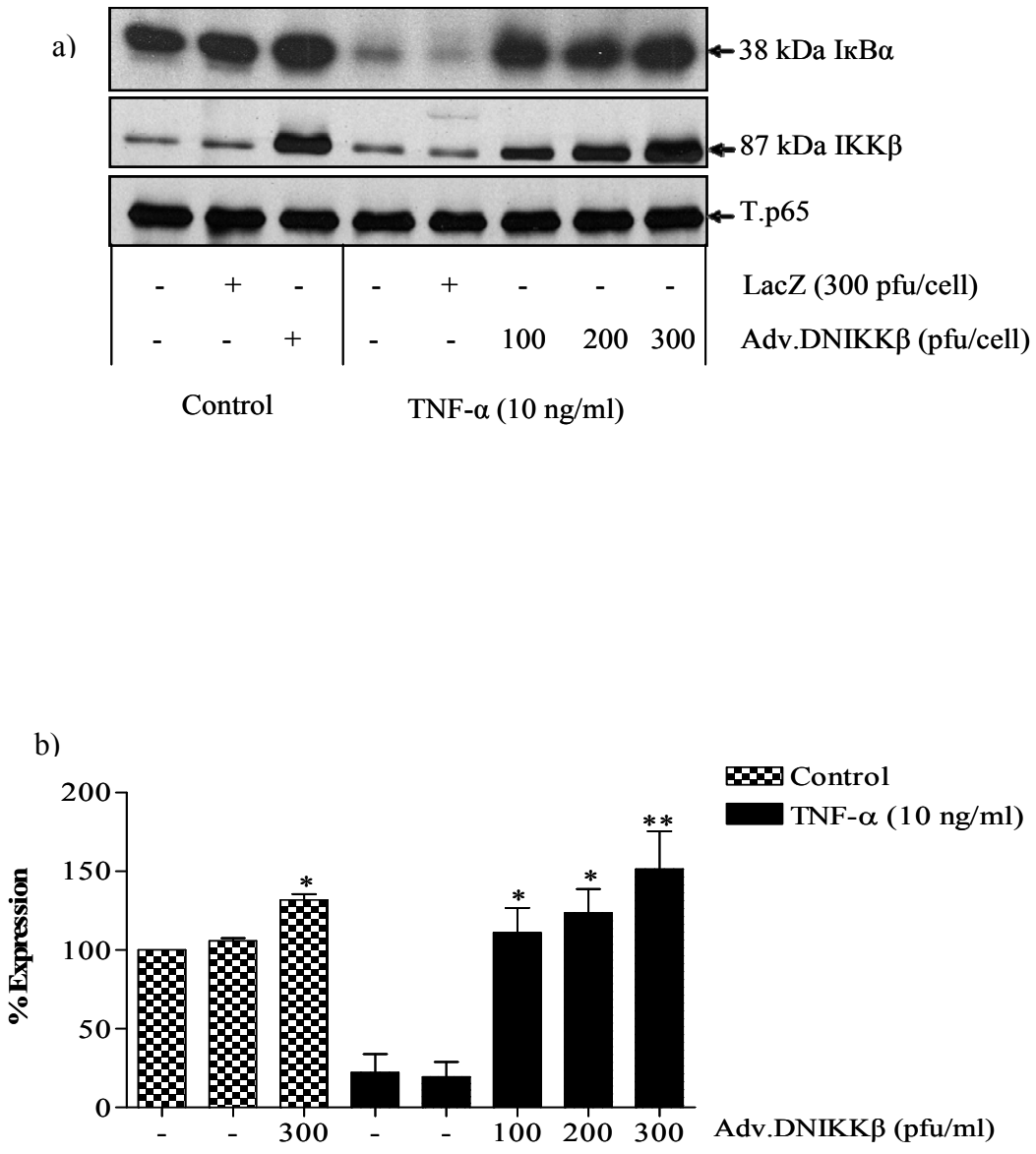


Figure 3.4: The effect of Adv.DNIKKβ upon TNF-α-mediated IκBα loss in HUVECs
Cells were infected with Adv.DNIKKβ for 40 hr prior stimulation with TNF-α (10 ng/ml) for 15 min. Whole cell lysates were prepared, separated by SDS PAGE and assessed for **a**) IκBα (38 kDa), IKKβ (87 kDa) and T.p65 as outlined in section 2.3. Blots were quantified for **b**) % expression by scanning densitometry; each value represents the mean ± s.e.m. The results are representative of 3 independent experiments. *P<0.05 compared with un-stimulated control, **P<0.01 compared with TNF-α stimulation.

3.3.2. The effect of Adenovirus encoding DNIKKβ on TNF-α-mediated JNK and c-Jun phosphorylation in HUVECs

A number of studies have shown that in HUVECs and other cell types JNK activation by TNF-α is strongly controlled by NF-κB. Blocking NF-κB activity however, by either knockout deletion of RelA or IKKβ or ectopic expression of IκBαM, a degradation-resistant variant of IκBα, markedly impairs the normal shutdown of TNF-α-mediated JNK signalling, thereby unveiling an additional, prolonged phase of JNK signalling. Such a prolonged phase of JNK induction has been implicated in activation of cell death (Luo et al., 2005, Papa et al., 2004b).

In order to reassess the kinetics of JNK phosphorylation following blockade of the NF-kB pathway, HUVECs were infected with Adv.DNIKKβ (300 pfu/cell) for 40 hr and then stimulated with 10 ng/ml TNF-α for up to 120 min. TNF-α alone has previously been shown to mediate a transient activation of JNK (section 3.2.1), however, in the presence of Adv.DNIKKβ, JNK phosphorylation was sustained for up to 120 min (fold stim. at 120 min = 18.08 ± 2.98, n=3) (Figure 3.5 **a**). The sustained phosphorylation of JNK correlated with the sustained phosphorylation of c-Jun (fold stim. at 120 min = 10.14 ± 2.82, n=3) (Figure 3.5 **b**).

3.3.3. The effect of Adenovirus encoding DNIKKβ on TNF-α- mediated activation of pro-apoptotic markers in HUVECs

Having established that Adv.DNIKKβ can result in prolonged JNK activation and nuclear c-Jun phosphorylation, we then examined the ability of TNF-α in the presence of Adv.DNIKKβ to initiate the activation of nuclear and cytoplasmic targets which may be involved in apoptosis including γ-H2AX, Poly (ADP-ribose) Polymerase-1 (PARP-1), caspase-3 and 9. HUVECs were infected with Adv.DNIKKβ (300 pfu/cell) for 40 hr and then stimulated with 10 ng/ml TNF-α for up to 24 hr (Figure 3.6 **a**). A slight increase in phosphorylation of γ-H2AX was observed at 0.5 hr (fold stim. at 0.5 hr = 7.49 ± 4.42, n=3), which returned back to basal level. Only at 24 hr, did γ-H2AX phosphorylation reach a significant level (fold stim. at 24 hr = 26.38 ± 6.01, n=3). A similar effect was

observed on another nuclear enzyme that responds to DNA damage and facilitates DNA repair, PARP-1 (Figure 3.6 **b**), with the response being approximately 20 fold by 24 hr (fold stim. at 24 hr = 19.48 ± 6.62, n=3). The phosphorylation of PARP-1 was represented by the presence of an 89 kDa cleaved PARP-1 form.

In addition, the effects of Adv.DNIKKβ on cytosolic pro-apoptotic targets were also investigated. Caspase-3 which is the most widely studied of the effector caspases and plays a key role in both the death receptor pathway and the mitochondrial pathway was activated by cleavage to 17 and 19 kDa active fragments (fold stim. at 24 hr = 35.27 ± 10.89, n=3) (Figure 3.7 **a**). A similar effect was observed for caspase-9. The phosphorylation of caspase-9 represented by the presence of a 35 and 37 kDa cleaved fragments was also observed at 24 hr with the response being approximately 20 fold of basal values (fold stim. at 24 hr = 20.83 ± 8.43) (Figure 3.7 **b**). The caspase-9 antibody also detected the total procaspase-9 form which is represented by a 47 kDa band.

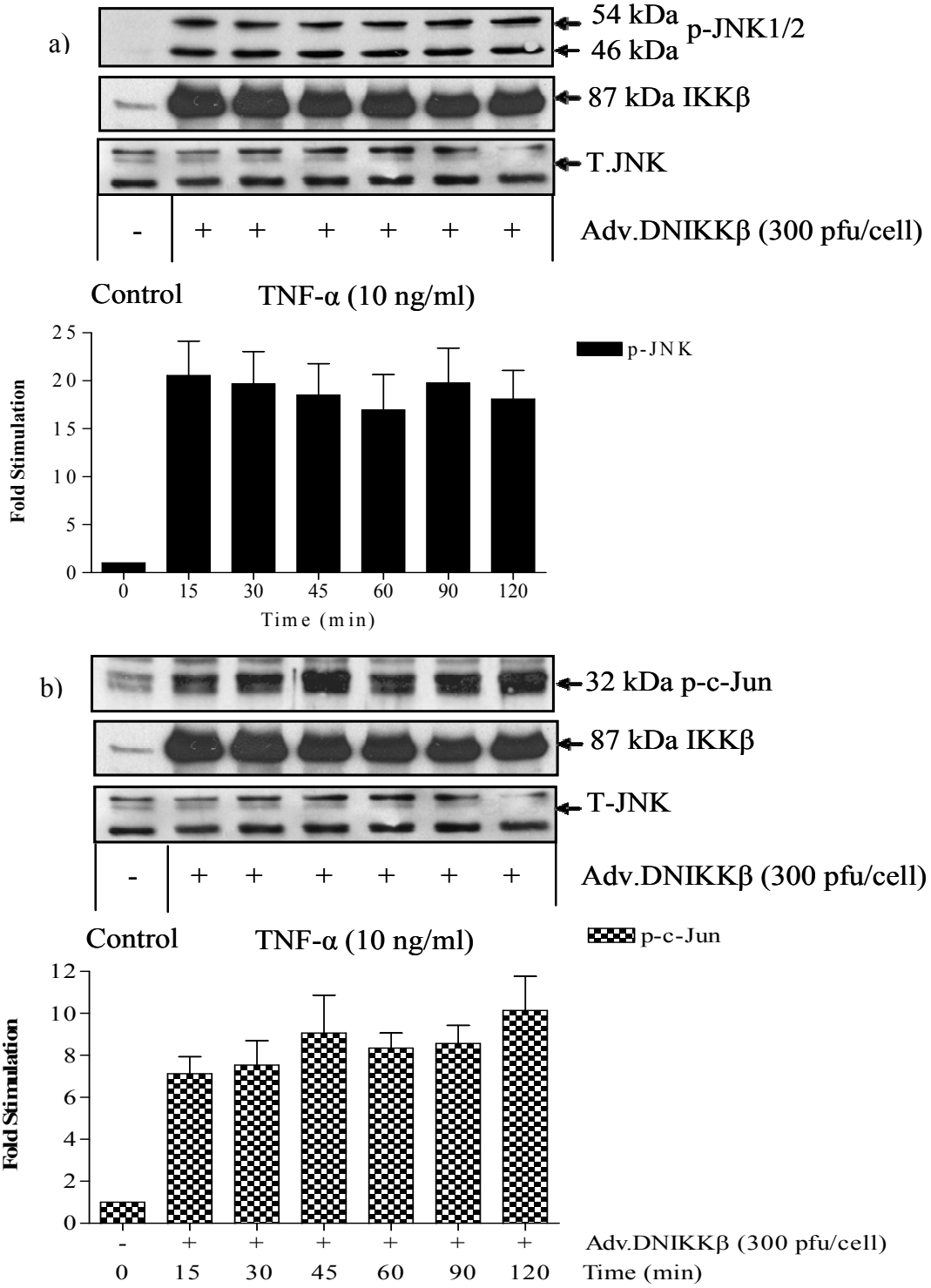


Figure 3.5: The effect of IKKβ inhibition upon JNK and c-Jun phosphorylation in TNF-α stimulated HUVECs: Cells were infected with Adv.DNIKKβ (300 pfu/cell) for 40 hr prior stimulation with TNF-α (10 ng/ml) for the times indicated. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for **a**) p-JNK1/2 (46/54 kDa), T-JNK and **b**) p-c-Jun (32 kDa) and T-JNK as outlined in section 2.3. The results are representative of 3 independent experiments. Blots were quantified for fold stimulation by scanning densitometry; each value represents the mean ± s.e.m.

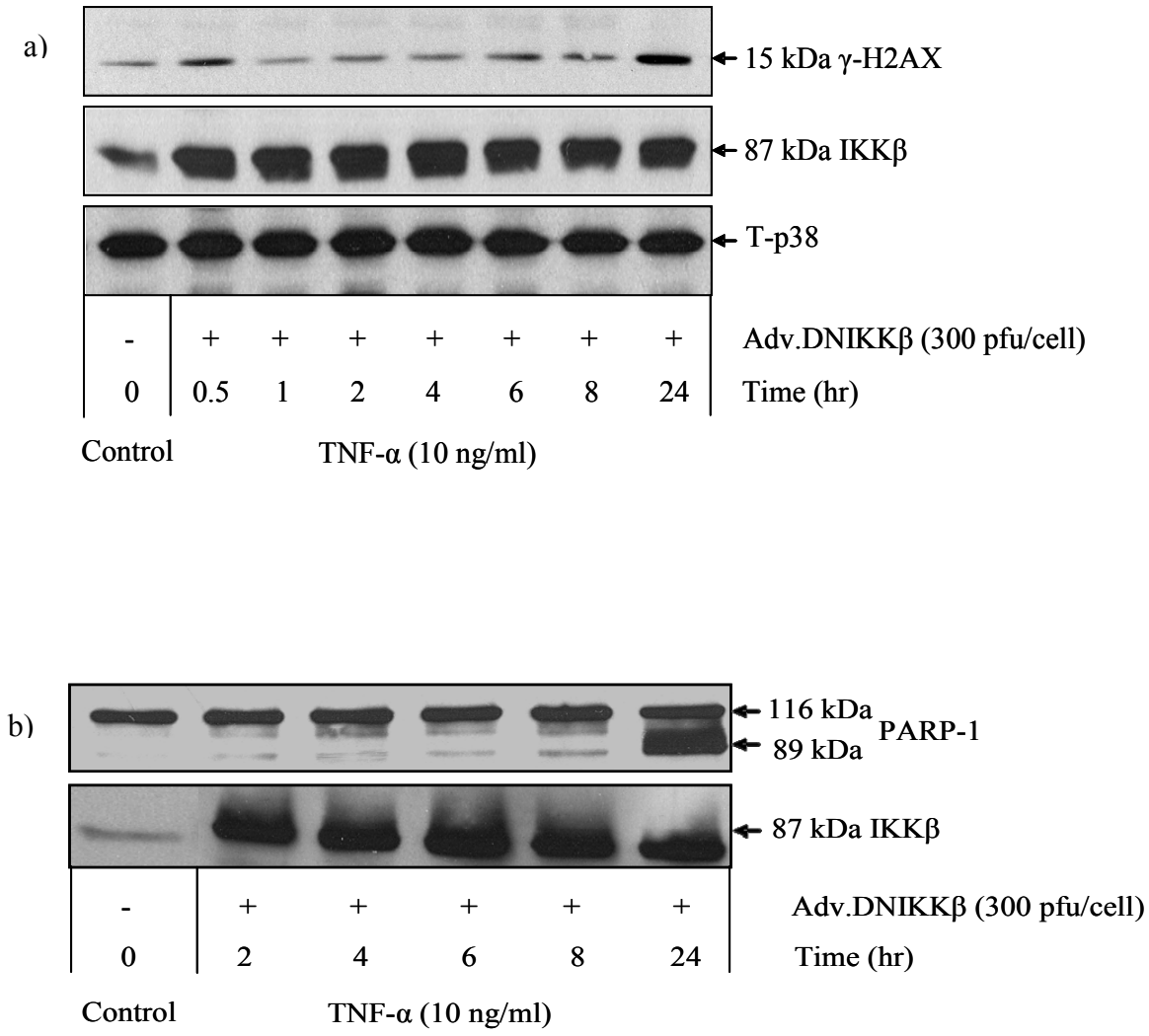


Figure 3.6: The effect of IKKβ inhibition upon H2AX phosphorylation (γ-H2AX) and PARP-1 in TNF-α-stimulated HUVECs: Cells were infected with Adv.DNIKKβ (300 pfu/cell) for 40 hr prior stimulation with TNF-α (10 ng/ml) for the times indicated. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for **a**) γ-H2AX (15 kDa), IKKβ (87 kDa), T-p38 and for **b**) PARP-1 (116 kDa), cleaved PARP-1 (35/37 kDa) and IKKβ (87 kDa) as outlined in section 2.3. The results are representative of 3 independent experiments.

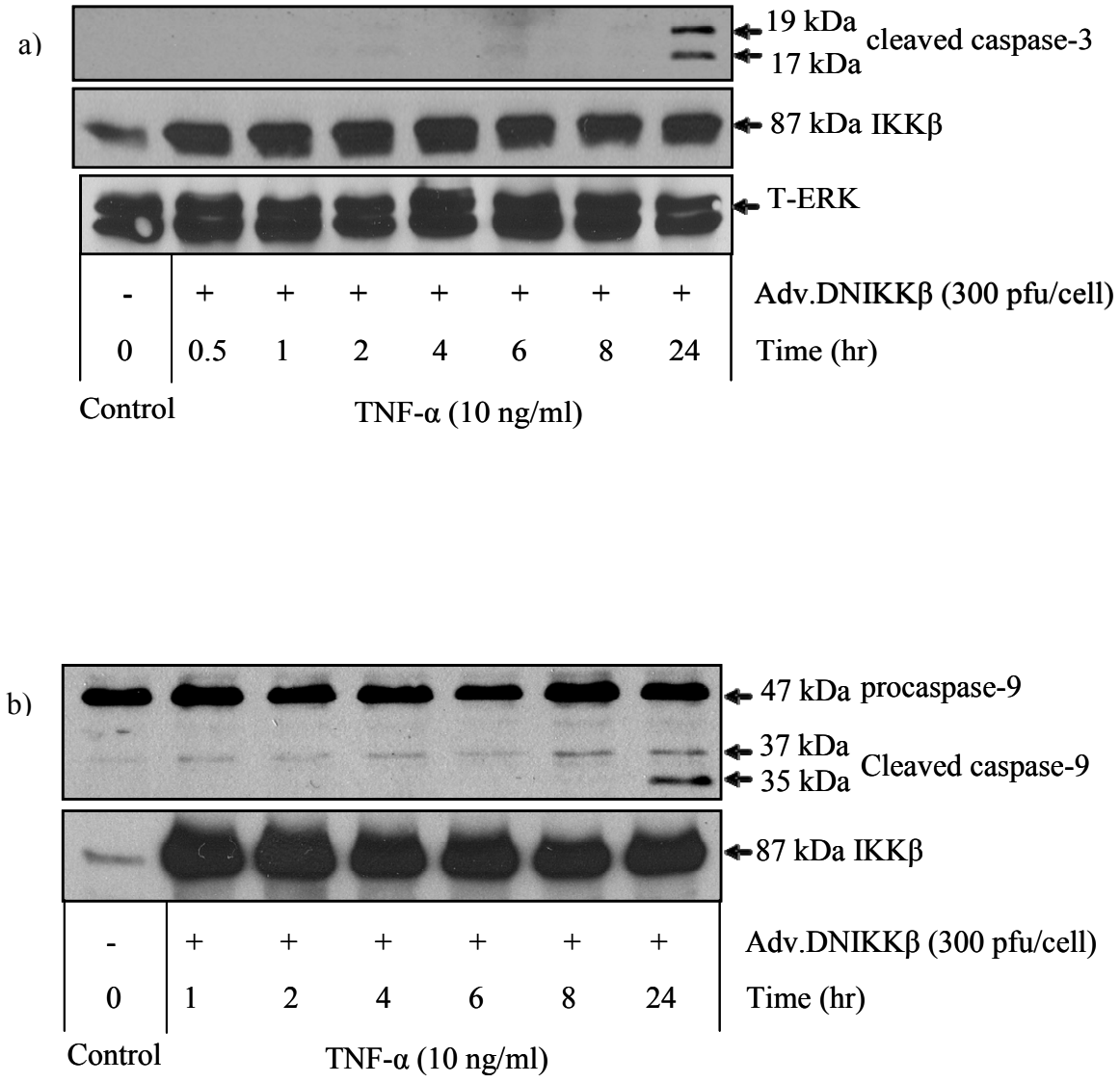


Figure 3.7: The effect of IKKβ inhibition upon caspase-3 and caspase-9 cleavage phosphorylation in TNF-α-stimulated HUVECs: Cells were infected with Adv.DNIKKβ (300 pfu/cell) for 40 hr prior stimulation with TNF-α (10 ng/ml) for the times indicated. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for **a**) cleaved caspase-3 (17/19 kDa), IKKβ (87 kDa) and T-ERK and for **b**) cleaved caspase-9 (35/37 kDa), pro-caspase-9 (47 kDa) and IKKβ (87 kDa) as outlined in section 2.3. The results are representative of 3 independent experiments.

3.4. THE EFFECT OF MKP-2 OVER-EXPRESSION ON PRO-APOPTOTIC PROTEINS STIMULATED BY TNF-α

In order to investigate the potential of MKP-2 to regulate JNK MAP kinase activation, an adenoviral construct encoding MKP-2, generated and purified in the laboratory (see chapter 2, section 2.4), was employed as an experimental tool in this study. The effect of this adenoviral construct on JNK signalling mediated pro-apoptotic protein expression in response to TNF-α was assessed. In some experiments the effectiveness of Adenovirus encoding MKP-2 gene was compared with the JNK inhibitor, SP600125. This JNK inhibitor is a reversible ATP competitive inhibitor which has been established in many cell types by inhibiting the actions of JNK mediated effects such as apoptosis, proliferation (Bogoyevitch and Arthur, 2008).

3.4.1. Sub-cellular localisation of MKP-2 in HUVECs

To confirm the nuclear localisation of MKP-2 in HUVECs, cells were infected with adenovirus encoding LacZ gene (300 pfu/cell) or increasing concentrations of Adenovirus encoding MKP-2 gene for 40 hr and then investigated using fluorescence microscopy (section 2.10) (Figure 3.8). No staining of MKP-2 was observed in cells infected with LacZ alone, however at increasing pfu MKP-2 gave enhanced cellular staining. At a pfu of 300, greater than 95% of the cells were infected and staining was strictly located to the nucleus. This was confirmed by nuclear staining with DAPI.

3.4.2. The effect of Adv.MKP-2 on TNF-α mediated JNK and c-Jun phosphorylation in HUVECs

Figure 3.9 shows the effect of increasing Adv.MKP-2 on TNF-α stimulated JNK phosphorylation. Stimulation of cells with 10 ng/ml TNF-α caused an increase in JNK phosphorylation (fold stim at 15 min = 36.71 ± 10.62, n=3) which was not changed by infection with LacZ adenovirus (300 pfu/cell fold stim. = 35.62 ± 10.09, n=3). Similarly, infecting cells with 50 pfu/ml of Adv.MKP-2 did not modify the TNF-α response, however, in response to 100 pfu/cell, phosphorylation was reduced by approximately

30% with further concentration dependent inhibition at 2 and 300 pfu/cell (% inhibition of 300 pfu/cell = 94.31 ± 2.13, P<0.001) (Figure 3.9). Total JNK was unchanged, indicating equal protein loading.

A similar effect was observed for c-Jun (Figure 3.10). Stimulation with TNF-α induced a significant increase of c-Jun phosphorylation (fold stim. at 30 min = 145 ± 10.81, n=3), which was slightly, but not significantly increased by infecting cells with LacZ (300 pfu/ml fold stim at 30 min = 156.76 ± 12.54, n=3). However, infection with Adv.MKP-2 (300 pfu/ml) significantly inhibited the phosphorylation of c-Jun by approximately 90 % (% inhibition = 91.78 ± 4.94, P< 0.001).

3.4.3. Inhibition of c-Jun phosphorylation induced by TNF-α using SP600125 in HUVECs

In order to determine the involvement of JNK in MKP-2 mediated effects, SP600125, an anthrapyrazole, identified as a novel pharmacological inhibitor of JNK (Bogoyevitch and Arthur, 2008), was used in comparison. HUVECs were pre-treated with SP600125 (10 μM) or vehicle (DMSO) for 1 hr before stimulation with TNF-α (10 ng/ml) for 15 min (Figures 3.11). SP600125 at concentration of 10 μM gave a significant inhibition of JNK phosphorylation mediated by TNF-α of approximately 80% (% inhibition= 82.66 ± 6.07, P< 0.01, n=3). The specificity of SP600125 was checked by investigating its effect on the endogenous protein expression of JNK, SP600125 appeared to have no effect (Figure 3.11). Since phosphorylation of c-Jun is a consequence of JNK activation, the effect of SP600125 was investigated. Similarly, c-Jun phosphorylation was significantly inhibited by pre-treatment with SP600125, giving a similar degree of blockade as for JNK (% inhibition at 30 min= 84.19 ± 2.27, P<0.01, n=3) (Figure 3.12).

3.4.4. The effect of MKP-2 over-expression on p38 MAP kinase and ERK1/2 phosphorylation mediated by TNF-α in HUVECs

Figure 3.13 demonstrates the effect of Adv.MKP-2 on TNF-α induced p38 MAP kinase and ERK1/2 phosphorylation. TNF-α stimulated a substantial increase in p38 MAP

kinase phosphorylation (fold stim. at 30 min = 7.40 ± 1.70, n=3) and a more moderate increase in ERK1/2 phosphorylation (fold stim. at 30 min = 3.67 ± 0.71, n=3). However, in response to 300 pfu/ml virus no significant change in either of those responses was observed (300 pfu/ml fold stim. at 30 min = 7.54 ± 2.13 and 3.72 ± 0.47, n=3, respectively). Total p38 and ERK1/2 were unchanged, indicating equal protein loading.

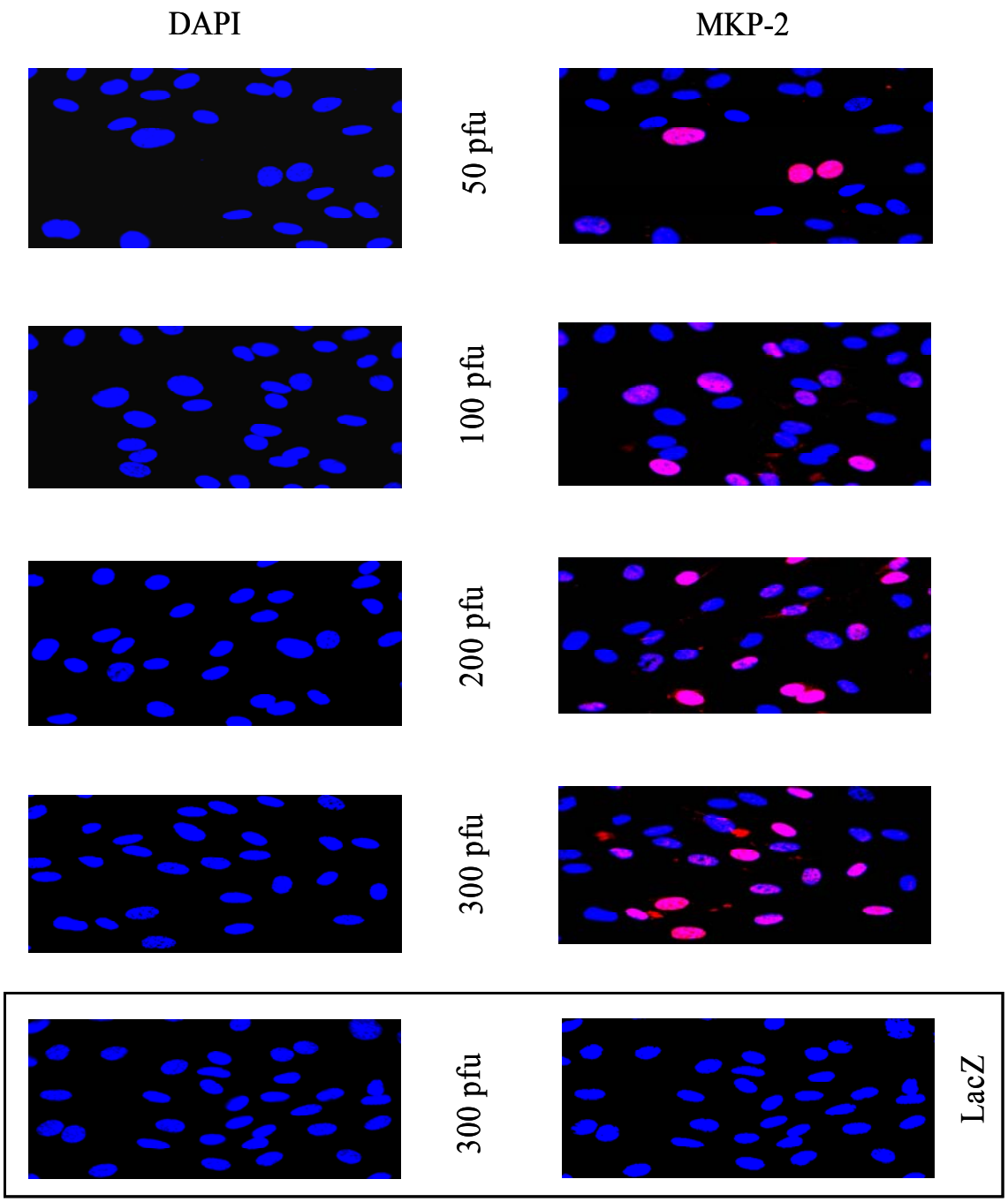


Figure 3.8: Sub-cellular localisation of MKP-2 in HUVECs. Cells were infected with different pfu/cell of Adv.MKP-2 for 40 hr then cells were fixed and stained for MKP-2 (1:100) as described in section 2.10. Nuclei were visualised by DAPI (blue) staining and MKP-2 sublocalisation was visualized using TRITC (red) staining using magnification of x40. The results are representative of 2 independent experiments.

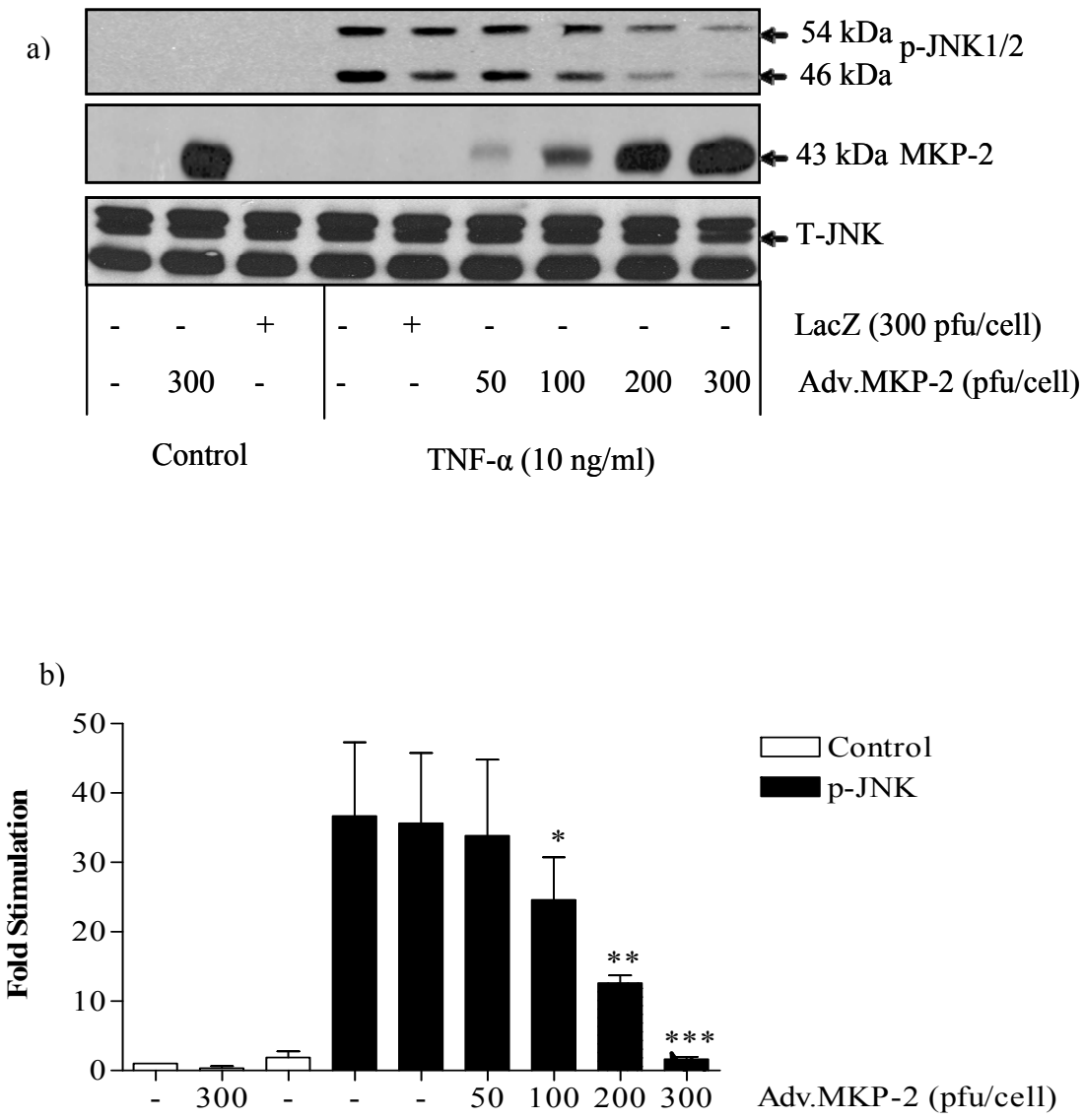


Figure 3.9: The effect of Adv.MKP-2 on TNF-α stimulated JNK phosphorylation in HUVECs: Cells were infected with Adv.MKP-2 for 40 hr prior stimulation with TNF-α (10 ng/ml) for 15 min. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for **a**) p-JNK(46/54 kDa), MKP-2 (43 kDa) and T-JNK as outlined in section 2.3. Blots were quantified for **b**) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. The results are representative of 4 independent experiments.*<0.05, **<0.01, ***<0.001 compared with stimulated control.

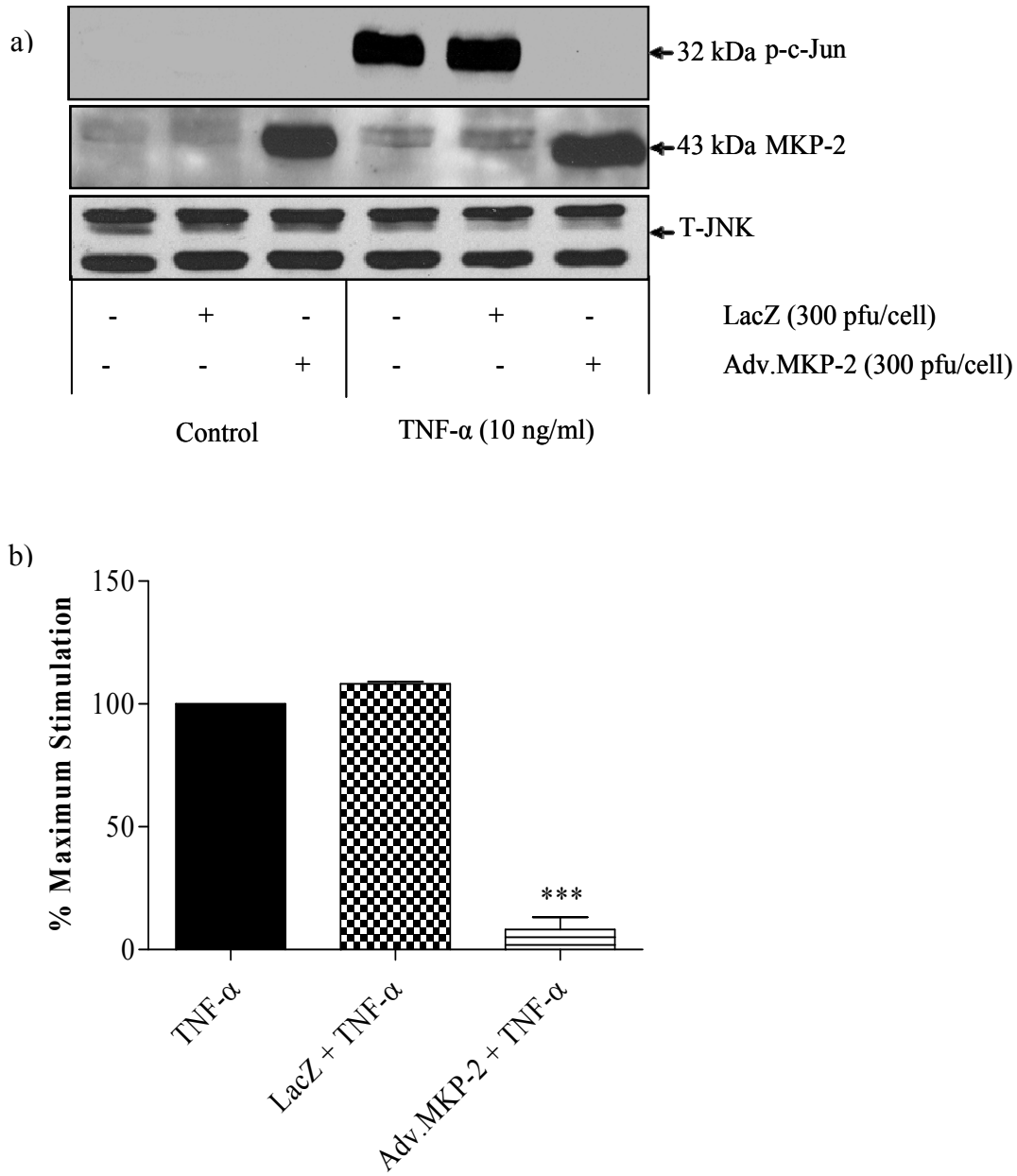


Figure 3.10: The effect of Adv.MKP-2 on TNF-α mediated c-Jun phosphorylation mediated in HUVECs: Cells were infected with Adv.MKP-2 for 40 hr prior stimulation with TNF-α (10 ng/ml) for 30 min. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for **a**) p-c-Jun (32 kDa), MKP-2 (43 kDa) and T-JNK as outlined in section 2.3. Blots were quantified for **b**) % maximum stimulation by scanning densitometry; each value represents the mean ± s.e.m. The results are representative of 3 independent experiments. ***P<0.001 compared with stimulated control.

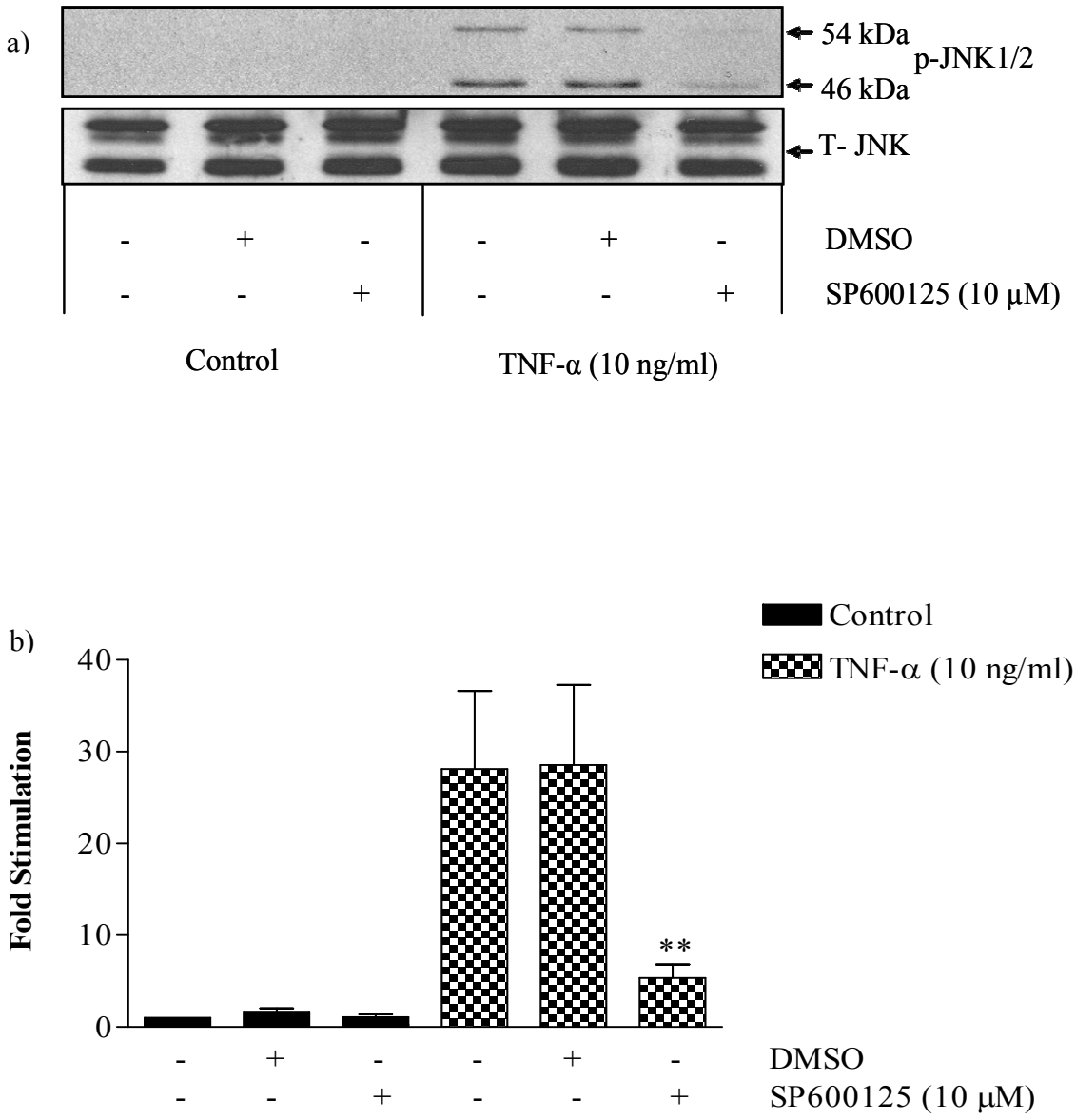


Figure 3.11: The effect of JNK inhibitor, SP600125 on TNF-α stimulated JNK phosphorylation in HUVECs: Cells were pre-treated with SP600125 (10 μM) for 1 hr before stimulation with TNF-α (10 ng/ml) for a period of 15 min. Whole cell lysates were prepared, separated by SDS PAGE, and assessed for **a**) p-JNK (46/54 kDa) and T-JNK as outlined in section 2.3. Blots were quantified for **b**) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. The results are representative of 3 independent experiments. **P<0.01 compared with agonist- stimulated control.

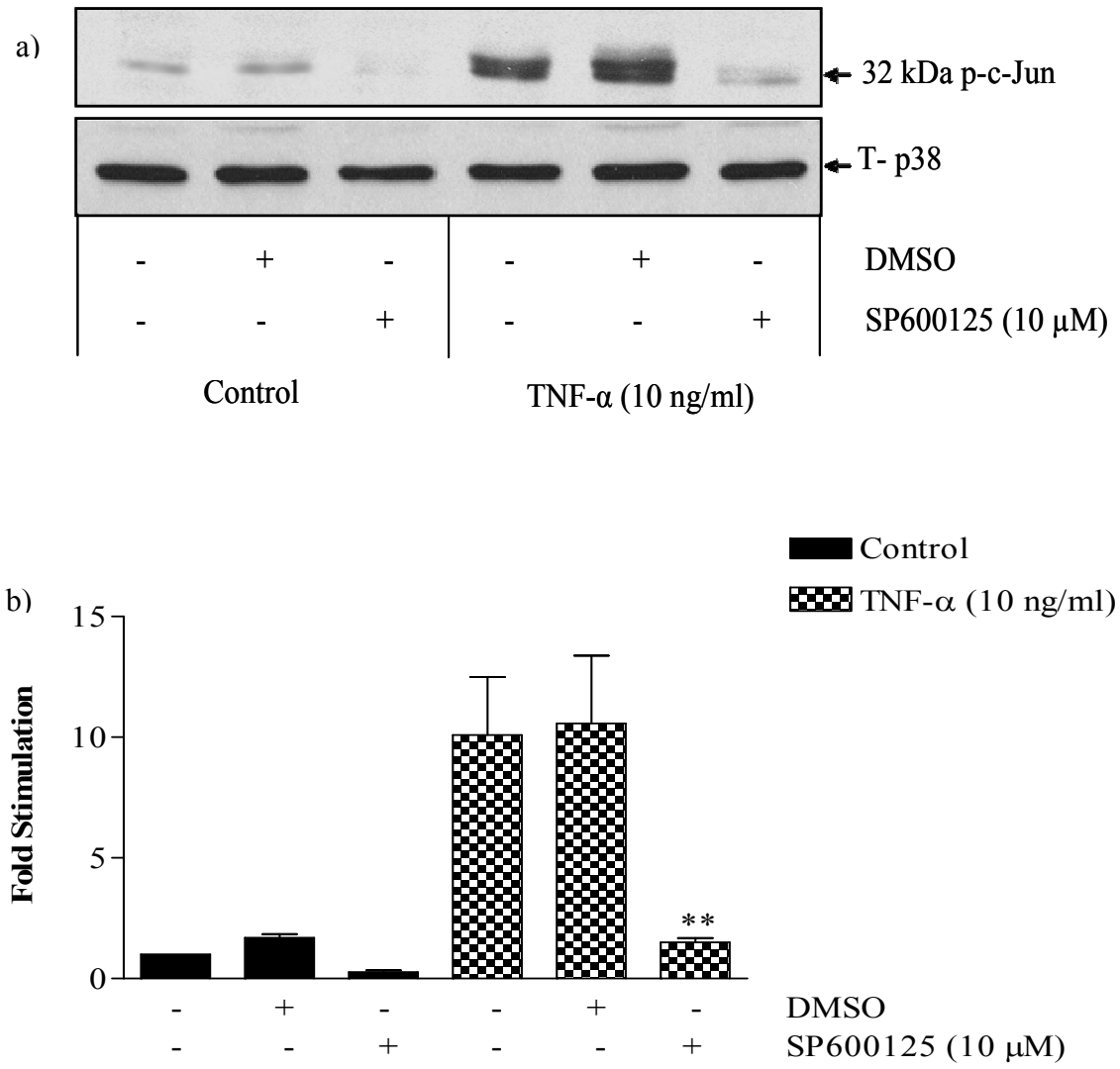


Figure 3.12: The effect of JNK inhibitor, SP600125 on TNF-α stimulated c-Jun phosphorylation in HUVECs: Cells were pre-treated with SP600125 (10 μM) for 1 hr before stimulation with TNF-α (10 ng/ml) for a period of 30 min. Whole cell lysates were prepared, separated by SDS PAGE, and assessed for **a**) p-c-Jun (32 kDa) and T-p38 as outlined in section 2.3. Blots were quantified for **b**) fold stimulation of p-c-Jun by scanning densitometry; each value represents the mean ± s.e.m. The results are representative of 3 independent experiments. **P<0.01 compared with agonist-stimulated control.

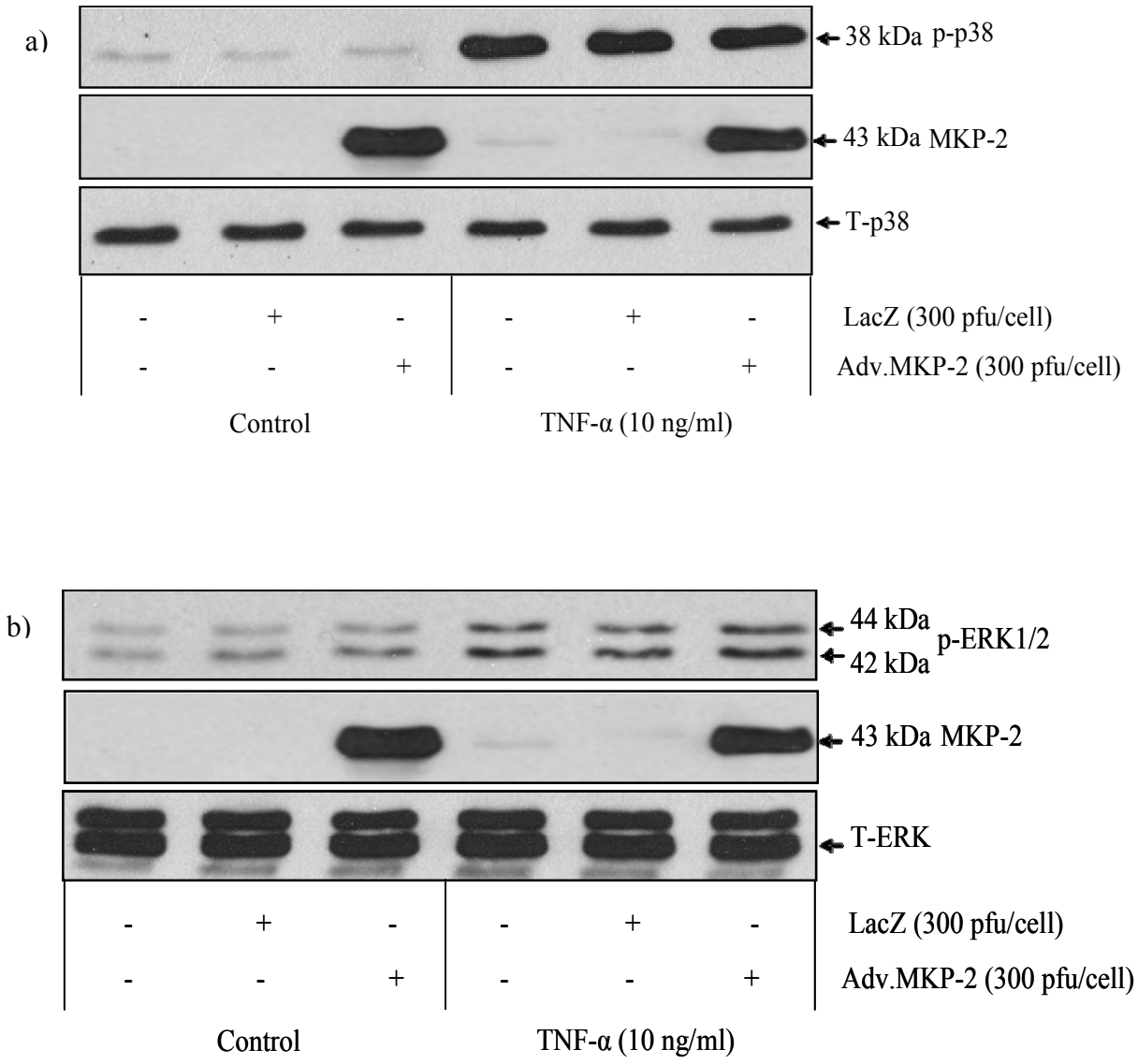


Figure 3.13: The effect of Adv.MKP-2 on p38 MAP kinase and ERK1/2 phosphorylation mediated by TNF-α in HUVECs: Cells were infected with Adv.MKP-2 for 40 hr prior to stimulation with TNF-α (10 ng/ml) for 30 min. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for **a**) p-p38, MKP-2 (43 kDa) and T-p38 and for **b**) p-ERK1/2 (42/44 kDa), MKP-2 (43 kDa) and T-ERK as outlined in section 2.3. The results are representative of 3 independent experiments.

3.4.5. The effect of MKP-2 over-expression on TNF-α mediated activation of pro-apoptotic markers in HUVECs

The effect of adenoviral encoding MKP-2 gene (Adv.MKP-2) on the phosphorylation of pro-apoptotic nuclear substrates was examined. As outlined above (section 3.3.3), TNF-α mediated phosphorylation of γ-H2AX was observed at 24 hr following infection with Advenovirus encoding DNIKKβ (300 pfu/cell) therefore, similar conditions were used to additionally assess the effect of Adv.MKP-2. TNF-α in combination with Adv.DNIKKβ increased phosphorylation of γ-H2AX by approximately 10 fold of basal values (fold stim. at 24 hr = 12.33 ± 2.36, $P<0.01$, n=3) (Figure 3.14). When the cells were co-incubated with Adv.MKP-2, phosphorylation of γ-H2AX was significantly inhibited reducing stimulated values by approximately 65% (% inhibition = 66.41 ± 5.22, n=3, $P<0.01$).

Similarly, Adv.MKP-2 had a marked inhibitory effect on PARP-1 cleavage. TNF-α in combination with Adv.DNIKKβ gave substantial cleavage of 116 kDa precursors to give the 89 kDa fragment (Figure 3.15). Whilst LacZ without effect, cleavage was almost completely reversed following co-incubation of cells with Adv.MKP-2. For both effects this was not due to any difference in the relative expression of both Adv.MKP-2 and Adv.DNIKKβ following co-infection, which was comparable.

3.4.6. The effect of SP600125 on TNF-α-stimulated H2AX phosphorylation (γ-H2AX) in HUVECs

Several studies have shown that γ-H2AX is a new JNK substrate that is phosphorylated in apoptotic cells (Lu et al., 2006). Therefore, to confirm the specificity of Adv.MKP-2, the effect of the JNK inhibitor SP600125 on γ-H2AX phosphorylation in response to TNF-α was also assessed. As Figure 3.16 illustrates, infecting cells with Adv.DNIKKβ (300 pfu/cell) for 40 hr prior to stimulation with TNF-α for further 24 hr period caused an increase in γ-H2AX phosphorylation (fold stim. at 24 hr = 27.88 ± 6.27, n=3). This response was significantly decreased upon pre-treatment of the cells with 10 μM of SP600125, γ-H2AX phosphorylation fell by approximately 50% (fold stim. at 24 hr = 15.98 ± 3.90, $P< 0.05$, n=3).

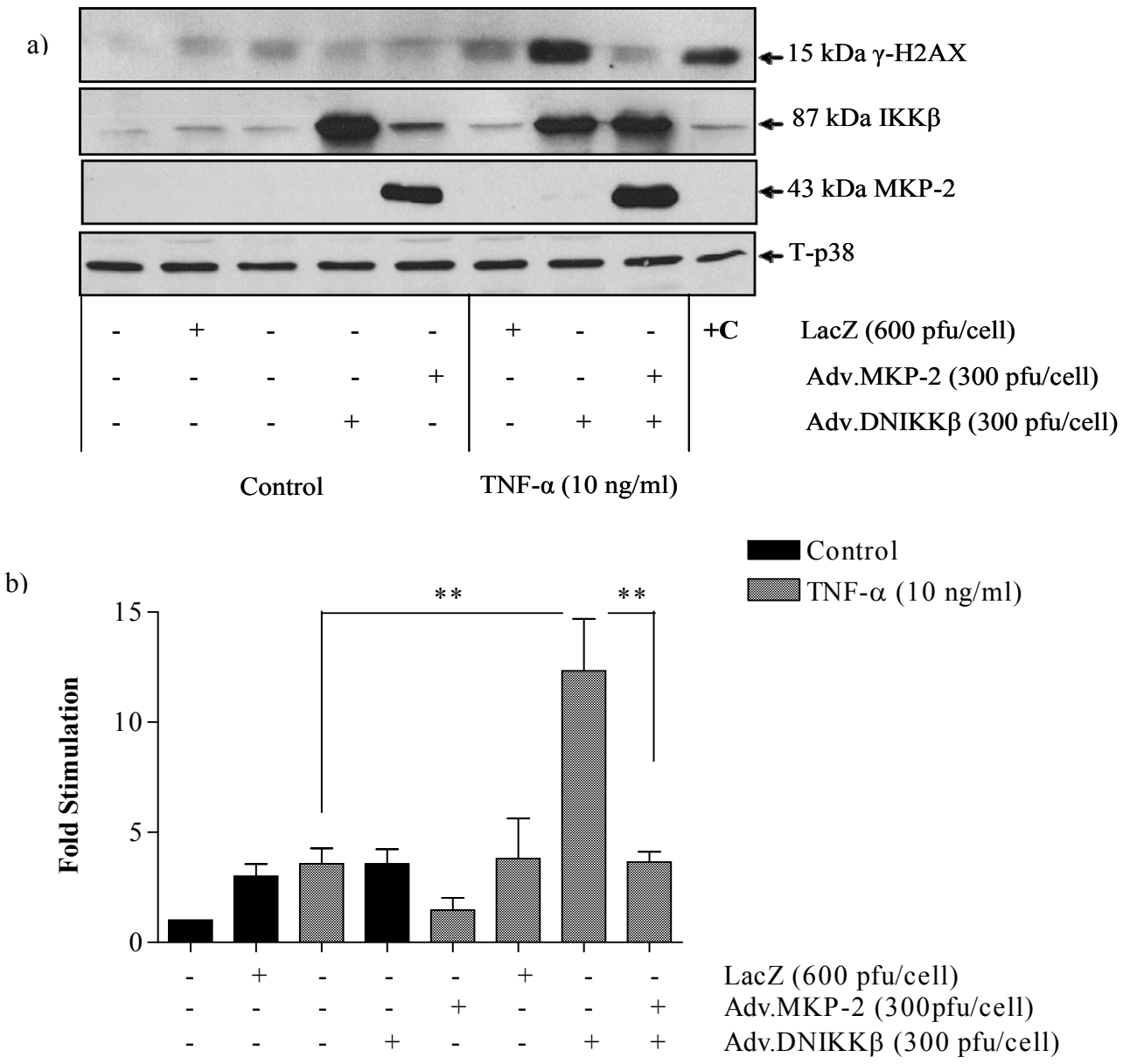


Figure 3.14: The effect of Adv.MKP-2 on H2AX phosphorylation (γ-H2AX) mediated by TNF-α in HUVECs: Cells were infected with 300 pfu/cell Adv.DNIKKβ or Adv.MKP-2 alone or in combination for 40 hr prior to stimulation with TNF-α (10 ng/ml) for a further 24 hr. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for **a**) γ-H2AX (15 kDa), IKKβ (87 kDa), MKP-2 (43 kDa) and T-p38 as outlined in section 2.3. Blots were quantified for **b**) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. The results are representative of 3 independent experiments. **P<0.01 compared with agonist-stimulated control and cells were infected with Adv.DNIKKβ alone. **+C** (positive control, UV-C 60 J/m^2).

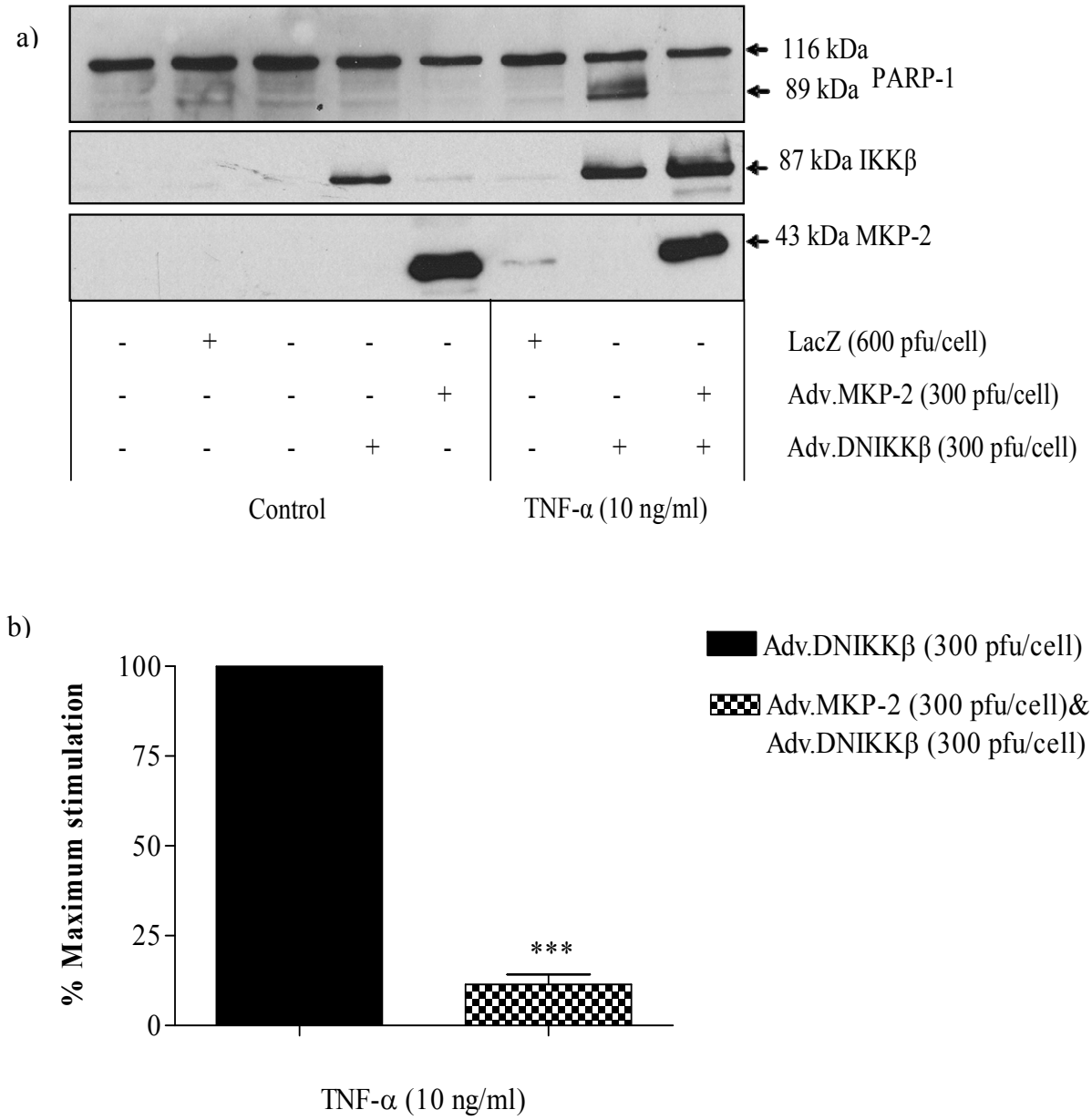


Figure 3.15: The effect of Adv.MKP-2 upon PARP-1 phosphorylation in TNF-α stimulated HUVECs: Cells were infected with 300 pfu/cell Adv.DNIKKβ or Adv.MKP-2 alone or in combination for 40 hr prior to stimulation with TNF-α (10 ng/ml) for a further 24 hr. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for **a**) PARP-1 (116kDa), cleaved PARP-1 (89 kDa), IKKβ (87 kDa) and MKP-2 (43 kDa) as outlined in section 2.3. Blots were quantified for **b**) % maximum stimulation by scanning densitometry; each value represents the mean ± s.e.m. The results are representative of 3 independent experiments. ***P<0.001 compared with agonist- stimulated control.

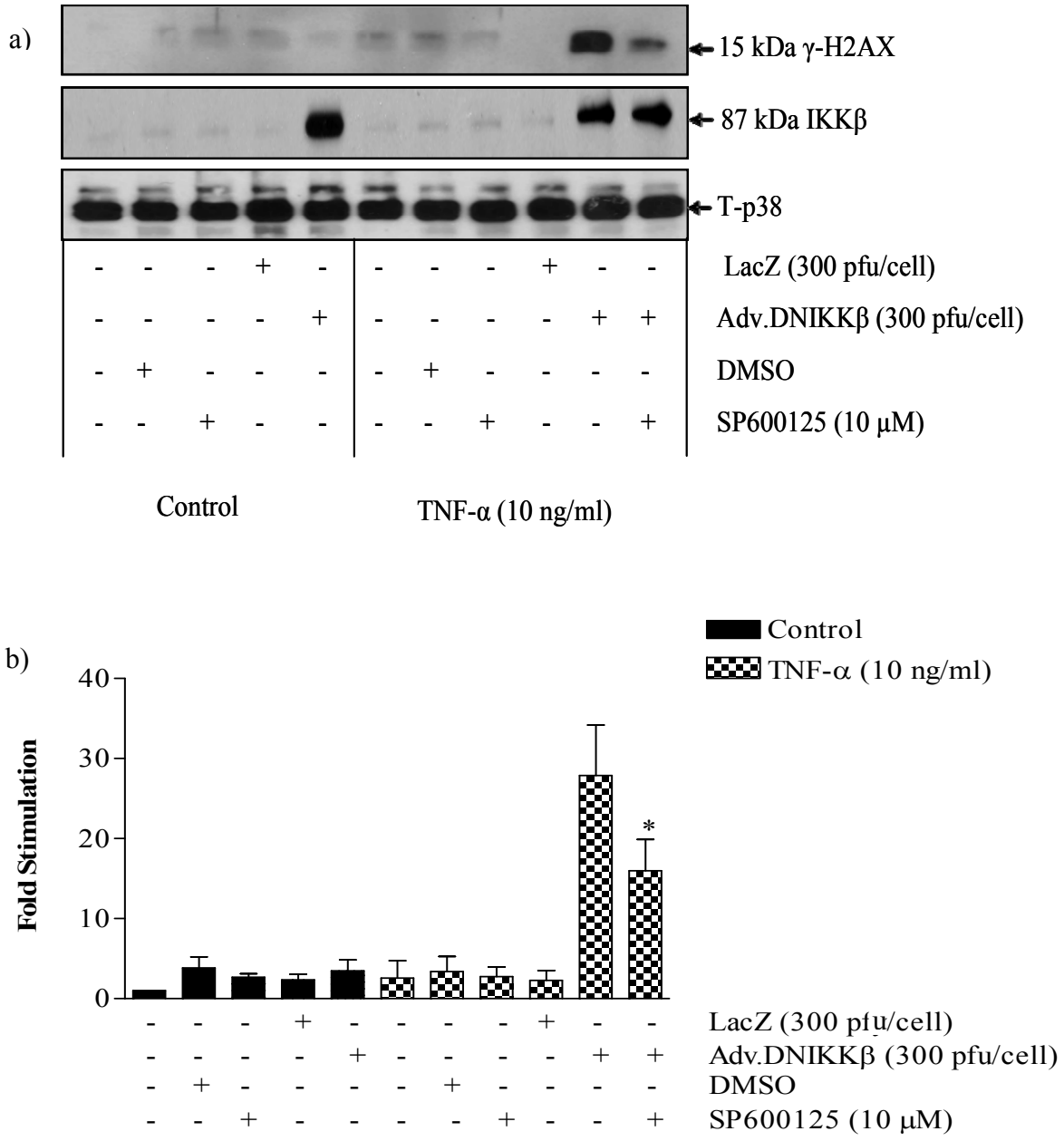


Figure 3.16: The effect of JNK inhibitor SP600125 on γ-H2AX phosphorylation mediated by TNF-α stimulated HUVECs: Cells were infected with 300 pfu/cell Adv.DNIKKβ for 40 hr prior to pre-treated with SP600125 (10 μM) for 1 hour before stimulation with TNF-α (10 ng/ml) for a period of 24 hr. Whole cell lysates were prepared, separated by SDS PAGE, and assessed for **a**) γ-H2AX (15 kDa), IKKβ (87 kDa) and T-p38 as outlined in section 2.3. Blots were quantified for **b**) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. The results are representative of 3 independent experiments. *P<0.05 compared with agonist- stimulated control.

3.4.7. The effect of MKP-2 over-expression after infection with an adenovirus expressing MKP-2 on TNF-α mediated Caspase-3 and 9 Cleavage in HUVECs

To further confirm the nuclear mediated effect of Adv.MKP-2, a number of cytosolic substrates within the apoptosis pathway were examined. Surprisingly, MKP-2 over-expression was able to strongly inhibit the formation of cleaved caspase-3 proteins (p17 and p19). Results revealed that infecting cells with Adv.DNIKKβ (300 pfu/cell) for 40 hr prior to stimulation with TNF-α (10 ng/ml) for further 24 hr increased caspase-3 cleavage by approximately 10 fold (fold stim. at 24 hr = 9.33 ± 2.36, $P<0.01$, n=3) (Figure 3.17). This response was essentially abolished when the cells were additionally infected with Adv.MKP-2 (% inhibition = 91.03 ± 2.88, $P<0.001$, n=3). A similar inhibition was observed for caspase-9 (Figure 3.18). Again infection with Adv.DNIKKβ in combination with TNF-α resulted in the cleavage of procaspase-9 to generate 35 and 37 kDa fragments. However, when cells were infected with both Adv.DNIKKβ (300 pfu/cell) and Adv.MKP-2 (300 pfu/cell) in combination, the formation of cleaved fragments was essentially abolished ($P<0.001$).

3.4.8. The effect of MKP-2 over-expression on TNF-α mediated Cytochrome C release in HUVECs

Having confirmed that Advenovirus encoding MKP-2 gene, a nuclear phosphatase, reverses cleavage of cytosolic effector caspases, the effect on mitochondrial integrity was also assessed by analysing release of cytochrome c into the cytosol using indirect immunofluorecence. Firstly, to confirm the co-localisation of MitoTracker dye with cytochrome c, cells stained with cytochrome c antibody were additionally incubated with MitoTracker Red CMXROS as described in section 2.8 (Figure 3.19 **a**). Staining was co-localised to an extra nuclear compartment suggesting the presence of cytochrome c within the mitochondria. HUVECs were then infected with Adv.DNIKKβ (300 pfu/cell) alone for 40 hr and then stimulated with TNF-α for 24 hr. This resulted in increased translocation of cytochrome c from the mitochondria into the cytosol as compared to un-stimulated cells (Figure 3.19 **b** indicated by the arrows). Following co-infection with Adv.MKP-2, release of cytochrome c was visibly impaired.

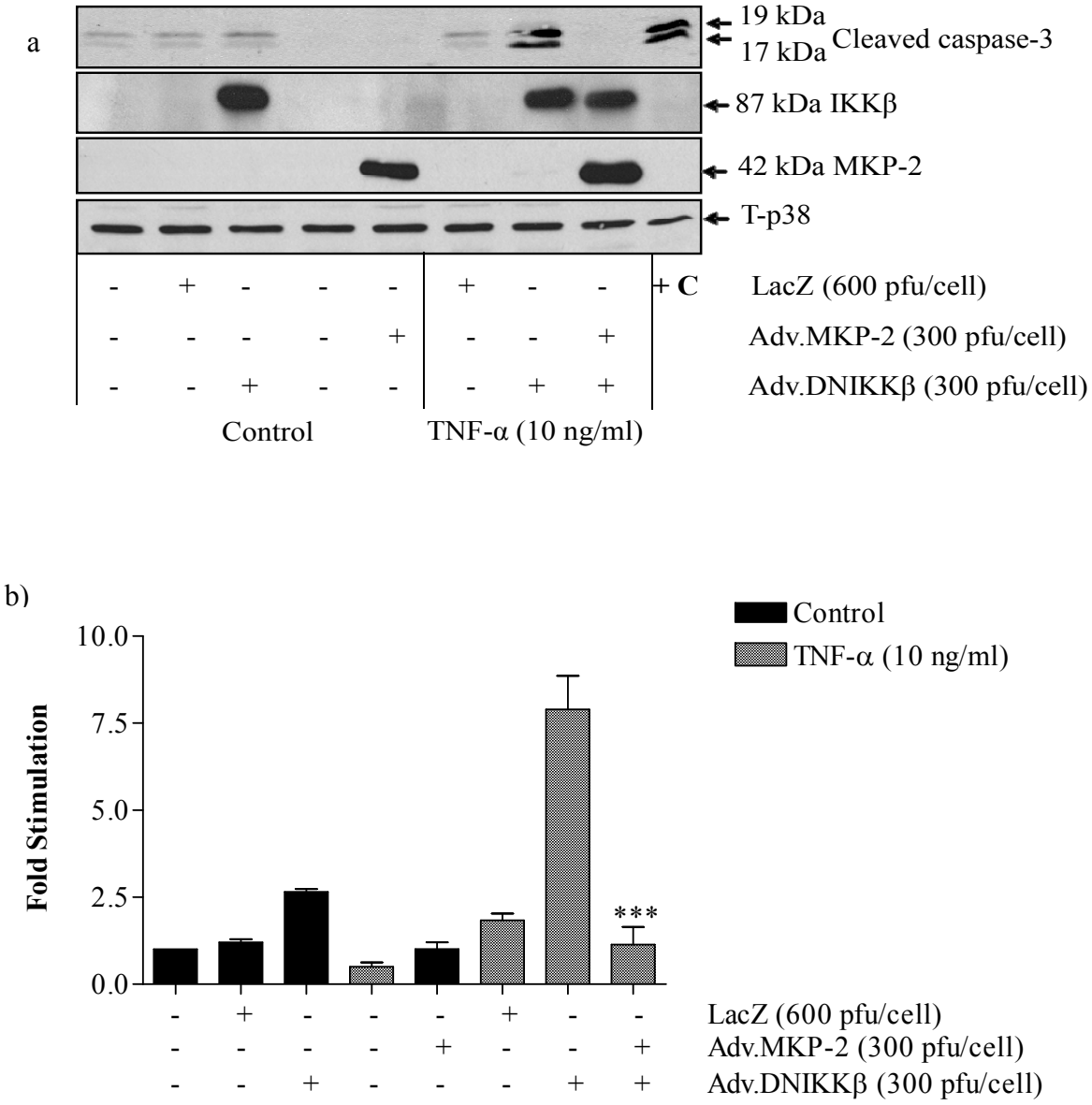


Figure 3.17: The effect of Adv.MKP-2 on Caspase-3 cleavage in TNF-α stimulated HUVECs: Cells were infected with 300 pfu/cell Adv.DNIKKβ or Adv.MKP-2 alone or in combination for 40 hr prior to stimulation with TNF-α (10 ng/ml) for a further 24 hr. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for **a**) cleaved caspase-3 (17/19 kDa), IKKβ (87 kDa) MKP-2 (43 kDa) and T-p38 as outlined in section 2.3. Blots were quantified for **b**) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. The results are representative of 3 independent experiments. ***P<0.001 compared with agonist- stimulated control. **+C** (positive control, staurosporine 1μM).

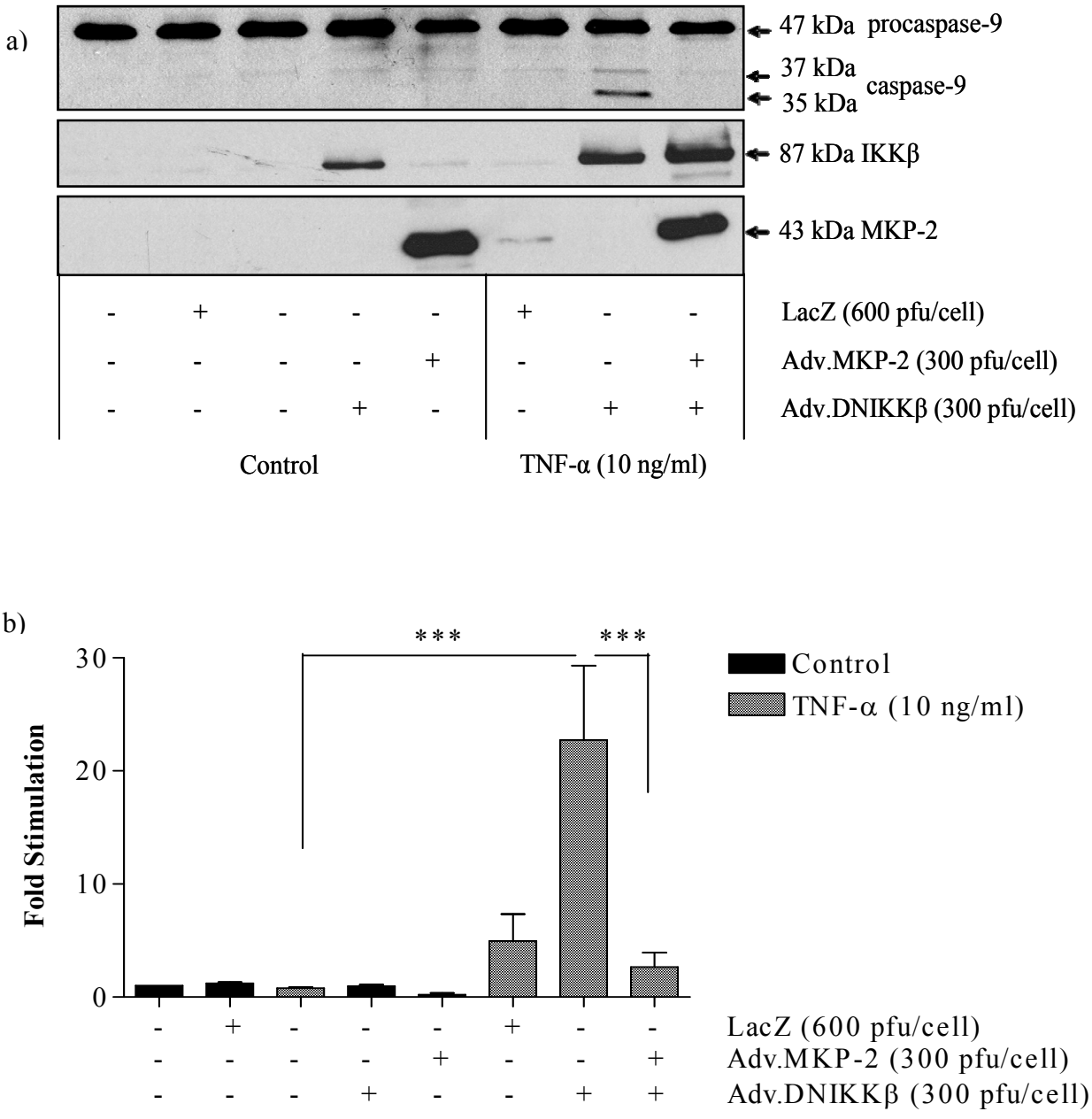
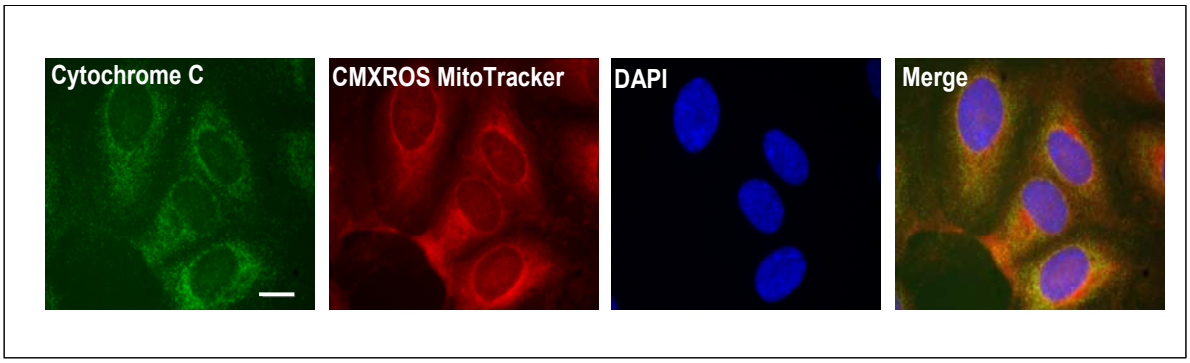


Figure 3.18: The effect of Adv.MKP-2 on Caspase-9 cleavage in TNF-α stimulated HUVECs: Cells were infected with 300 pfu/cell Adv.DNIKKβ or Adv.MKP-2 alone or in combination for 40 hr prior to stimulation with TNF-α (10 ng/ml) for a further 24 hr. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for **a**) procaspase-9 (47 kDa), cleaved caspase-9 (35/37 kDa), IKKβ (87 kDa) and MKP-2 (43 kDa) as outlined in section 2.3. Blots were quantified for **b**) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. The results are representative of 3 independent experiments. ***P<0.001 compared with agonist- stimulated control or infected with DNIKKβ.

a)



b)

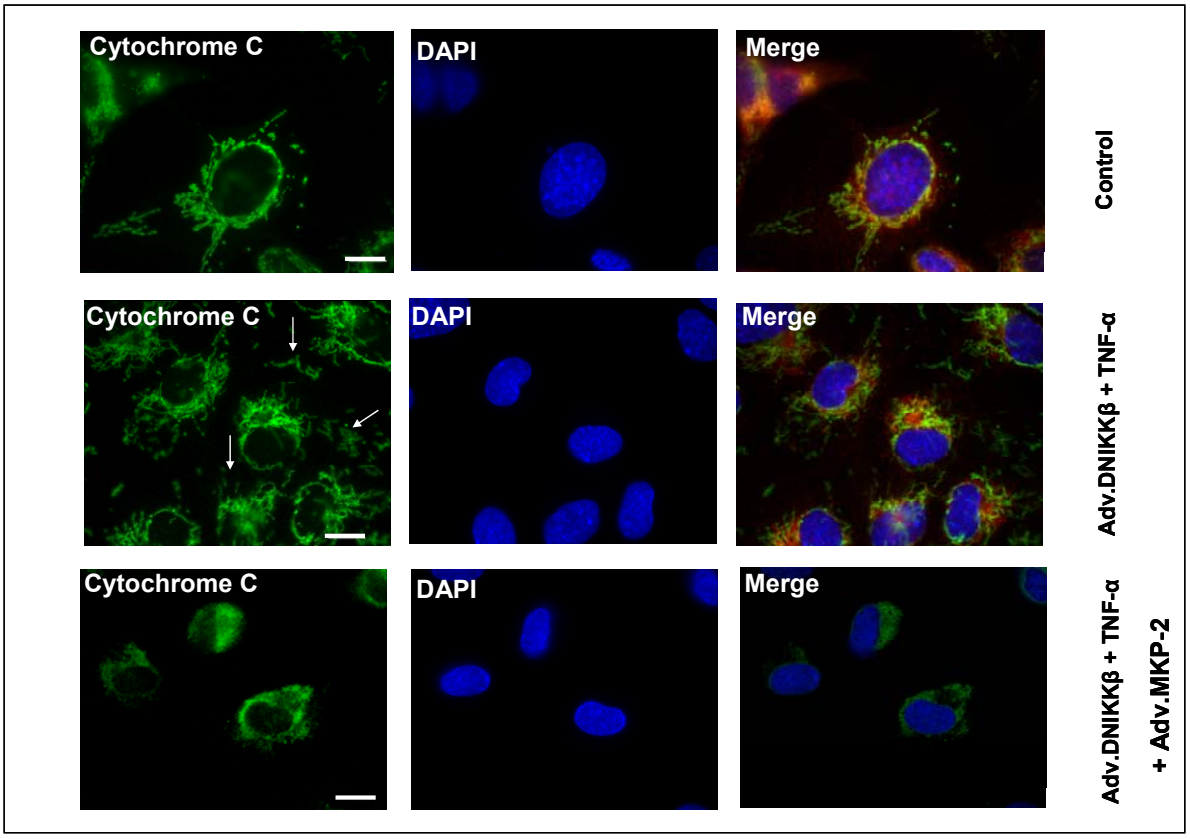
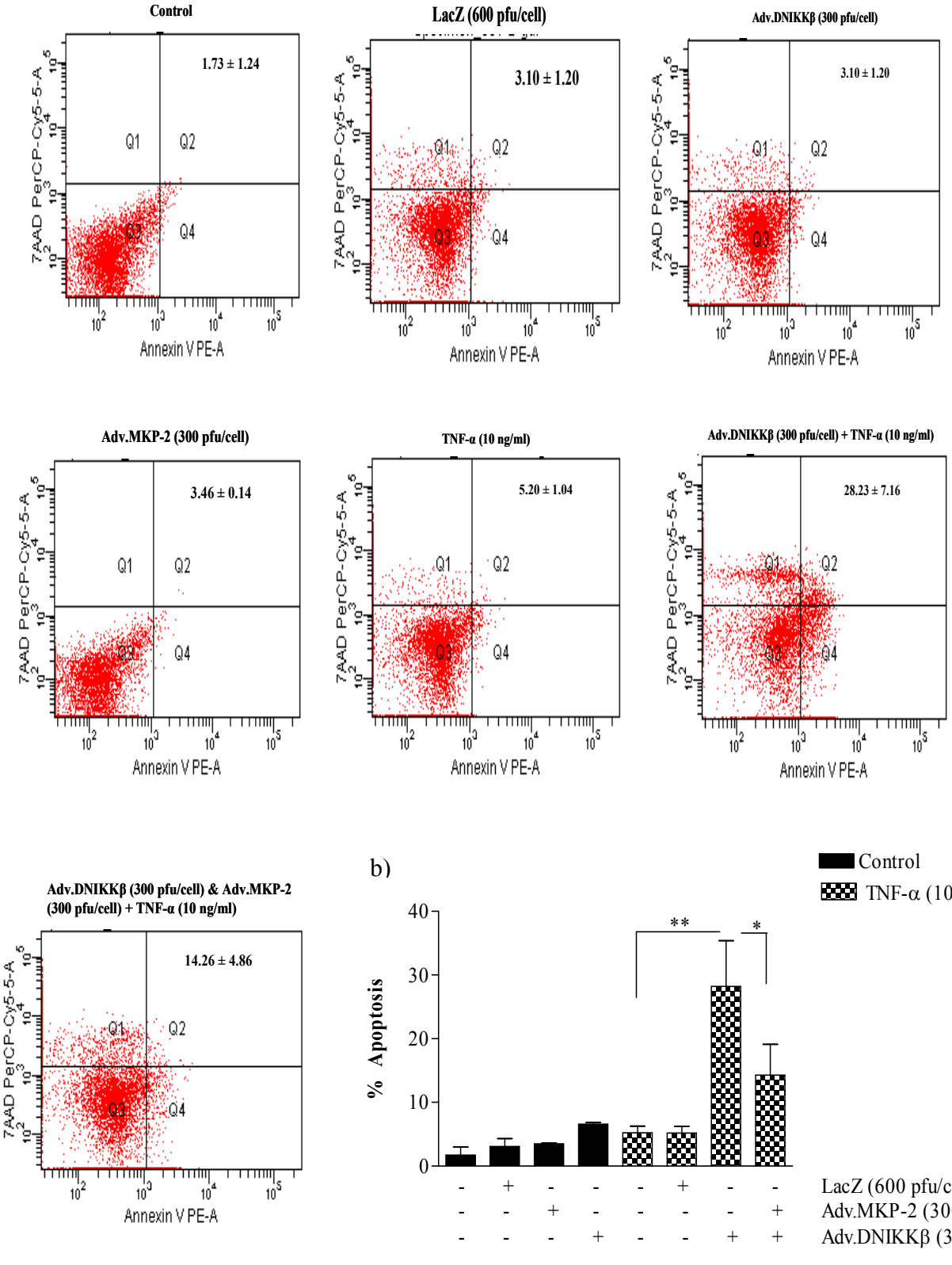


Figure 3.19: The effect of Adv.MKP-2 on cytochrome c release in TNF-α stimulated HUVECs: a) HUVECs were fixed and stained for cytochrome c (FITC, green) and CMXROS MitoTracker (TRITC, red) colocalisation as described in section 2.10. **b**) Control cells or cells were infected with 300 pfu/cell Adv.DNIKKβ alone or in combination with Adv.MKP-2 for 40 hr prior to stimulation with TNF-α (10 ng/ml) for a further 24 hr. Cells were also fixed and stained for cytochrome c (FITC) and DAPI (blue). Arrows indicate cytochrome c in the cytosol. All cells were viewed x100 magnification, using a Nikon EP-1 fluorescent microscope. The results are representative of a single experiment (scale bar=10μm).

3.4.9. MKP-2 over-expression infection reverses TNF-α mediated apoptosis in HUVECs

Having established in section 3.4 that MKP-2 over-expression was able to reverse activation of pro-apoptotic proteins; the potential for Adv.MKP-2 to reverse endothelial cell apoptosis using flow cytometry analysis was assessed. HUVECs were infected with Adv.DNIKKβ (300 pfu/cell) or Adv.MKP-2 (300 pfu/cell) alone or in combination for 40 hr prior to stimulation with TNF-α for a further 24 hr. Samples were analysed for Annexin V-phycoerythin and 7-Amino-Actinomycin-D (AAD) staining as outlined in section 2.7. (Figure 3.20). Cells stimulated alone with TNF-α showed no significant increase in apoptosis, although both viral constructs increased apoptosis to a small extent (% cell death, control =1.73 ± 1.24, LacZ = 3.10 ± 1.20, Adv.DNIKKβ = 6.60 ± 0.21, TNF-α = 5.20 ± 1.04, Adv.MKP-2 = 3.46 ± 0.14). However, infecting cells with Adv.DNIKKβ (300 pfu/cell) prior to stimulation with TNF-α resulted in a significant increase in apoptosis, (% cell death = 28.23 ± 7.16, $P<0.01$). Under these conditions co-expression of Adv.MKP-2 significantly reduced cell death by approximately 50 % (% cell death + Adv.MKP-2 = 14.26 ± 4.86, $P<0.05$). This effect was not due to competition between the two viruses for cellular uptake since expression of each protein did not affect the other.

a)


Control
1.73 ± 1.24
LacZ (600 pfu/cell)
3.10 ± 1.20
Adv.DNIKKβ (300 pfu/cell)
3.10 ± 1.20
Adv.MKP-2 (300 pfu/cell)
3.46 ± 0.14
TNF-α (10 ng/ml)
5.20 ± 1.04
Adv.DNIKKβ (300 pfu/cell) + TNF-α (10 ng/ml)
28.23 ± 7.16
Adv.DNIKKβ (300 pfu/cell) & Adv.MKP-2 (300 pfu/cell) + TNF-α (10 ng/ml)
14.26 ± 4.86
7AAD PerCP-Cy5-5-A
Annexin V PE-A
Q1
Q2
Q3
Q4

b)

Control
TNF-α (10 ng/ml)
% Apoptosis
0
10
20
30
40
**
*
- + - - - + - - LacZ (600 pfu/cell)
- - + - - - - + Adv.MKP-2 (300 pfu/cell)
- - - + - - + + Adv.DNIKKβ (300 pfu/cell)

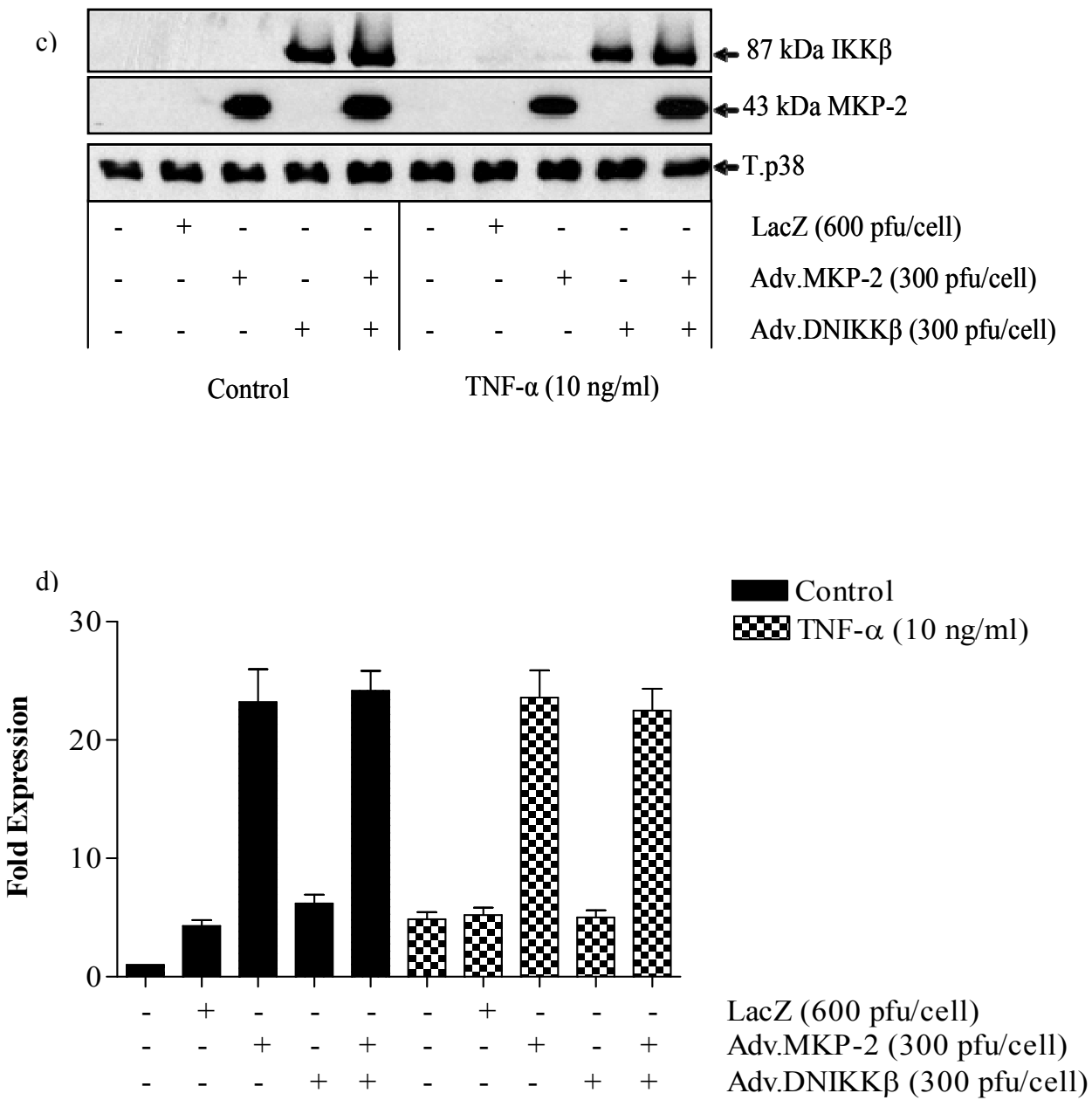


Figure 3.20: Adv.MKP-2 infection reverses TNF-α mediated apoptosis in HUVECs. HUVECs were infected with 600 pfu Lac Z (alone), 300 pfu Adv.DNIKKβ or Adv.MKP-2 alone or in combination for 40 hr prior to stimulation with TNF-α for a further 24 hr. Samples where assessed for Annexin V and 7-AAD staining as outlined in section 2.7. (Panels a and b), whilst in parallel control experiments cell samples were Western blotted for DNIKKβ and MKP-2 (Panel c). Each value represents the mean ± s.e.m. of at least 4 experiments (Panels b and d). *P<0.05 compared to TNF-α/Adv.DNIKKβ stimulation alone. **P<0.01 compared to TNF-α alone.

3.4.10. The effect of SP600125 on TNF-α mediated apoptosis in HUVECs

Having established that Adv.MKP-2 was able to significantly reverse endothelial cell apoptosis, the effect of SP600125 was also assessed. Cells infected with Adv.DNIKKβ prior to stimulation with TNF-α resulted in 8 fold increased in cell death (% cell death = 31.08 ± 4.91, n=3) (Figure 3.21). However pre-treatment with SP600125 for 1 hr before stimulation with TNF-α, gave only around 20% inhibition of apoptosis which was not statistically significant (% cell death = 24.95 ± 4.70, n=3).

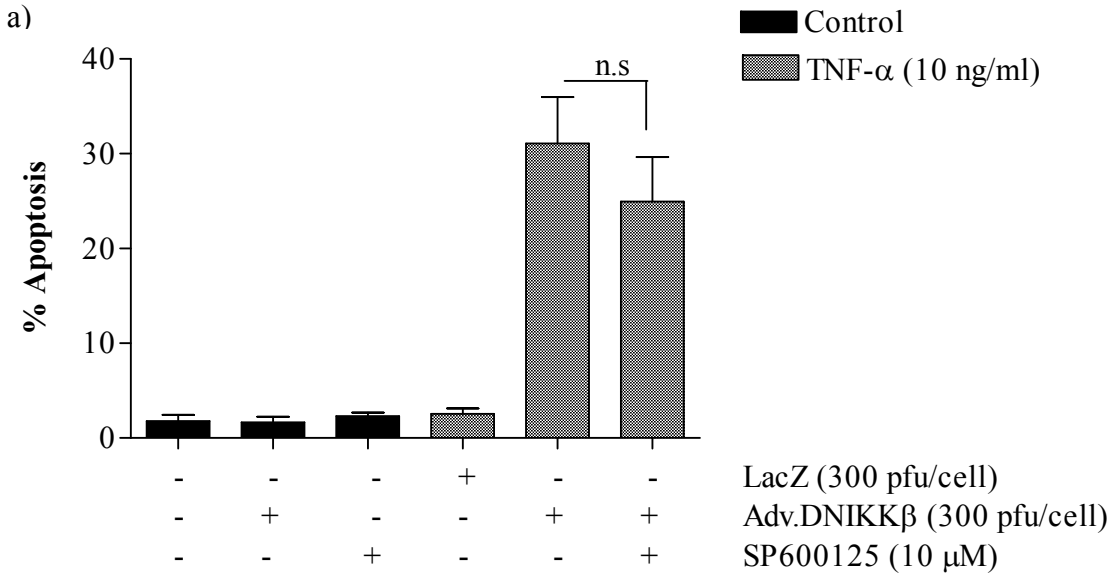


Figure 3.21: Effect of SP600125 on TNF-α mediated apoptosis in HUVECs.
HUVECs were infected with 300 pfu Lac Z or 300 pfu Adv.DNIKKβ for 40 hr prior to pre-treatment with SP600125 (10 μM) for 1 hr before stimulation with TNF-α (10 ng/ml) for a further 24 hr. Samples were assessed for Annexin V and 7-AAD staining as outlined in section 2.7. Each value represents the mean ± s.e.m of at least 4 experiments. n.s ($P>0.05$).

3.5. THE INTERACTION OF MKP-2 WITH THE NF-κB PATHWAY

The results from figures (3.20) showed that Adv.MKP-2 could reverse the pro-apoptotic effects of TNF-α under conditions of NF-κB inhibition. Recent studies have suggested this may involve in interaction between MKP-2 with NF-κB (Al-Mutairi et al., 2010). Therefore, the effect of Adv.MKP-2 on components of the NF-κB pathway was examined.

3.5.1. The effect of Adv.MKP-2 on IκBα loss mediated by TNF-α

Following the study that demonstrated that MKP-2 may interact with NF-κB as mentioned in section 3.5, this interaction was examined at the level of IκBα loss (Figure 3.22). Infecting HUVECs with 300 pfu/cell of Adv.DNIKKβ resulted in an increase in basal expression of IκBα, however, infection with an equivalent concentration of Adv.MKP-2 reduced the basal expression significantly (% inhibition = 78.70 ± 6.75, $P<0.0$, n=3). Similarly, TNF-α (1 ng/ml) caused an increased in IκBα loss which was inhibited in the presence of Adv.DNIKKβ. However, this inhibition was reversed by the over-expression of Adv.MKP-2 ($P<0.05$).

3.5.2. The effect of Adv.MKP-2 on NF-κB translocation into the nucleus mediated by TNF-α

To ascertain the effect of MKP-2 on p65 translocation into the nucleus, nuclear extracts were prepared after infecting cells with Adv.MKP-2 (300 pfu/cell) and stimulated with two concentrations of TNF-α (1 ng/ml and 10 ng/ml) for 1 hr. As figure 3.23 shows, both TNF-α concentrations stimulated an increase in p65 NF-κB protein expression in the nucleus. Following infection with Adv.MKP-2, nuclear p65 NF-κB expression was not altered. Also the control virus LacZ caused an increase in NF-κB expression.

Another approach to confirm the effect of Adv.MKP-2 on NF-κB activity was also employed. Cells were infected with an adenovirus encoding NF-κB-luciferase (Adv.NF-κB-Luc) and additionally infected with Adv.DNIKKβ and Adv.MKP-2 prior to stimulation with TNF-α for 8 hr as shown in Figure 3.24. As expected, Adv.DNIKKβ gave a significant inhibition in NF-κB-driven gene expression over approximately 60 fold (% inhibition = 63.73 ± 9.40, $P<0.001$, n=3). However, when cells were co-incubated with Adv.MKP-2 no reversal in the NF-κB reporter activity was observed.

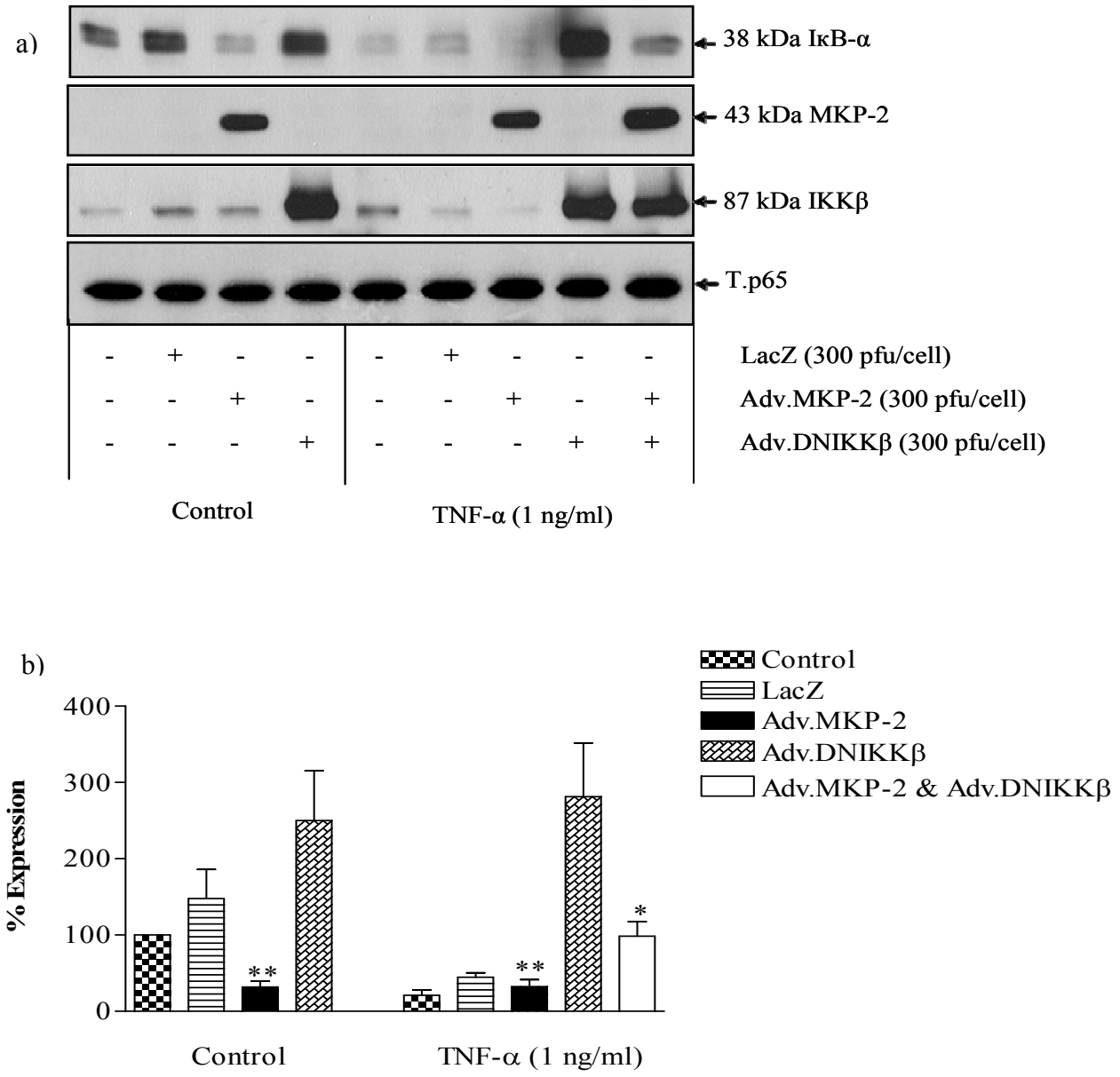


Figure 3.22: The effect of Adv.MKP-2 on Adv.DNIKKβ inhibition of IκBα loss mediated by TNF-α in HUVECs. Cells were infected with Adv.MKP-2 (300 pfu/cell), Adv.DNIKKβ (300 pfu/cell) or both for 40 hr prior to stimulation with TNF-α (1 ng/ml) for 15 min. Whole cell lysates were prepared, separated by SDS PAGE, and assessed for **a**) IκBα (38 kDa), IKKβ (87 kDa), MKP-2 (43 kDa) and T.p65 as outlined in section 2.3. Blots were quantified for **b**) % expression by scanning densitometry; each value represents the mean ± s.e.m. The results are representative of 3 independent experiments. **P<0.01 compared with control cells infected with Adv.DNIKKβ. **P<0.01 compared with agonist-stimulated cells infected with Adv.DNIKKβ alone. *P<0.05 compared with agonist-stimulated cells infected with Adv.DNIKKβ alone.

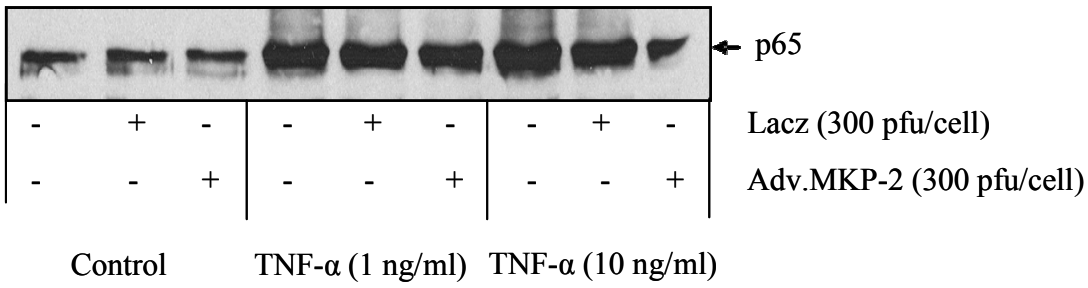


Figure 3.23: The effect of Adv.MKP-2 on TNF-α-mediated nuclear p65 NF-κB expression in HUVECs. Cells were infected with Adv.MKP-2 (300 pfu/cell) for 40 hr prior to stimulation with TNF-α (10 ng/ml) for 1 hr and nuclear extracts were prepared as outlined in section 2.4, then separated by SDS PAGE and assessed for p65 NF-κB as outlined in section 2.3. Blot is representative of single experiment.

a)

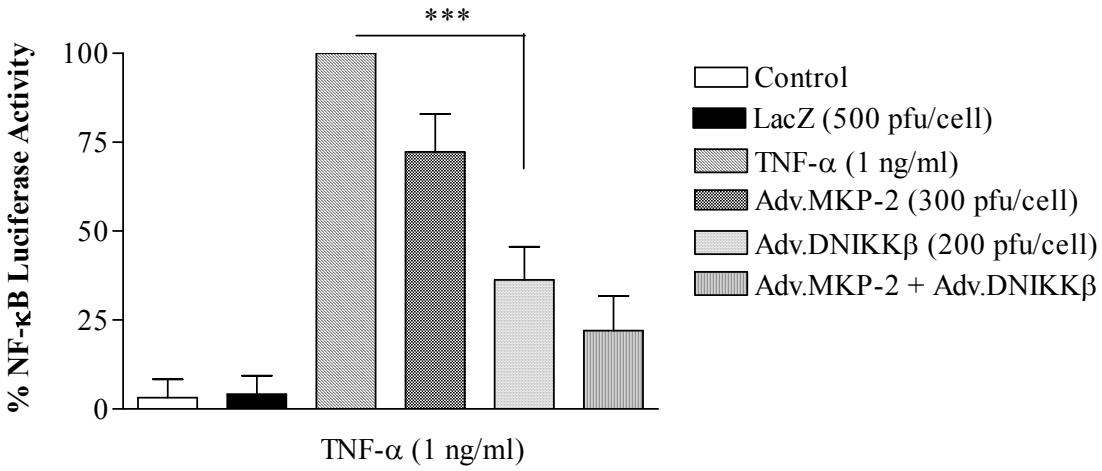


Figure 3.24: The effect of Adv.MKP-2 on NF-κB luciferase reporter activity mediated by TNF-α in HUVECs. Cells were infected with Adv.NF-κB-Luc and additionally Adv.MKP-2 (300 pfu/cell) or Adv.DNIKKβ (300 pfu/cell) as indicated for 40 hr prior to stimulation with TNF-α (1 ng/ml) for 8 hr. Cell lysates were then measured for luciferase activity as previously described in section 2.6. Data shown is expressed as % NF-κB luciferase activity and each value represents the mean ± s.e.m (n=3). ***P<.001 compared with agonist-stimulated control

3.6. THE EFFECT OF MKP-2 OVER-EXPRESSION ON MAP KINASE-INDUCED APOPTOTIC PROTEIN EXPRESSION IN HUVECs BY H_2O_2 AND SERUM DEPRIVATION

Following on from the previous experiments, which demonstrated that MKP-2 over-expression could reverse JNK mediated nuclear and mitochondrial dependent apoptosis in response to TNF-α plus Adv.DNIKKβ, additional agents such as H_2O_2 and serum deprivation which are known strongly activate JNK were utilised to confirm previous observations.

3.6.1. H_2O_2 and serum deprivation mediated activation of c-Jun N-terminal kinase (JNK) in HUVECs

Oxidative stress is known to activate multiple signal transduction pathways in many experimental systems (Pober et al., 2008, Martindale and Holbrook, 2002). Therefore, H_2O_2 was used as an agent that could act as a paradigm of oxidative stress. To evaluate the relative magnitude of JNK phosphorylation by H_2O_2, cells were treated with 0.2 mM H_2O_2 in endothelial basal media (EBM-2) in the absence of an antioxidant, ascorbic acid, over a period of 8 hr. The data presented in Figure (3.25 panel **a**) shows a representative Western blot demonstrating that H_2O_2 stimulated a substantial phosphorylation of JNK after 1 hr approximately 10 fold of basal values which then increased reaching a maximum level by 2 hr (fold stim. at 2 hr = 22.99 ± 4.52, n=3). The activation of JNK then returned towards basal values after 6 hr.

The effect of serum and growth factor withdrawal on JNK phosphorylation in HUVECs was investigated (Figure 3.25 panel **b**). Cells were washed with serum free medium and incubated over a period of 8 hr. Under these conditions a significant increase in the phosphorylation of JNK was observed, reaching a maximum between 1 and 2 hr with the response observed to be approximately 60-70 fold of basal values (fold stim = 65.96 ± 4.10 and 65.68 ± 5.88, n=3). This response gradually declined thereafter and fell further towards basal levels by 8 hr.

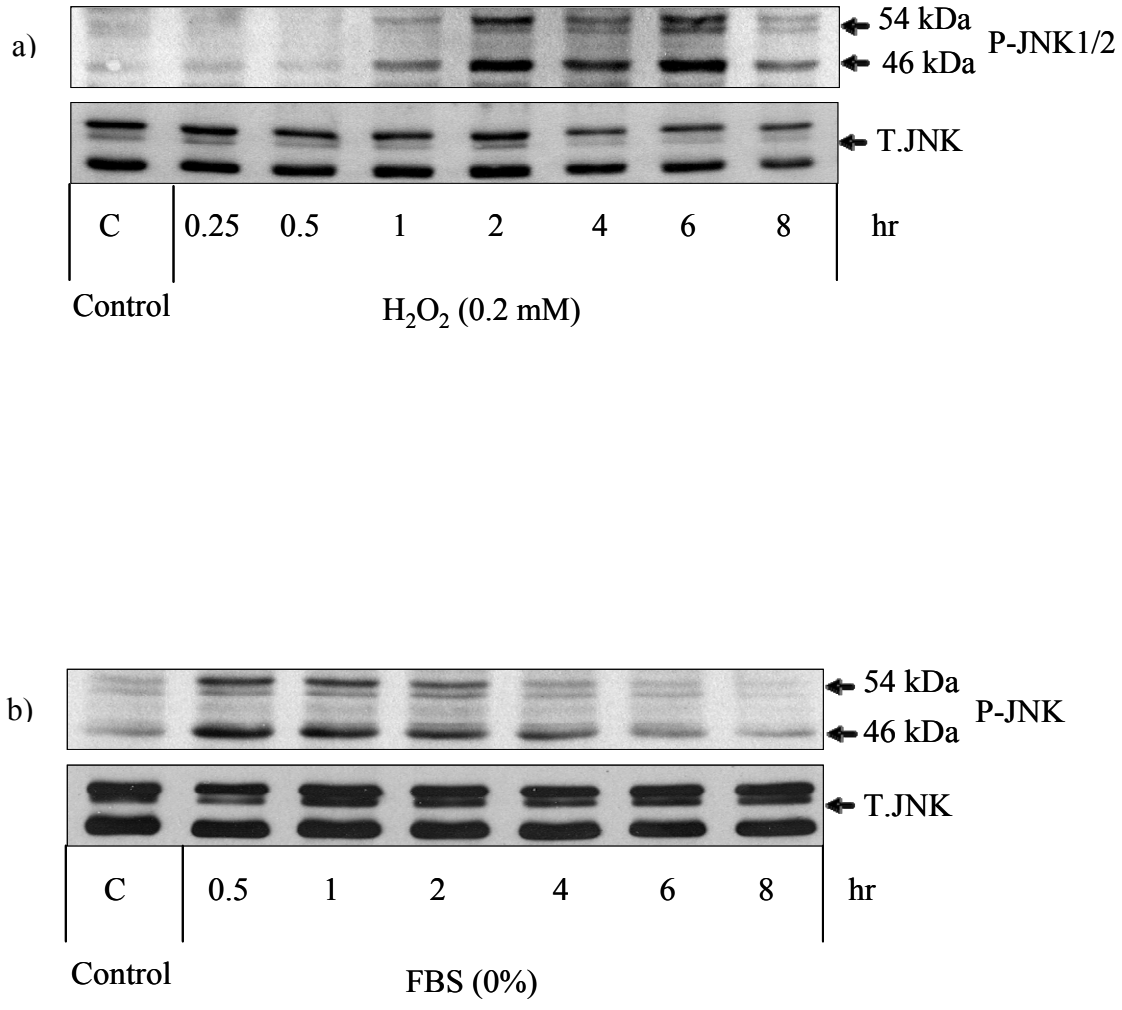


Figure 3.25: The effect of H_2O_2 and serum & growth factor deprivation upon JNK phosphorylation in HUVECs. a) Cells were treated with H_2O_2 (0.2mM) or **b**) incubated with serum free media (FBS 0%) for the times indicated. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for p-JNK (46/54 kDa) and T.JNK as outlined in section 2.3. The results are representative of 3 independent experiments.

3.7. THE EFFECT OF OVER-EXPRESSION OF MKP-2 BY INFECTION WITH ADV.MKP-2 ON PRO-APOPTOTIC PROTEINS STIMULATED BY H_2O_2 AND SERUM DEPRIVATION

3.7.1. The effect of over-expression of MKP-2 on H_2O_2 mediated JNK and c-Jun phosphorylation in HUVECs

Figure 3.26 shows the effect of Adv.MKP-2 (300 pfu/cell) upon JNK signalling in response to H_2O_2. Stimulation with H_2O_2 induced a significant increase of JNK phosphorylation (fold stim. at 2 hr = 49.97 ± 15.06, n=3), which was slightly but not significantly decreased by infecting cells with LacZ (300 pfu/ml fold stim. at 2 hr = 48.84 ± 15.28, n=3). However, infection with Adv.MKP-2 (300 pfu/ml) significantly inhibited the phosphorylation of JNK by approximately 90% (fold stim. at 2 hr = 1.72 ± 0.12, $P< 0.01$). Similarly, infection with Adv.MKP-2 (300 pfu/cell) essentially abrogated the phosphorylation of c-Jun (% inhibition = 98.60 ± 0.48, $P<0.001$, n=3) (Figure 3.27).

To confirm that the effect of Adv.MKP-2 was mediated through JNK inhibition, HUVECs were pre-treated with SP600125 (10 μM) or vehicle (DMSO) for 1 hr before stimulation with H_2O_2. At a concentration of 10 μM, SP600125 caused a significant inhibition of c-Jun phosphorylation mediated by H_2O_2 (Figure 3.28), the response dropped from 24 to 4 fold of basal values (fold stim. H_2O_2 at 2 hr = 24.21 ± 6.32, H_2O_2 + SP600125 at 2 hr = 4.30 ± 0.67, $P< 0.01$, n=3).

3.7.2. The effect of over-expression of MKP-2 on serum deprivation induced JNK and c-Jun phosphorylation in HUVECs

The effect of FBS deprivation on JNK phosphorylation was also examined (Figure 3.29), HUVECs were infected with Adv.MKP-2 (300 pfu/cell) for 40 hr and then stimulated with serum free EBM-2 medium for 30 min. Under these conditions a significant increase in JNK phosphorylation was observed (fold stim. at 30 min = 39.53 ± 9.95, n=3), which was slightly but not significantly decreased by infecting cells with LacZ. However, infection with Adv.MKP-2 significantly inhibited the phosphorylation of JNK reducing levels back to near basal values (Adv.MKP-2 fold stim. at 30 min = 0.75 ± 0.33, P<

0.001, n=3). Similarly infection with Adv.MKP-2 (300 pfu/cell) significantly inhibited c-Jun phosphorylation by approximately 95% (Adv.MKP-2 % inhibition at 30 min = 94.78 ± 1.99, $P<0.001$, n=3) (Figure 3.30).

The effect of Adv.MKP-2 was also compared to SP600125. Figure 3.31 shows serum deprivation treatment resulted in the usual substantial increase in JNK phosphorylation (fold stim. at 30 min = 15.79 ± 5.33, n=3), which was slightly but not significantly increased by the vehicle, DMSO. In contrast, as a result of pre-treatment with 10 μM of SP600125, a significant reduction in c-Jun phosphorylation was observed, the response falling to approximately 2 fold of basal values (SP600125 fold stim. at 30 min = 2.29 ± 0.60, $P< 0.01$, n=3).

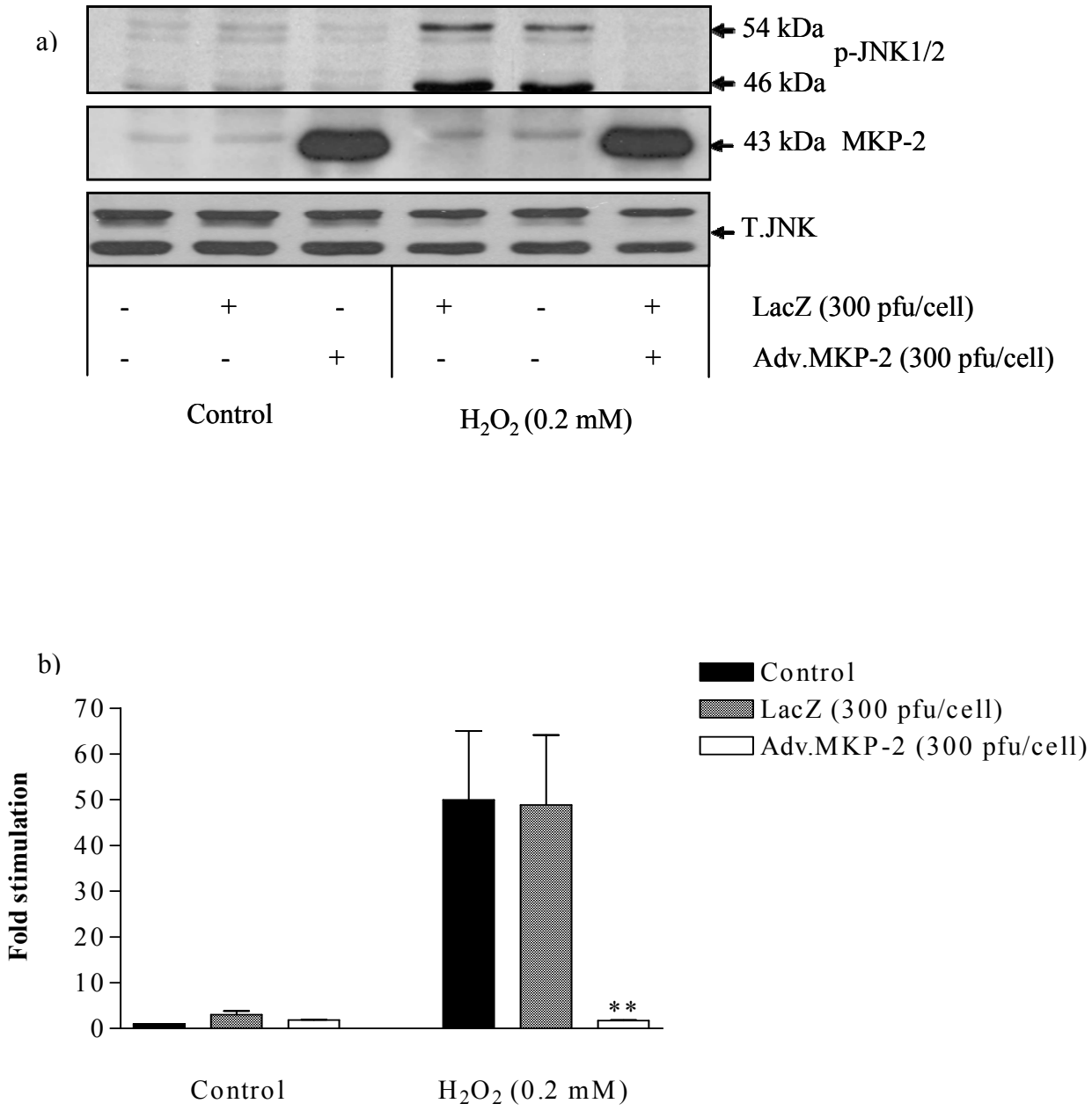


Figure 3.26: The effect of Adv.MKP-2 upon H_2O_2 stimulated JNK phosphorylation in HUVECs: Cells were infected with Adv.MKP-2 for 40 hr prior to stimulation with H_2O_2 (0.2 mM) for 2 hr. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for **a**) p-JNK (46/54 kDa), MKP-2 (43 kDa) and T.JNK as outlined in section 2.3. Blots were quantified for **b**) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. The results are representative of 3 independent experiments. **P<0. 01 compared with agonist-stimulated control.

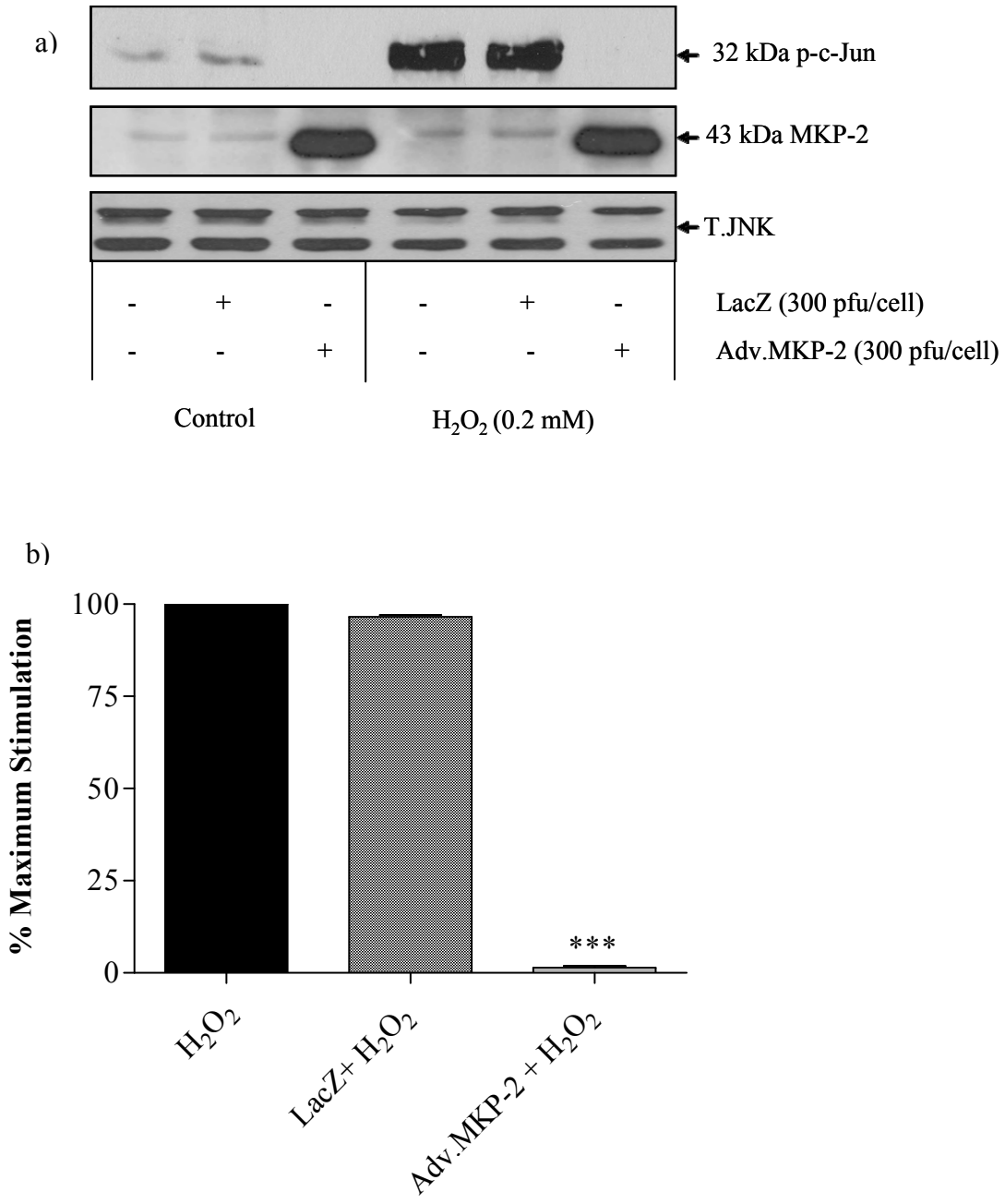


Figure 3.27: The effect of Adv.MKP-2 upon H_2O_2 stimulated c-Jun phosphorylation in HUVECs: Cells were infected with Adv.MKP-2 for 40 hr prior to stimulation with H_2O_2 (0.2 mM) for 2 hr. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for **a**) p-c-Jun (32 kDa), MKP-2 (43 kDa) and T.JNK as outlined in section 2.3. Blots were quantified for **b**) % maximum stimulation by scanning densitometry; each value represents the mean ± s.e.m. The results are representative of 3 independent experiments. ***P<0. 001 compared with agonist-stimulated control.

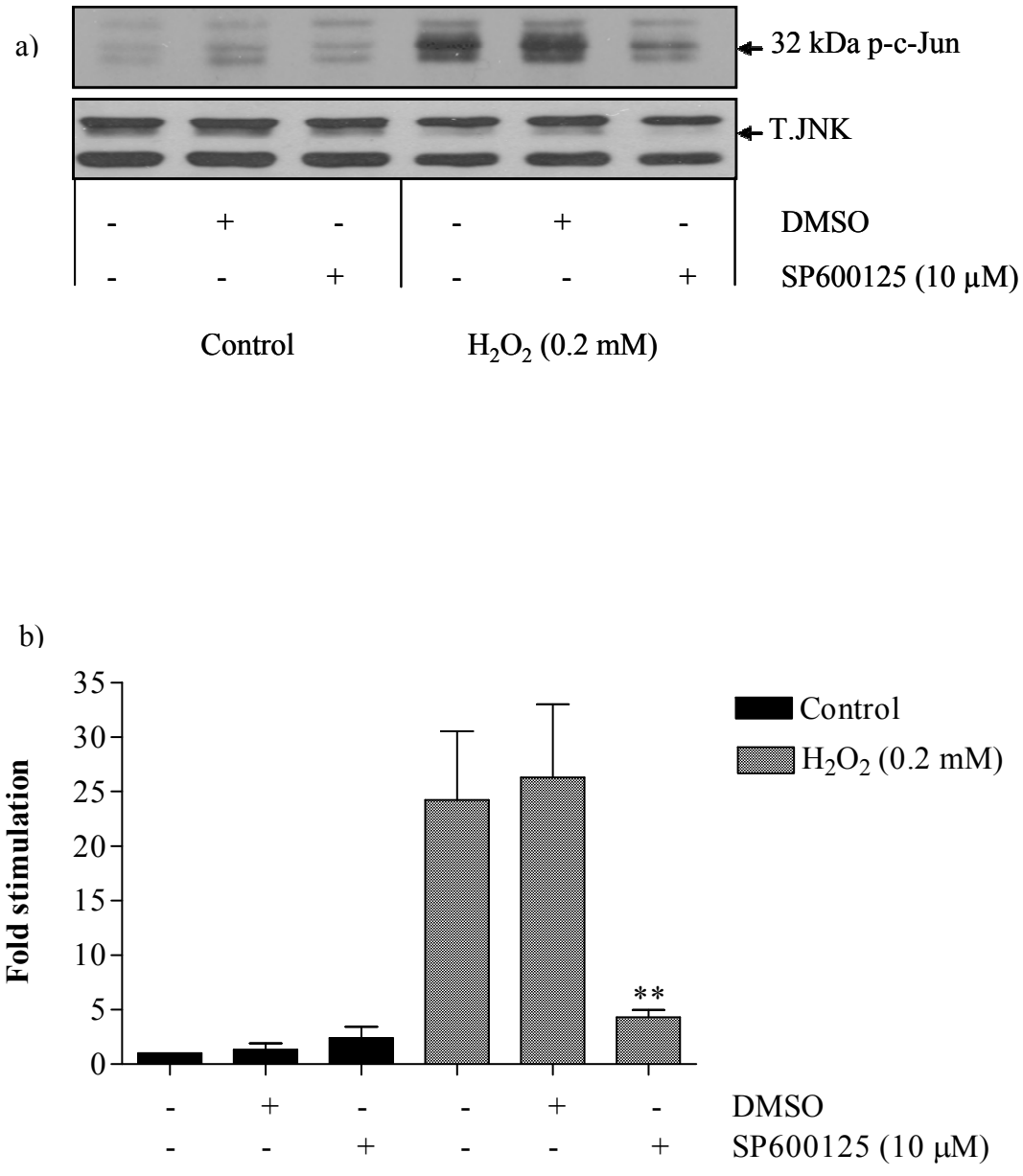


Figure 3.28: The effect of JNK inhibitor, SP600125 on c-Jun phosphorylation mediated by H_2O_2 in HUVECs: Cells were pre-treated with SP600125 (10 μM) for 1 hr before stimulation with H_2O_2 (0.2 mM) for 2 hr. Whole cell lysates were prepared, separated by SDS PAGE, and assessed for **a**) p-c-Jun (32 kDa) and T.JNK as outlined in section 2.3. Blots were quantified for **b**) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. The results are representative of 3 independent experiments. **P<0.01 compared with agonist- stimulated control.

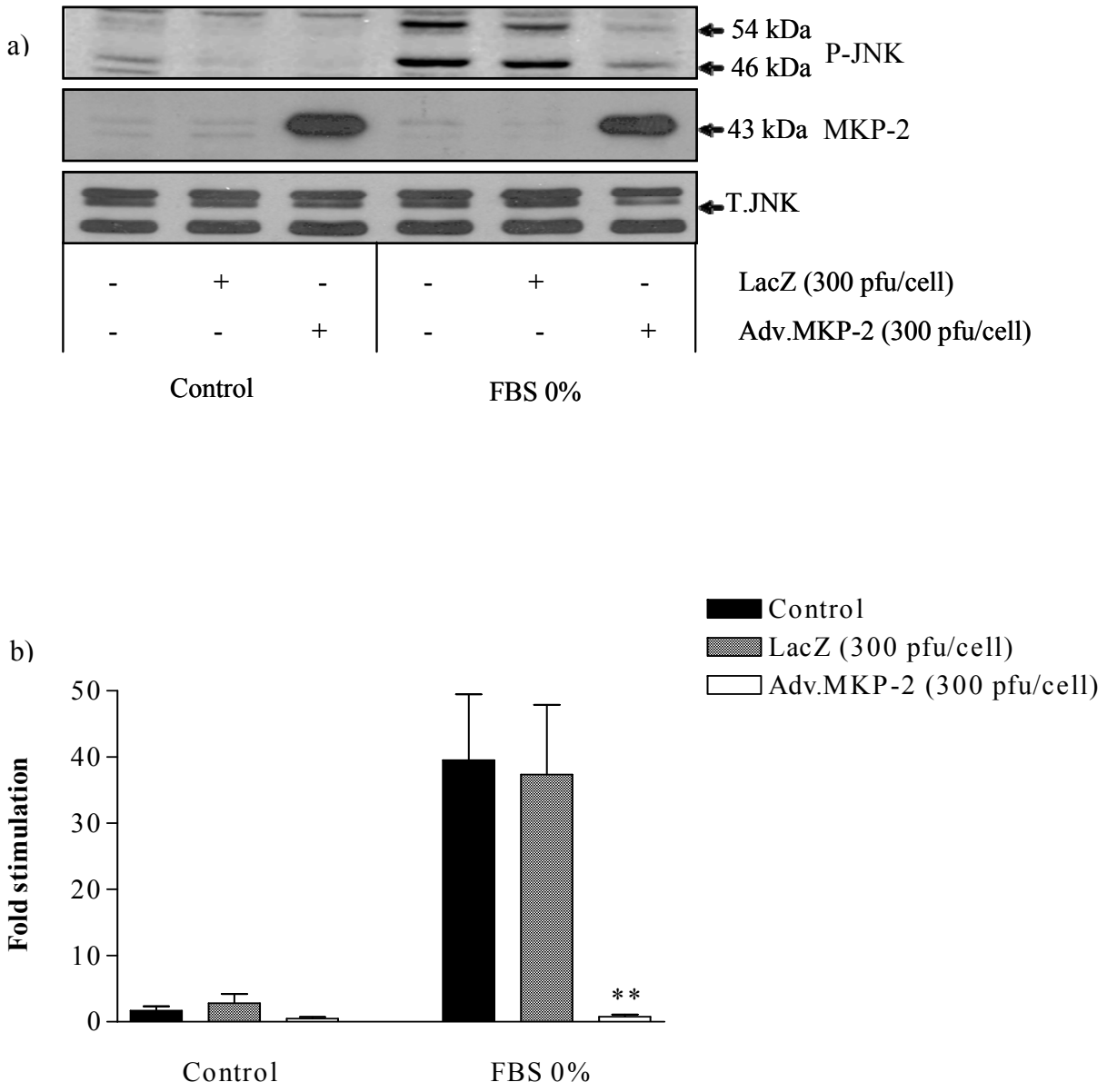


Figure 3.29: The effect of Adv.MKP-2 upon serum deprivation induced JNK phosphorylation in HUVECs: Cells were infected with Adv.MKP-2 for 40 hr prior to incubation in serum free media (FBS 0%) for 30 min. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for **a**) p-JNK (46/54 kDa), MKP-2 (43 kDa) and T.JNK as outlined in section 2.3. Blots were quantified for **b**) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. The results are representative of 3 independent experiments. **P<0. 01 compared with agonist-stimulated control.

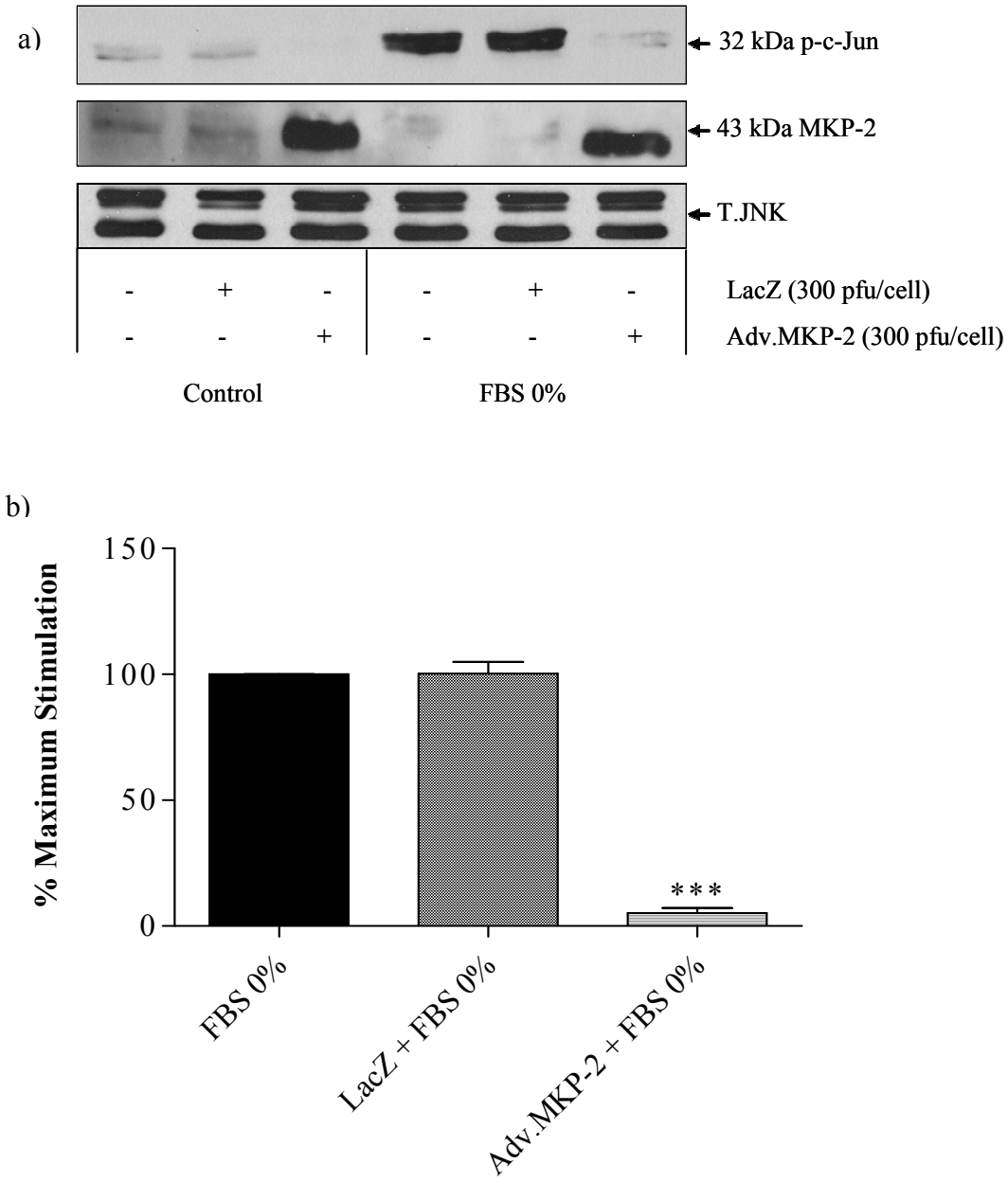


Figure 3.30: The effect of Adv.MKP-2 upon serum deprivation induced c-Jun phosphorylation in HUVECs: Cells were infected with Adv.MKP-2 for 40 hr prior to stimulation with H_2O_2 (0.2 mM) for 30 min. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for **a**) p-c-Jun (32 kDa), MKP-2 (43 kDa) and T.JNK as outlined in section 2.3. Blots were quantified for **b**) % maximum stimulation by scanning densitometry; each value represents the mean ± s.e.m. The results are representative of 3 independent experiments. ***P<0.001 compared with agonist-stimulated control.

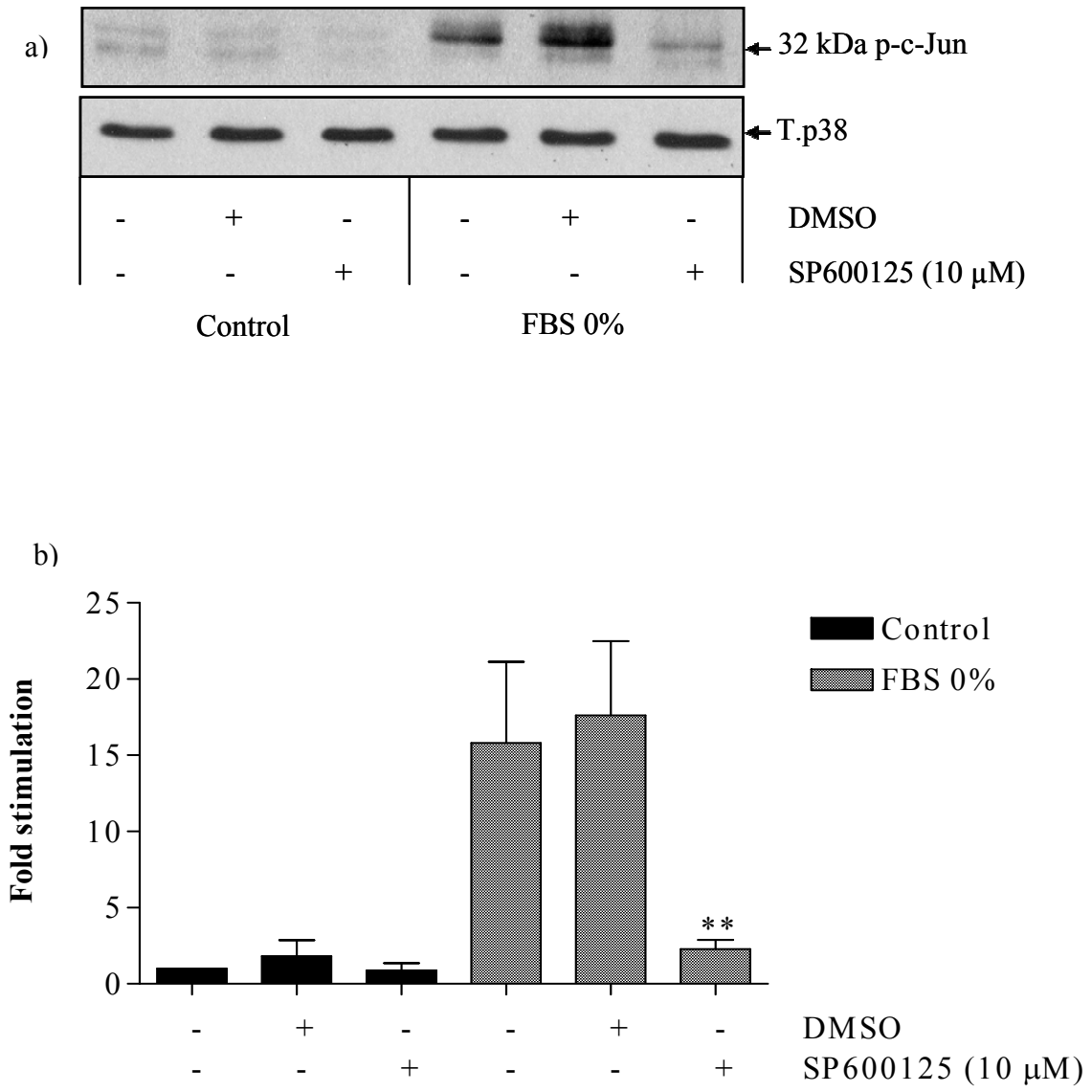


Figure 3.31: The effect of JNK inhibitor, SP600125 on c-Jun phosphorylation stimulated by serum deprivation in HUVECs: Cells were pre-treated with SP600125 (10 μM) for 1 hr prior incubation in serum free media (FBS 0%) for 30 min. Whole cell lysates were prepared, separated by SDS PAGE, and assessed for **a**) p-c-Jun (32 kDa) and T.p38 as outlined in section 2.3. Blots were quantified for **b**) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. The results are representative of 3 independent experiments. **P<0.01 compared with agonist- stimulated control.

3.7.3. The effect of over-expression of MKP-2 by infection with Adv.MKP-2 on γ-H2AX and PARP-1 phosphorylation in response to H_2O_2 or serum deprivation in HUVECs

Having established the effect of MKP-2 over-expression on γ-H2AX in response to TNF-α in combination with Adv.DNIKKβ (section 3.4.5), the effects upon H_2O_2 and serum deprivation were investigated. Figure 3.32 shows that cells stimulated with H_2O_2 (0.2 mM) and serum deprivation for 6 hr resulted in increased γ-H2AX phosphorylation of approximately 30 and 28 fold of basal values, respectively (FBS 0% fold stim. at 6 hr = 29.48 ± 5.98 and H_2O_2 fold stim. at 6 hr = 27.80 ± 1.5, n=3). These responses were not altered by infecting with LacZ, however, the response declined markedly when cells were infected with Adv.MKP-2 (Adv.MKP-2, FBS 0% fold stim. at 6 hr = 2.37 ± 0.29 and H_2O_2 fold stim. at 6 hr = 2.55 ± 0.14, $P<0.001$, n=3). Similarly, Adv.MKP-2 had a marked inhibitory effect on PARP-1 cleavage. Cells stimulated with H_2O_2 and FBS 0% for 24 hr gave substantial cleavage of the 116 kDa precursor to give the 89 kDa product (Figure 3.33). Whilst LacZ was without effect, cleavage was almost completely reversed following incubation of cells with Adv.MKP-2.

Furthermore, HUVECs were pre-treated with SP600125 for 1 hr before stimulation with H_2O_2 (0.2 mM) or FBS 0% for 6 hr. Figure 3.34 show that SP600125 at concentration of 10 μM significantly inhibited γ-H2AX phosphorylation mediated by H_2O_2 or FBS 0% treatment (H_2O_2 and FBS 0% fold stim. at 6 hr = 28.21 ± 5.09 and 24.73 ± 5.75 respectively, n=3), However, SP600125 as JNK inhibitor resulted in reducing the level of γ-H2AX phosphorylation by approximately 50% (SP600125 with H_2O_2 and FBS 0% fold stim. at 6 hr = 12.23 ± 3.64 and 13.73 ± 2.34, respectively, $P< 0.05$, n=3).

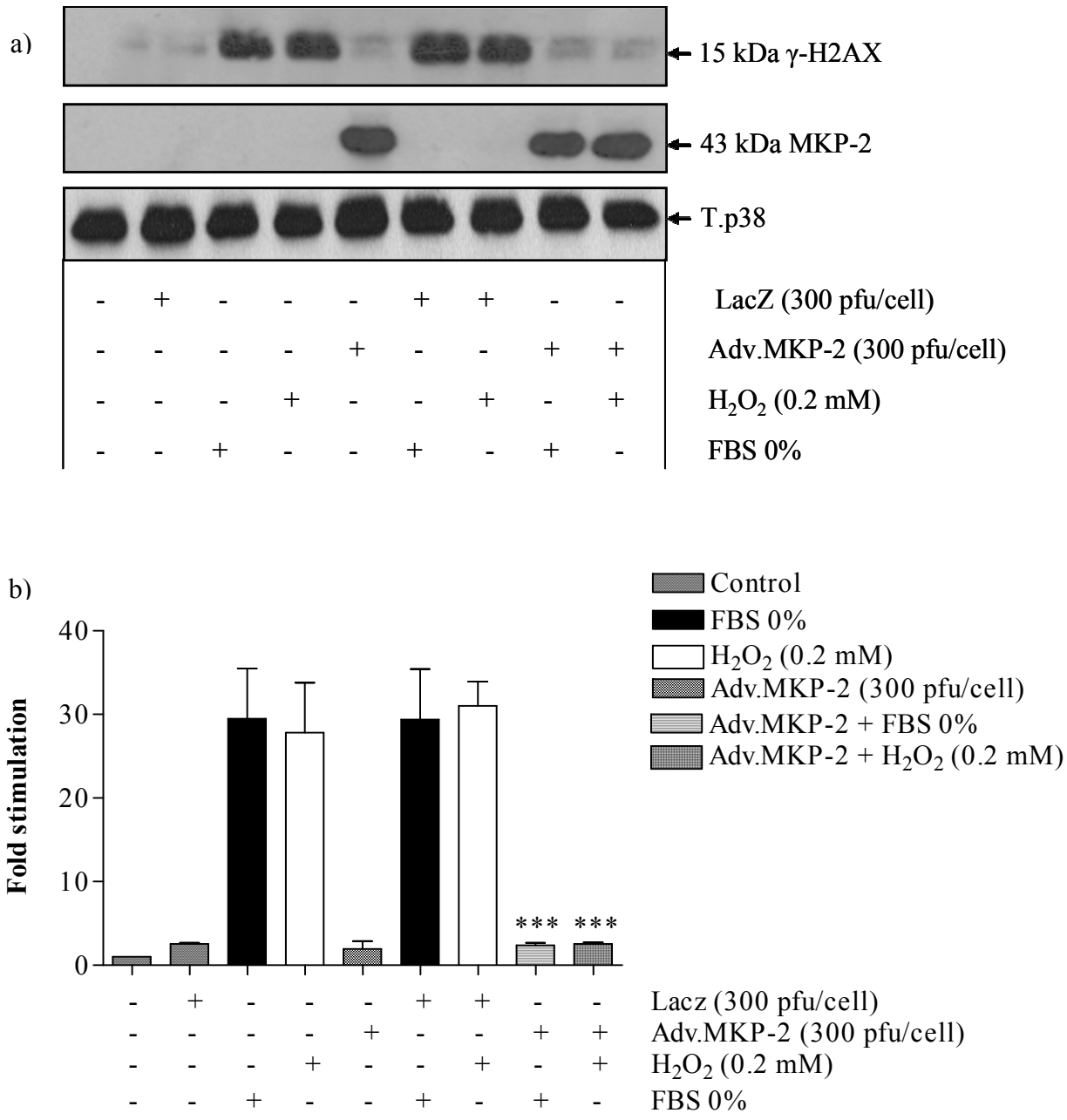


Figure 3.32: The effect of Adv.MKP-2 on H_2O_2 stimulated γ-H2AX phosphorylation in HUVECs: Cells were infected with Adv.MKP-2 for 40 hr prior to stimulation with H_2O_2 (0.2 mM) for 6 hr. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for **a**) γ-H2AX (15 kDa), MKP-2 (43 kDa) and T.p38 and as outlined in section 2.3. Blots were quantified for **b**) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. The results are representative of 3 independent experiments. ***P<0.001 compared with agonist-stimulated control.

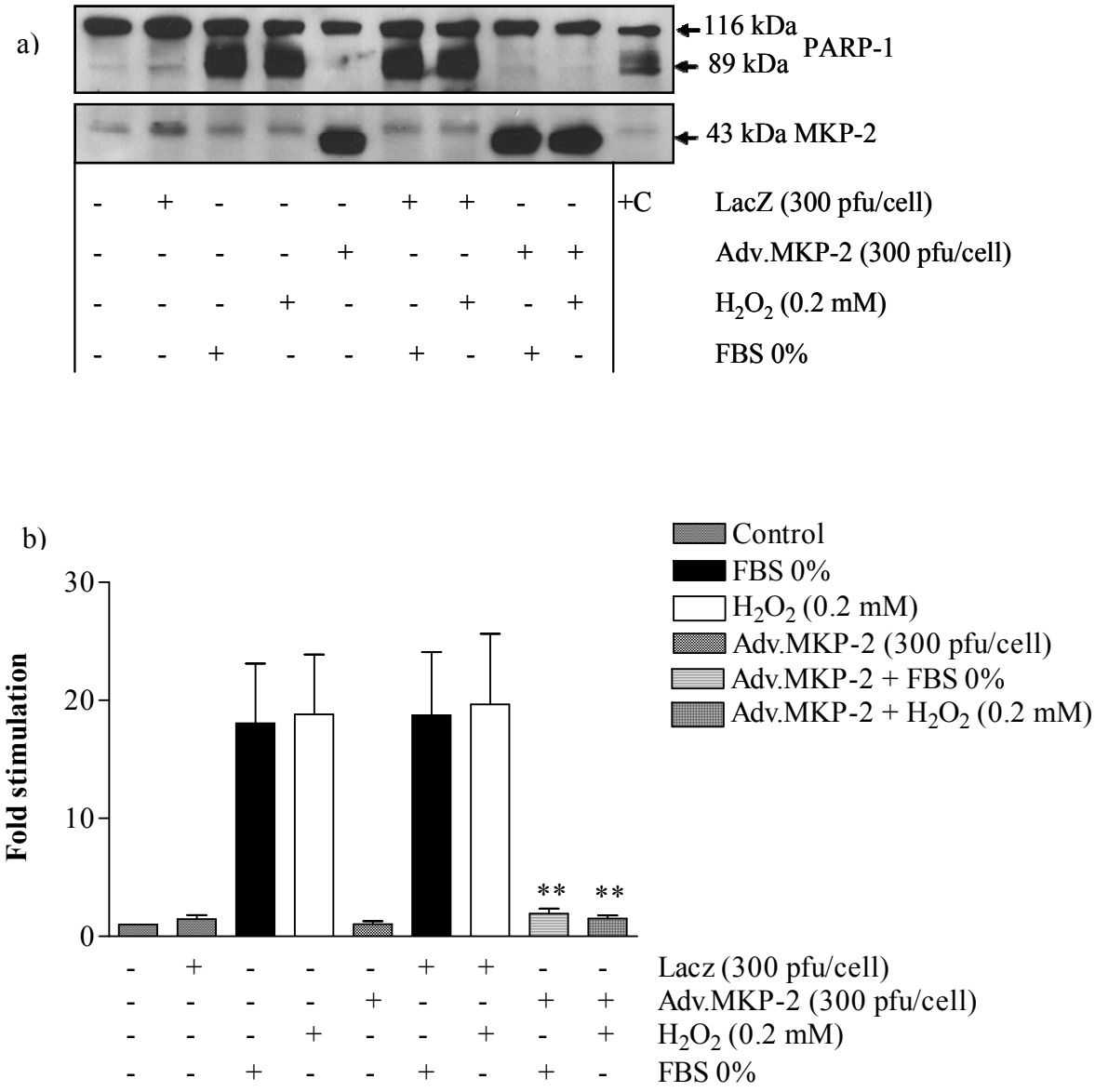


Figure 3.33: The effect of Adv.MKP-2 on PARP-1 phosphorylation in H_2O_2 and serum deprivation stimulated HUVECs: Cells were infected with 300 pfu/cell Adv.MKP-2 for 40 hr prior to stimulation with H_2O_2 (0.2 mM) or incubated in serum free media (FBS 0%) for further 24 hr. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for **a**) PARP-1 (116kDa), cleaved PARP-1 (89 kDa) and MKP-2 (43 kDa) as outlined in section 2.3. Blots were quantified for **b**) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. The results are representative of 3 independent experiments. **P<0.01 compared with agonist-stimulated control. **+C** (positive control, staurosporine 1 μM)

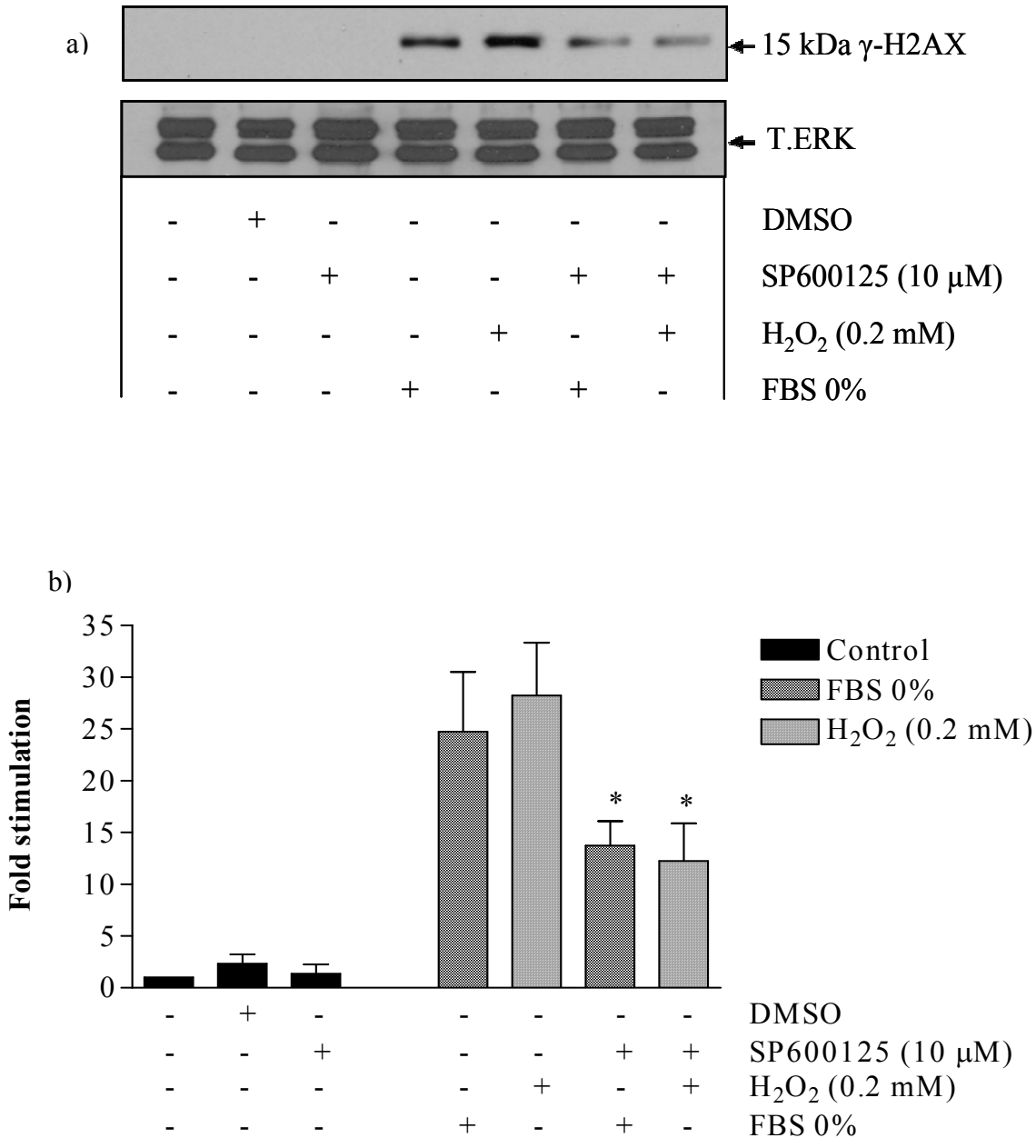


Figure 3.34: The effect of JNK inhibitor, SP600125 on γ-H2AX phosphorylation mediated by H_2O_2 and serum deprivation stimulated HUVECs: Cells were pre-treated with SP600125 (10 μM) for 1 hr before incubated with serum free media (FBS 0%) for 6 hr. Whole cell lysates were prepared, separated by SDS PAGE, and assessed for **a**) γ-H2AX (15 kDa) and T.ERK as outlined in section 2.3. Blots were quantified for **b**) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. The results are representative of 3 independent experiments. *P<0.05 compared with agonist-stimulated control.

3.7.4. The effect of over-expression of MKP-2 by infection with Adv.MKP-2 on Caspase-3 and 9 Cleavage in HUVECs in response to H_2O_2 and serum deprivation

The effect of Adv.MKP-2 upon the cleavage of cytosolic pro-apoptotic substrates was also examined (Figure 3.35). Cells stimulated with H_2O_2 (0.2 mM) or FBS 0% for 24 hr resulted in increased caspase-3 cleavage by approximately 40 fold of basal values, respectively (H_2O_2, fold stim. at 24 hr = 41.45 ± 10.86 and FBS 0% = 37.24 ± 7.68, $P<0.01$, n=3, respectively). These responses were essentially abolished when the cells were additionally infected with Adv.MKP-2 (Adv.MKP-2 + H_2O_2, fold stim. at 24 hr = 4.03 ± 1.47 or Adv.MKP-2 + FBS 0% = 4.26 ± 1.79 $P<0.01$, n=3, respectively).

A similar inhibition was observed for caspase-9 (Figure 3.36). Again stimulation with H_2O_2 or FBS 0% resulted in the cleavage of procaspase-9 to generate 35 and 37 kDa fragments. However, when cells were infected with Adv.MKP-2 (300 pfu/cell), the formation of cleaved fragments was essentially abolished ($P<0.001$).

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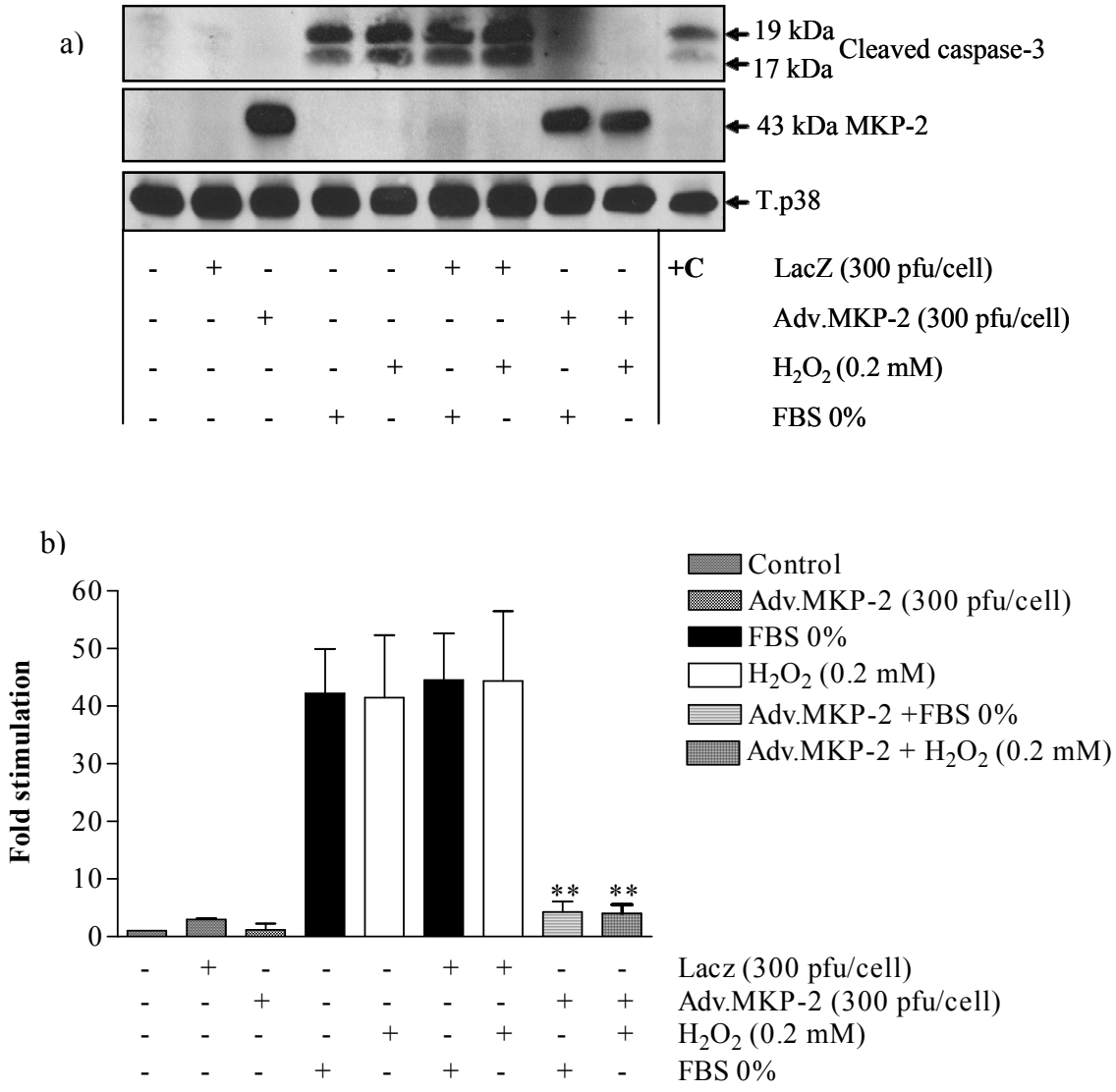


Figure 3.35: The effect of Adv.MKP-2 on Caspase-3 cleavage in H_2O_2 and serum deprivation stimulated HUVECs: Cells were infected with 300 pfu/cell Adv.MKP-2 for 40 hr prior to stimulation with H_2O_2 or incubation in serum free media (FBS 0%) for a further 24 hr. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for **a**) cleaved caspase-3 (17/19 kDa), MKP-2 (43 kDa) and T.p38 as outlined in section 2.3. Blots were quantified for **b**) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. The results are representative of 3 independent experiments. **P<0.01 compared with agonist- stimulated control. **+C** (positive control, staurosporine 1μM).

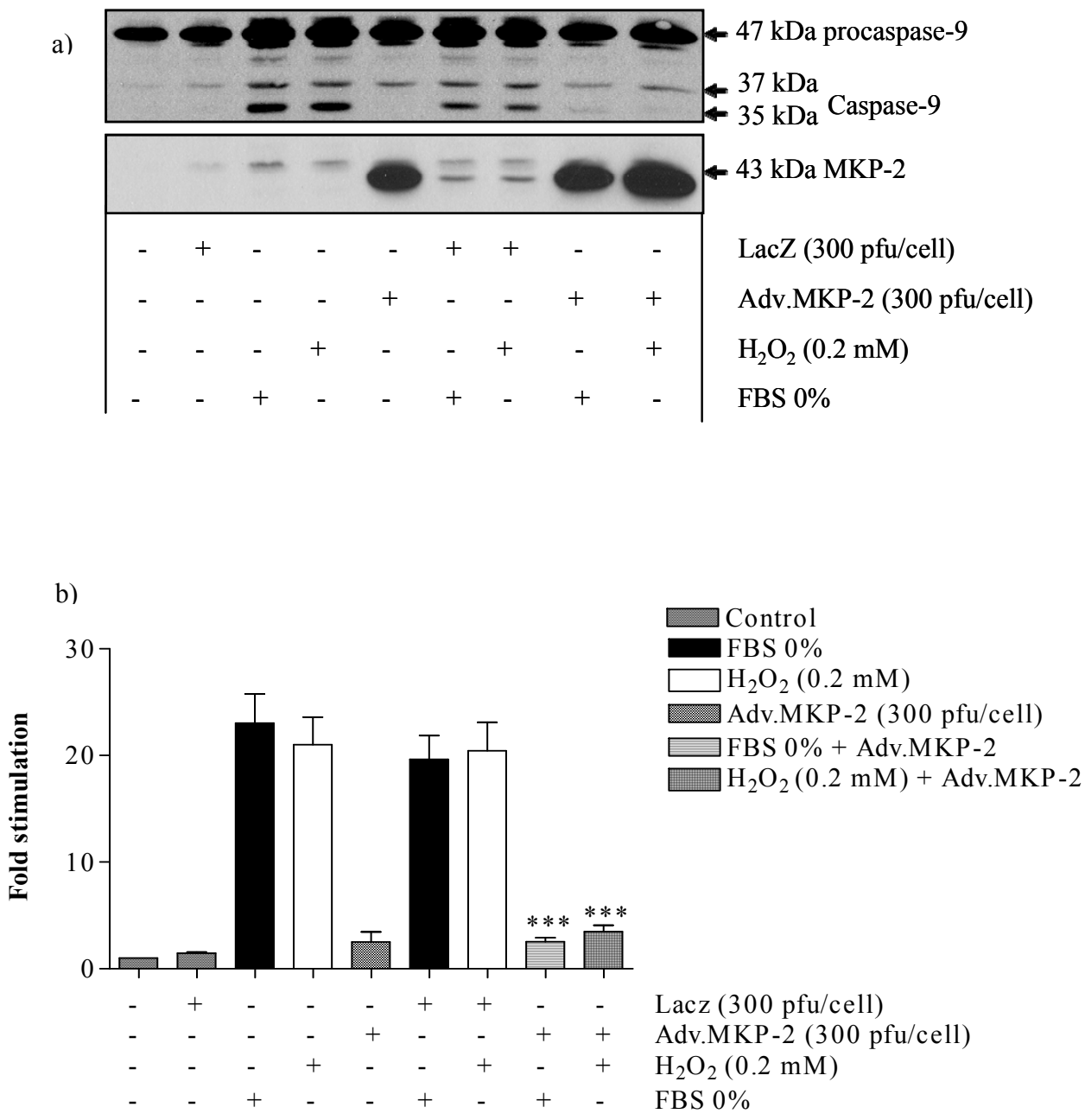


Figure 3.36: The effect of Adv.MKP-2 on Caspase-9 cleavage in H_2O_2 and serum deprivation stimulated HUVECs: Cells were infected with 300 pfu/cell Adv.MKP-2 for 40 hr prior to stimulation with H_2O_2 or incubation in serum free media (FBS 0%) for a further 24 hr. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for **a**) procaspase-9 (47 kDa), cleaved caspase-9 (35/37 kDa), MKP-2 (43 kDa) as outlined in section 2.3. Blots were quantified for **b**) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. The results are representative of 3 independent experiments. ***P<0.001 compared with agonist- stimulated control.

3.7.5. The effect of over-expression of MKP-2 by infection with Adv.MKP-2 on Cytochrome C release in HUVECs stimulated in response to H_2O_2 and serum deprivation

Having confirmed that TNF-α-induced HUVECs death involved translocation of JNK to the mitochondria where it triggers cytochrome c release into the cytosol (section 3.4.8), the effects of H_2O_2 and serum deprivation were also investigated (Figure 3.37). Cells stimulated with H_2O_2 (0.2 mM) or FBS 0% for 24 hr were fixed and stained with cytochrome c antibody as described in section 2.10. In control cells, cytochrome c staining was located in the mitochondrial (Figure 3.37 **a**). When cells were stimulated with H_2O_2 or FBS 0% for 24 hr, this resulted in release of cytochrome c into the cytosol (Figure 3.37 **b**). However, when cells were infected with Adv.MKP-2 (300 pfu/cell) for 40 hr and then stimulated with H_2O_2 or FBS 0% for a further 24 hr, this resulted in substantial inhibition of cytochrome c within the cytosol (Figure 3.37 **b & c)**.

3.7.6. Adv.MKP-2 infection reverses H_2O_2 and serum deprivation induced apoptosis in HUVECs

To confirm the potential for adenovirus encoding MKP-2 gene to reverse endothelial cell apoptosis, the effects upon H_2O_2 or serum deprivation (FBS 0%) were examined. HUVECs were infected with Adv.MKP-2 (300 pfu/cell) for 40 hr prior to stimulation with H_2O_2 (0.2 mM) or (FBS 0%) for a further 24 hr. Samples were assessed for Annexin V and 7-AAD staining as outlined in section 2.7 (Figure 3.38). Cells stimulated with H_2O_2 (0.2 mM) or FBS 0% showed significant increase in apoptosis (Control % cell death: H_2O_2 = 49.10 ± 10.00, FBS 0% = 37.06 ± 1.83, n=3). However, infecting cells with Adv.MKP-2 (300 pfu/cell) prior to stimulation with H_2O_2 or FBS 0% for a further 24 hr resulted in a significant decrease in apoptosis (% cell death, Adv.MKP-2 + H_2O_2 = 18.00 ± 1.49 and Adv.MKP-2 + FBS 0% = 9.53 ± 2.61, $P<0.01$, n=3).

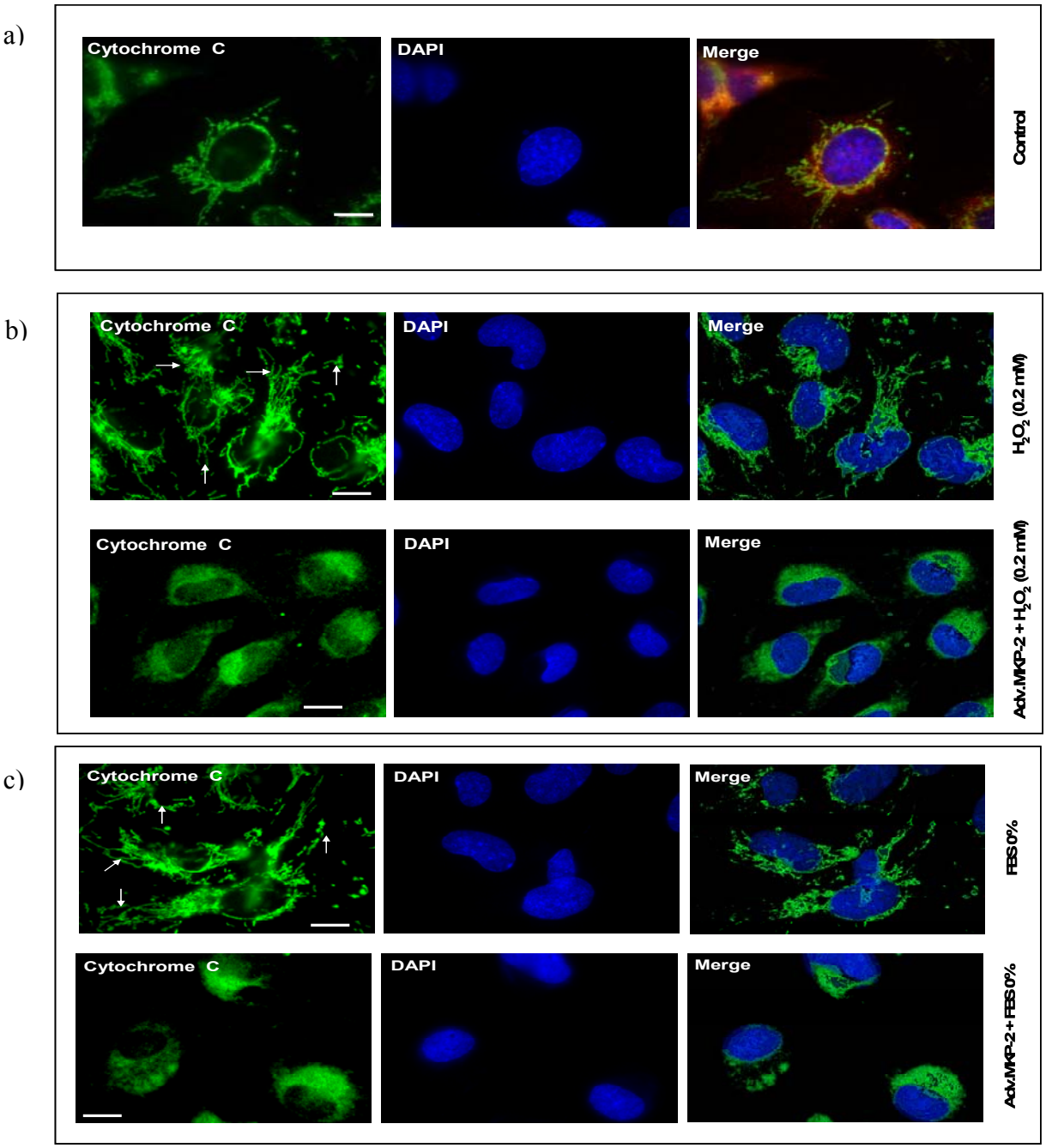


Figure 3.37: The effect of Adv.MKP-2 on Cytochrome c release in H_2O_2 and serum deprivation stimulated HUVECs: a) Control cells were fixed and stained with cytochrome c antibody (FITC, green) and DAPI (blue) as described in section 2.10. Cells were infected with 300 pfu/cell Adv.MKP-2 for 40 hr prior to stimulation with **b**) H_2O_2 (0.2 mM) or **c**) serum deprivation (FBS 0%) for a further 24 hr. Cells were then fixed and stained with cytochrome c antibody (FITC), DAPI. Arrows indicate cytosolic cytochrome c release into the cytosol. All cells were viewed x100 magnification, using a Nikon EP-1 fluorescent microscope. The results are representative of a single experiment (scale bar =10μm).

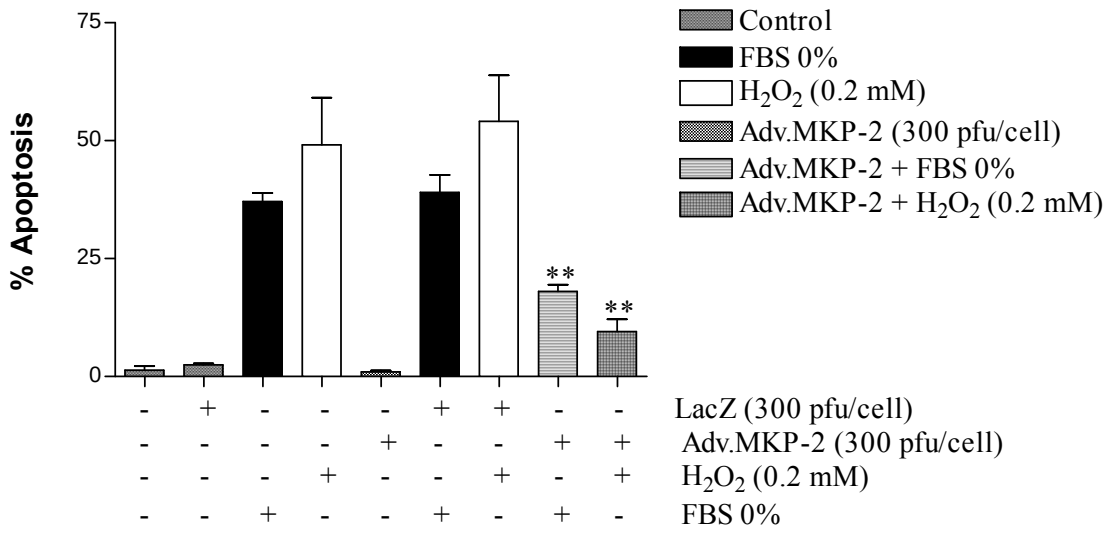


Figure 3.38: Adv.MKP-2 infection inhibits H_2O_2 and serum deprivation induced apoptosis in HUVECS.

HUVECs were infected with Lac Z (300 pfu/cell) or Adv.MKP-2 (300 pfu/cell) for 40 hr prior to stimulation with H_2O_2 (0.2 mM) or incubated in serum free media (FBS 0%) for a further 24 hr. Samples assessed for Annexin V and 7-AAD staining as outlined in section 2.7. Results were quantified for % apoptosis; each value represents the mean ± s.e.m. of at least 4 experiments. **P<0.01 compared with agonist- stimulated control.

3.7.7. The effect of SP600125 on H_2O_2 and serum deprivation induced apoptosis in HUVECs

The effect of SP600125 on endothelial cell apoptosis was also investigated using H_2O_2 (0.2 mM) or serum deprivation (FBS 0%). Cells stimulated with H_2O_2 or FBS 0% for 24 hr gave an 8-10 fold increase endothelial cell death (Control % cell death: H_2O_2 = 51.43 ± 6.36 and FBS 0% = 41.57 ± 7.16, respectively, n=3) (Figure 3.39). However, when cells were pre-treated with SP600125 (10 μM) for 1 hr prior to stimulation with H_2O_2 (0.2 mM) or FBS (0%) for a further 24 hr, there was a substantial and significant decrease in apoptosis of approximately 50% (Control % apoptosis: H_2O_2 = 28.18 ± 8.56 and FBS 0% = 20.30 ± 8.35, respectively, $P<0.05$, n=3).

Taken, together these studies suggest that inhibition of JNK is sufficient to partially inhibit apoptosis but pharmacological blockade is clearly less effective than with Adv.MKP-2.

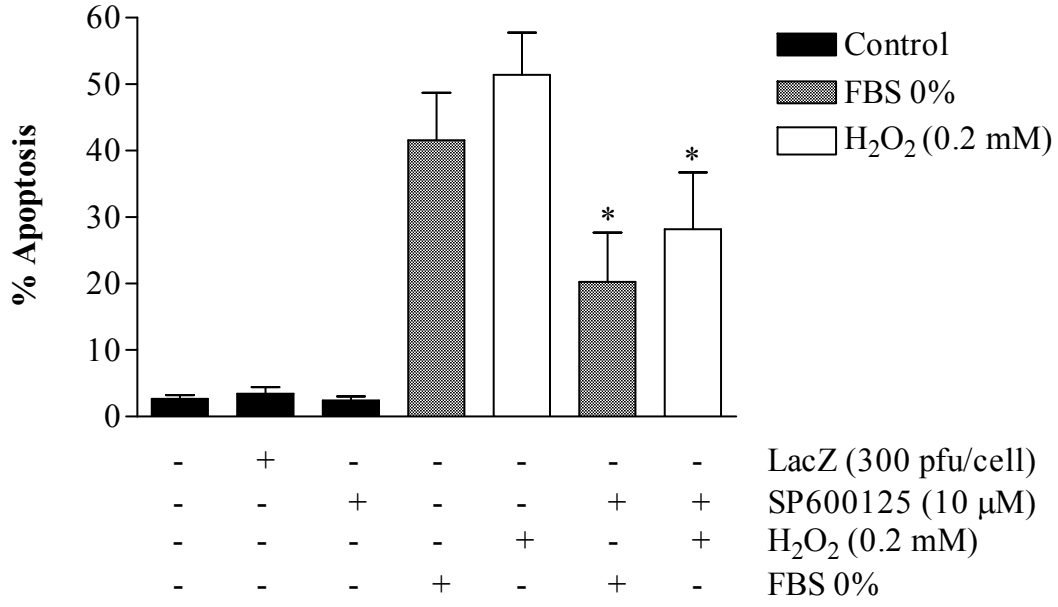


Figure 3.39: Effect of SP600125 on H_2O_2 and serum deprivation induced apoptosis in HUVECS.

HUVECs were pre-treated with SP600125 (10 μM) for 1 hr before stimulation with H_2O_2 (0.2 mM) or incubated in serum free media (FBS 0%) for a further 24 hr. Samples where assessed for Annexin V and 7-AAD staining as outlined in section 2.7. Results were quantified for % apoptosis; each value represents the mean ± s.e.m. of at least 3 independent experiments. *P<0.05 compared with agonist- stimulated control.

3.8. Discussion

In this study the adenoviral mediated over-expression of MKP-2 was utilised as an experimental pharmacological tool to reverse JNK-mediated responses in HUVECs and thus determine its potential use in clinical conditions involving endothelial cell apoptosis. The effect of TNF-α on the up-regulation of pro-apoptotic protein expression under conditions of NF-κB inhibition were extensively investigated. Over-expression of MKP-2 showed a substantial reduction in the up-regulation of several pro-apoptotic proteins and at the same time indicated a possible cross-talk mechanism involving an interaction with the NF-κB signalling pathway. Moreover, this work also demonstrated that over-expression of MKP-2 could be equally effective in regulating JNK mediated apoptosis in response to other stressful agents such as H_2O_2 or serum deprivation. This effect was more effective compared with JNK inhibitor SP600125. Furthermore, our results revealed important information about the location of JNK during apoptosis implying a strictly nuclear action.

Initial studies in HUVECs demonstrated that TNF-α stimulated a very strong activation of JNK but a much smaller stimulation of either p38 MAP kinase or ERK. These observations were in agreement with previous results in endothelial cells and other cell lines (Kuldo et al., 2005, Yoshizumi et al., 2004, Hippenstiel et al., 2000). The pattern of JNK phosphorylation induced by TNF-α was essentially similar, TNF-α mediated a transient activation of JNK. This data was again similar to previous findings in endothelial cells and other systems (Karmann et al., 1996, Rumora et al., 2001, Ventura et al., 2004, Westwick et al., 1994, Gao and Ji, 2008, Mong et al., 2008). JNK has been shown to phosphorylate members of the AP-1 transcription factor complex such as c-Jun, Jun B and Jun D. It has also been established that JNK regulated AP-1 transcriptional activity *in vivo* is due, in part, to an effect of JNK (Davis, 2000), however, this was not directly investigated in this chapter. Rather, the activation of c-Jun in response to TNF-α was demonstrated and again the pattern of phosphorylation was similar. The importance

of c-Jun activation in regulating signalling through JNK has been previously described; fibroblasts containing a mutant form of c-Jun demonstrated impaired activation of JNK in response to number of stimuli (Sprowles et al., 2005). This and other studies have confirmed the importance of c-Jun in controlling the efficiency of MAP kinases by transcriptional repression of MAP kinase phosphatases.

Previous studies have addressed the hypothesis that prolonged JNK activation is important in TNF-α induced apoptosis. Pober et al. (2008) showed that ligand engagement of TNF-R1 leads to recruitment to this receptor of the death domain-containing proteins TRADD and RIP1, which can then form one of two complexes. Complex1, which contains TRAF2, binds to the cytosolic tail of TNF-R1 to promote activation of NF-κB and thereby cell survival, whilst complex II, which is assembled when TRADD and RIP1 are ubiquitinated, localizes instead to cytosol where it associates with FADD, which in turn recruits and activates pro-caspase-8 and -10, leading in the end to cell death (Pober et al., 2008). However the efficacy of activation of this part of the cascade is tightly controlled by NF-κB which regulates the expression of a number of anti-apoptotic proteins. TNF-α in combination with Adv.DNIKKβ was shown to mediate sustained activation of both JNK and c-Jun, consistent with previous studies which have shown TNF-α mediated apoptosis in HUVECs, but only in conditions where NF-κB is directly inhibited (Stehlik et al., 1998), or where protein synthesis is prevented (Wadgaonkar et al., 2004). Moreover, blocking NF-κB by ablation of IKKβ impaired the shut-down of TNF-α mediated JNK signalling (De Smaele et al., 2001). Furthermore, a number of studies have shown that in endothelial and other cell types, activation of NF-κB inhibited prolonged JNK activation in response to TNF-α through the increased expression of a number of protective proteins including gadd45β (De Smaele et al., 2001, Papa et al., 2004a, Papa et al., 2008) and XIAP (Tang et al., 2001). This intervention prolonged JNK1 activation, resulting in apoptosis (Tang et al., 2002). Other recent studies also demonstrated that several anti-oxidant enzymes such as Mn-SOD and Feritin heavy chain (FHC) are under NF-κB regulation and may also contribute to limiting JNK activity through inhibiting ROS formation (Papa et al., 2006). Thus, the NF-κB mediated suppression of prolonged JNK activation is crucial for inhibition of TNF-α induced

apoptosis. Indeed, studies have demonstrated that transient activation of JNK is normally a signal for cell growth and differentiation (Papa et al., 2006, Karin, 2006). Deletion of NF-κB/RelA or pre-treatment with inhibitors of IKKβ caused liver apoptosis, and this was inhibited by mutation of JNK-1(Chang et al., 2006).

In this chapter sustained JNK activation was observed in response to H_2O_2 and serum deprivation agents which also initiate apoptosis. These results were consistent with several other studies (Xu et al., 2004, Robinson et al., 2001, Murakami et al., 2005). The concentration of H_2O_2 used in this study was 0.2 mM (Murakami et al., 2005, Jiang et al., 2009) which is low in comparison to other studies which physiological stimuli, such as PDGF, can induce a transient increase in the intracellular concentration of H_2O_2 in vascular smooth muscle cells which is equivalent to an extracellular concentration in the range of 0.1-1.0 mM (Meng et al., 2002, Sundaresan et al., 1995). Thus, the concentration of H_2O_2 used in this study would be likely to reflect a physiologically relevant condition. This is important as dual effects may be manifest dependent on the concentration used. In endothelial cells, rather than contribute to oxidative stress, endogenous ROS which are generated from mitochondria may have normal physiological signalling functions, activating "second messengers" that regulate EC growth, proliferation and vascular remodelling (Pober and Min, 2006). However, several another studies implicate ROS production in the cellular apoptosis (Robinson et al., 2001, Papa et al., 2004b, Stehlik et al., 1998, Ho et al., 2000)

Differential sensitivity to ROS is relevant to the activation of JNK. For example, under normal conditions the redox regulatory protein thioredoxin (Trx) binds to and inhibits the activity of apoptosis signal-regulating kinase 1 (ASK1), a MAPKK kinase involved in both JNK and p38 MAP kinase activation. However, severe oxidative stress causes dissociation of the Trx-ASK1 complex leading to activation of JNK and p38 MAP kinase (Saitoh et al., 1998, Adler et al., 1999, Song and Lee, 2003, Liu and Min, 2002). Pro-apoptotic stimuli such as TNF-α and oxidative stress dissociate Trx1/Trx2 from ASK1, leading to enhanced mitochondrial-dependent apoptosis characterized by cytochrome c release, caspase-3 activation, and nuclear fragmentation (Zhang et al., 2004). Also, ROS

may induce the release of ASK1 from its cellular inhibitors such as protein 14-3-3, which in unactivated ECs binds to phosphoserine ^{967} on ASK1 (Zhang et al., 1999). H_2O_2 also mediates the phosphorylation of PKD, translocation form the EC plasma membrane to the cytoplasm, and association with ASK1, which facilitates ASK1 oligomerization and autophosphorylation at Thr-845, leading to ASK1 activation. At the same time H_2O_2 induces 14-3-3 binding which is important for PKD-mediated ASK1-JNK signalling. Inhibition of PKD by pharmacological inhibitors or siRNA blocks H_2O_2 induced ASK1-JNK activation and EC apoptosis (Zhang et al., 2005).

Moreover, biochemical evidence indicates that under normal conditions glutathione S-transferase (GST) binds to JNK and inhibits its activation, but that interaction is disrupted by oxidative stress (Adler et al., 1999). Several studies have provided evidence that JNK activation by H_2O_2 and/or stresses that affect the cellular redox state occurs in part through suppression of phosphatases involved in JNK inactivation (Kamata and Hirata, 1999). Also, through phosphorylation of Bcl-2 and Bax (Murakami et al., 2005). Recent study has also suggested other mechanisms for JNK activation which can be activated following H_2O_2 treatment through an EGFR and PDGF-dependent mechanism (Chen et al., 2001a, Chen et al., 2004). Thus, ROS might act at multiple levels in the JNK signalling to regulate its activities.

The results in this chapter also revealed that depriving HUVECs of serum for up to 24 hr elicited a significant increase in JNK phosphorylation. In this study serum deprivation was used to generate a component of ischemia *in vitro* (Bielawska et al., 1997, Fujio et al., 2000). These results confirmed previous studies which showed that these kinases become active in response to the stress of serum deprivation in a variety of cells, including human microvascular ECs (Gupta et al., 1999). A more recent study showed that dominant negative mutant of JNK inhibited serum deprivation-induced apoptosis in HUVECs (Harfouche et al., 2003).

The results in this chapter showed that prolonged JNK activation led to sustained c-Jun phosphorylation mediated transcription of genes that are involved in apoptosis including

γ-H2AX, PARP-1 and also the cytosolic targets caspases 3 and 9 and cytochrome c. Evidence has been accumulated indicating a role for c-Jun/AP-1 in apoptosis, functioning as both a positive and a negative modulator of apoptotic pathways in different cell types (Liebermann et al., 1998). For example, when rat sympathetic neurons undergo apoptosis during nerve growth factor withdrawal, the levels of c-Jun mRNA and protein increase (Estus et al., 1994, Ham et al., 1995). Microinjection of neutralizing antibodies specific for c-Jun, or over-expression of a dominant-negative c-Jun mutant, is able to protect nerve growth factor–deprived sympathetic neurons from apoptosis. However, somewhat contradictory evidence has also been published. It has been shown that c-Jun is not essential for apoptosis *in vivo* during normal development, as c-Jun$^{-/-}$ mouse embryos exhibited increased rather than reduced apoptosis in their livers. These authors concluded that AP-1 may have a protective role against apoptosis (Roffler-Tarlov et al., 1996). Several studies in other cell types also indicate that c-Jun induction either has no direct relationship to apoptosis or has an inhibitory role (Shimizu et al., 1996, Sabapathy and Wagner, 2004). Moreover, TNF-α induced apoptosis in IKKβ$^{-/-}$ fibroblasts is independent of *de novo* protein synthesis as it occurs in the presence of the protein synthesis inhibitor CHX (Lin and Dibling, 2002).

Nevertheless, the results obtained from other types of cell system indirectly lead to speculation that c-Jun activation may be involved in H_2O_2-mediated EC apoptosis. Firstly, c-Jun/AP-1 has been known as a major transcription factor responsive to the cellular redox state. Secondly, both c-Jun expression and AP-1 binding activity can be induced by H_2O_2. In addition, in glomerular mesangial cells, disruption of c-Jun/AP-1 inhibits H_2O_2- initiated apoptosis (Ishikawa et al., 1997). Consistent with this work, other studies reveal an up-regulation of c-Jun in HUVECs after H_2O_2 treatment (Wang et al., 1999). This data is consistent with previous observations in human microvascular ECs that show H_2O_2 induces an AP-1 binding complex containing c-Jun (Shono et al., 1996). The activity of c-Jun is also known to be increased in response to serum deprivation in HUVECs (Liu et al., 2001) and this regulates HUVECs viability (Chen et al., 2006). Therefore, prolonged JNK activation may exert its pro-apoptotic effect, at least in part, through c-Jun but it's likely that other proteins may be involved.

Having established that TNF-α (in the presence of Adv.DNIKKβ), H_2O_2 and serum deprivation can result in sustained JNK and c-Jun activation, the ability of these apoptotic agents to initiate activation of others targets which may be involved in apoptosis was examined. All three conditions resulted in the cleavage of PARP-1, a well recognised DNA damage sensor (Krishnakumar and Kraus, 2010) and phosphorylation of the histone protein H2AX which has recently been shown to be involved in regulating cellular responses to DNA damage (Kinner et al., 2008). Previous studies indicated that γ-H2AX was phosphorylated by the phosphatidylinositol-3 kinase-related kinases (PIKK) group of kinases at Ser^{139} (Daniel et al., 2004a, Fernandez-Capetillo et al., 2004). However, this chapter demonstrated γ-H2AX phosphorylation by JNK in agreement with recent studies in UV-treated Hela cells and IL-1β stimulated human chondrocytes (Nieminen et al., 2005, Lu et al., 2006). Phosphorylation of γ-H2AX by JNK activation has been found to be associated with the formation of the DNA double strand breaks (DSB) in jurkat cells following UVC exposure (Sluss and Davis, 2006, Rogakou et al., 2000). Phosphorylation of γ-H2AX through the JNK pathway has also recently been demonstrated in TNF-α stimulated fibroblasts (de Feraudy et al., 2010) and neurons (Dadakhujaev et al., 2009). Furthermore, it has been shown that JNK1 siRNA effectively suppressed UVA induced γ-H2AX phosphorylation in MEFs (Lu et al., 2006). These findings suggested that γ-H2AX phosphorylation is essential for DNA fragmentation associated with apoptosis through JNK activation.

Experiments in this chapter show that phosphorylation of γ-H2AX was correlated with caspase cleavage in response to TNF-α /Adv.DNIKKβ, H_2O_2 or serum deprivation. This was in agreement with a recent study which reported increased level of γ-H2AX phosphorylation coincident with activation of caspase-3 (Karreman et al., 2009), whilst other work has demonstrated JNK-mediated H2AX phosphorylation through the caspase-3/caspase-activated DNase (CAD) pathway (Lu et al., 2006). In addition, a key finding was that γ-H2AX phosphorylation could be blocked by the treatment of Jurkat and HL60 cell lines with a caspase inhibitor (Rogakou et al., 2000). These observations suggest that γ-H2AX is a consequence of apoptosis and downstream of the major effect of caspases.

This study also demonstrated that caspases are activated in response to the treatments as outlined above. This included the generation of p17 and p19 caspase-3 fragments and the generation from caspase-9, of 35 and 37 kDa fragments. These findings were consistent with previous studies which have implicated caspase-3 and caspase-9 in endothelial cell apoptosis in response to TNF-α in the presence of CHX (Daniel et al., 2004b, Hermann et al., 1997) and in response to a number of stressful agents such as high glucose and H_2O_2 (Ho et al., 2000, Ramachandran et al., 2002), mediated in turn by JNK activation. Immunofluorescence staining demonstrated release of cytochrome c from the mitochondria into the cytosol in response to TNF-α /Adv.DNIKKβ, H_2O_2 or serum deprivation. This finding was consistent with previous studies which have demonstrated that JNK is required for UV-induced apoptosis in primary murine embryonic fibroblasts (MEFs), due to activation of the mitochondrial death signalling pathway, including the release of cytochrome c (Tournier et al., 2000). Consistent with these findings it has been shown recently that translocation of JNK to the mitochondria was essential in triggering cytochrome c release into the cytosol in HEK293 cells in response to genotoxic stress (Cadalbert et al., 2005). Serum deprivation for 24 hr also caused release of cytochrome c from the mitochondria to the cytosol in HUVECs (Williams et al., 2006). This release resulted in apoptosome formation followed by the activation of pro-caspase-9, which in turn cleaves downstream effector caspase-3. Taken together these studies suggest that in addition to nuclear substrates JNK can influence apoptosis by targeting the mitochondria.

In order to assess the involvement of JNK activation in TNF-α, H_2O_2 or serum deprivation induced endothelial cells apoptosis, an adenoviral construct of mitogen-activated protein kinase phosphatase-2 (MKP-2) was utilised. Over-expression of MKP-2 via this route gave rise to a functional protein which was able to dephosphorylate and inactivate JNK and prevent downstream phosphorylation of c-Jun. Previous studies performed in our laboratory have shown that stable over-expression of MKP-2, resulted in a similar inhibition of JNK activation in EAhy 926 endothelial cells (Robinson et al., 2001). These results were also in agreement with recent studies which demonstrated that induction of MKP-2 in an *in vivo* mouse model as well as in cell lines led to inhibition of

JNK activity stimulated in response to gonadotropic hormones (Zhang and Roberson, 2006). Another recent study showed MKP-2 over-expression resulted in an inhibition of apoptosis in U937 myeloid leukaemia cells by dephosphorylating JNK (Wang et al., 2007a) and that conditional expression of MKP-2 protected against genotoxic stress mediated apoptosis in HEK293 cells (Cadalbert et al., 2005). Moreover, another recent study demonstrated that suppressing MKP-2 by siRNA in HEK 293 cells led to prolonged JNK activation in response to H_2O_2 treatment (Teng et al., 2007).

These data and the experiments shown in this chapter suggest that the specificity of MKP-2 is towards the JNK group of MAP kinases but not ERK or p38 MAP kinase. One possible reason for this is the lack of nuclear translocation of ERK and p38 MAP kinase stimulated by TNF-α, however, this possibility was not examined in the present study. However, a lack of ERK dephosphorylation is observed in cells either constitutively expressing MKP-2 (Robinson et al., 2001) or as demonstrated previously in the laboratory, following adenoviral infection (Al-Mutairi at al. 2010, submitted for publication). Other studies have clearly demonstrated that MKP-2 is expressed exclusively within the nucleus due to the presence of two nuclear localisation sequences (Chen et al., 2001b, Sloss et al., 2005), confirming that inhibition of JNK is likely to have occurred within the nucleus. As outlined in chapter-1, MKP-2 is able to bind strongly to p38 MAP kinase whilst being unable in the majority of studies to enhance dephosphorylation of this kinase (Chen et al., 2001b, Robinson et al., 2001, Cadalbert et al., 2005). One approach to ensure more selective specificity of MKP-2 would be to generate a mutated version of the protein which would lack the MKB binding domain (see Introduction). Recently a novel variant of MKP-2 lacking the ERK binding domain has been identified (Cadalbert et al., 2010) which despite being unable to affect ERK activity nevertheless retains the ability to dephosphorylate JNK.

Having established that over-expression of MKP-2 displayed selectivity towards JNK, the effect upon signalling pathways leading to nuclear and cytosolic pro-apoptotic protein activation was investigated. As shown in the results section 3.3.4, MKP-2 essentially abolished the phosphorylation of γ-H2AX and PARP-1 in response to stimulation with

TNF-α in combination with Adv.DNIKKβ, H_2O_2 or serum deprivation. These findings were consistent with previous studies which have demonstrated that MEFs transfected with stable DN-JNK1 strongly inhibited UVA-induced phosphorylation of γ-H2AX through inhibition of JNK (Lu et al., 2006), implicating as expected, a nuclear action of MKP-2. Indeed, over-expression of MKP-1, a nuclear DUSP increased MCF-7 resistance to H_2O_2 induced cell death through inhibition of JNK and PARP-1 (Zhou et al., 2006).

Interestingly, the present study has also demonstrated that over-expression of MKP-2 was able to abolish the formation of cleaved caspase-3 proteins at Asp^{28} and Asp^{175} to generate p19 and p17 subunits. This finding was consistent with a recent study which showed that MKP-1-deficient MEFs responed to anisomycin with enhanced caspase-3 cleavage and apoptosis compared with wild type (Wu and Bennett, 2005). This same study suggested that MKP-1 may function in an anti-apoptotic manner. Moreover, it has been demonstrated that MKP-1 was over-expressed in the early stage of prostate cancer progression and this was associated with decreased caspase-3 cleavage and cell death (Srikanth et al., 1999). Data from these studies imply that both MKP-1 and 2 can regulate caspase cleavage suggesting the potential of caspase translocation to the nucleus. The translocation of caspase fragments has been shown to be dependent upon prior cleavage of procaspase-3 in the cytosol and association with recognised substrates (Kamada et al., 2005). This observation was consistent with previous studies which identified a possible link between cytosolic caspase activation and apoptotic nuclear events (Enari et al., 1998, Sakahira et al., 1998). Whilst the precursor form of caspase-3 was localised to the cytoplasm, caspase-3 played important roles in the nuclear changes in apoptotic cells (Zheng et al., 1998). The same study has shown that some cytoplasmic substrates translocated into the nucleus after cleavage by caspase-3 leading to nuclear morphological changes. In this scenario, caspase-activated DNase (CAD), DNA fragmentation factor (DFF) 40 and apoptotic chromatin condensation inducer in the nucleus (Acinus) were identified in the cytoplasmic fraction of apoptotic cells (Sahara et al., 1999). On the other hand, CAD/DFF40 and Acinus have been shown to be localised in the nucleus even before apoptosis induction and initiation of chromosomal degradation (Lechardeur et al., 2000). Thus, many substrates for caspase-3 have been shown to

translocate from the cytoplasm into the nucleus after apoptosis induction, and it has been proposed that the active caspase-3 is translocated into nuclei by simple diffusion after disruption of the nuclear-cytoplasmic barrier (Faleiro and Lazebnik, 2000). The mechanism regulating nuclear translocation of active caspase-3 is still unclear and was not assessed in this study but the evidence above leads to the expectation that caspase-3 remains cytosolically located prior to cleavage.

Therefore, as MKP-2 is strictly nuclear located, even after infection of the Adv.MKP-2 at high pfu, this suggests that to regulate caspase activation in endothelial cells, JNK must be firstly activated within the nucleus before translocation to the cytosol. As indicated previously current dogma suggests roles for JNK both within the nucleus, possibly via c-Jun, and mitochondria (Dhanasekaran and Reddy, 2008), however, a functional link between each compartment has not been defined. Recent studies have demonstrated the involvement of nuclear and mitochondrial phases of JNK activation in mediating apoptosis in response to stress challenge (Cadalbert et al., 2005) and the role of the different JNKs (JNK1 and 2) in this process (Eminel et al., 2004, Cadalbert et al., 2005). These studies suggested interdependence between the two phases of JNK activation, the latter phase depending upon the earlier nuclear phases. This hypothesis is in agreement with Tournier et al., (2000) who previously demonstrated that cytochrome c release from mitochondria is induced by nuclear JNK activation in response to DNA damage (Tournier et al., 2000). Moreover, it is possible that a pool of MKP-2 is located in the mitochondria, as is the case with MKP-1 which functions to directly regulate JNK activity within this compartment (Uzgare et al., 2003, Rosini et al., 2004, Vicent et al., 2004). To confirm this idea would require the use of confocal microscopy.

To further confirm the functional selectivity of MKP-2 for JNK in relation to apoptosis, a pharmacological JNK inhibitor, SP600125, was used. The findings replicate previous studies using SP600125 which show inhibition of JNK signalling (Huang et al., 2007, Shimada et al., 2007), phosphorylation of c-Jun (Bennett et al., 2001, Nieminen et al., 2006, Bogoyevitch and Arthur, 2008), inhibition of PARP-1 cleavage, caspase activation (Bain et al., 2003, Cameron et al., 2003, Bogoyevitch and Arthur, 2008), phosphorylation

of γ-H2AX and reversal of apoptosis (Lu et al., 2006). Interestingly, SP600125 also inhibited the activation of both nuclear and cytosolic substrates which may support the idea of JNK inhibition within two compartments with two pools of JNK present. A previous study however, used SP600125 treatment in combination with MKP-2 over-expression and observed no additive effects, suggesting both treatments were accessing a similar pool of JNK (Cadalbert et al., 2005). An additional feature of SP600125 treatment was the lack of effect relative to Adv.MKP-2 on TNF-α/DNIKKβ induced apoptosis, which was not replicated when either H_2O_2 or serum deprivation was used to initiate apoptosis. Under these conditions both SP600125 and MKP-2 were equally as effective. This again indicates a potential interaction between MKP-2 and the NF-κB pathway which is not based on short term JNK inhibition.

Work from different laboratories has demonstrated the possibility of cross talk between JNK and NF-κB in different type of cells (Chen et al., 2003, De Smaele et al., 2001, Maggirwar et al., 2000, Tang et al., 2001, Wang et al., 2007b) however, in the main cross talk is usually in one direction in that inhibition of IKKβ or NF-κB signalling potentiates JNK activity and promotes cell death. Studies utilizing a combination of MKP-2 and DNIKKβ viruses suggested a cross talk mechanism in the opposite direction in that MKP-2 over-expression reversed the inhibitory effect of DNIKKβ on cell death. To date only one study has described similar phenomena, revealling an enhancement in the activation of NF-κB pathway in fibroblasts derived from c-Jun knockout mice (Sanchez-Perez et al., 2002).

Unfortunately, further studies investigating the effect of MKP-2 over-expression upon NF-κB-dependent anti-apoptotic proteins expression were inconclusive. Over-expression of MKP-2 increased the loss in IκBα in either non stimulated or TNF-α treated cells and since IκBα degradation is normally regulated by IKKβ dependent pathway phosphorylation upon Ser^{32} and Ser^{36} (Viatour et al., 2005), the expectation from the results was that NF-κB activation would be increased and NF-κB gene transcriptional activity enhanced. Preliminary results showed that this was not the case, whilst TNF-α induced translocation of NF-κB which was further confirmed by p65 nuclear extract or

infection HUVECs with Adv.NF-κB-Luc, the transcriptional activity of NF-κB gene induced by TNF-α was enhanced. The reasons for these results were not clear however various possibilities could be examined. First is the potential for MKP-2 to bind NF-κB and inhibit DNA binding; moreover, NF-κB binding to MKP-2 may possibly have an effect on IκB exit to the cytosol, a hypothesis shown by Hay and his colleagues (Fujihara et al., 2005). This may result in the presence of free IκBα in the cytosol which may then be prone to proteosomal degradation. Free IκBα has been shown to be translocated to the nucleus, where it disengages NF-κB from DNA and exports the NF-κB/ IκBα complex back to the cytosol (Arenzana-Seisdedos et al., 1995). Otherwise, MKP-2 may have another mechanism by stimulating IKKβ activity to mediate the phosphorylation and ubiquitination and degradation of IκBα. In addition MKP-2 over-expression had a negative effect upon NF-κB reporter activity suggesting a direct interaction with NF-κB itself or an inhibitory effect on transcriptional transactivation. These possibilities require to be examined. It would also be important to determine if genes encoding NF-κB anti-apoptotic proteins were affected, but time considerations did not allow for these experiments to be conducted.

CHAPTER 4

THE EFFECT OF MKP-2 OVER-EXPRESSION ON MAP KINASE ACTIVATION IN HUMAN VASCULAR SMOOTH MUSCLE CELLS (HVSMCs)

4.1. Introduction

The results in chapter 3 demonstrated the potential for over-expression of MKP-2 via infection with Adv.MKP-2 to inhibit JNK signalling in endothelial cells and reverse apoptosis. This in itself may be a useful approach, as several cardiovascular diseases are known to be linked to endothelial dysfunction. However, it is well accepted that underlying human vascular smooth muscle cell (HVSMC) hypertrophic remodelling is also a key event in cardiovascular disease. This adaptive phenotype is a marker of atherosclerosis, (Brevetti et al., 2003), hypertension (Mulvany, 2008), balloon angioplasty and stent insertion (Coats et al., 2008), critical limb ischaemia (Coats et al., 2003) and other conditions related to impaired peripheral flow. In this chapter, HVSMC were used as models to compare the effect of MKP-2 over-expression on HVSMC function. VSMC are the predominant cellular elements of the vascular media, responsible for vasoconstriction and dilation in response to normal or pharmacologic stimuli (Rudijanto, 2007). During blood vessel formation, the phenotype of VSMC in the medial layer of the wall changes such that secretion of extracellular matrix (ECM) protein is reduced and the formation of intracellular myofilaments is increased (Schwartz, 1997, Libby et al., 1997). This transition, from synthetic to a contractile state, is required for the VSMC to perform its primary function, contraction and dilation of the blood vessel wall to regulate blood pressure and flow. Under pathological conditions, cells in mature vessels can undergo a reverse phenotypic shift from the normal contractile state to synthetic, proliferative cells that can migrate from the media into the intimal region. Moreover, VSMC can migrate to the intima and proliferate following vascular injury (Rudijanto, 2007).

The proliferative activities of VSMCs are regulated by growth promoters such as platelet derived growth factors (PDGF) (released by platelets adherent to the locus of endothelial leakage, ECs, macrophages, and VSMC), endothelin-1 (ET-1), thrombin, fibroblast growth factor (FGF) and Ang-II (Schwartz, 1997). Diverse signal transduction systems have been proposed to translate the mitogenic stimulus within VSMCs that control their proliferation; these include NF-κB (Hoshi et al., 2000), the MAP kinases (MAPKs) (Che et al., 2001, Koyama et al., 1998) or the PI3K pathway (Mehrhof et al., 2005). Evidence suggests that unlike ECs both ERK and JNK may play a positive role in proliferation and therefore both may be targets for inhibition by

MKP-2 over-expression. In this chapter the ability of Adv.MKP-2 to inhibit growth factor stimulated MAP kinase activity and resultant proliferation and apoptosis has been studied.

4.2. CHARACTERISATION OF FCS AND PDGF SIGNALLING PATHWAYS IN HVSMCs

Experiments were conducted in human smooth muscle cells derived from saphenous vein tissues provided by Dr. Paul Coats from patients undergoing angioplasty surgery. Using saphenous vein ensured no atherosclerotic lesion was present which may compromise cellular function. The identity of HVSMCs was confirmed by α-actin staining as a marker (Figure 4.1). Routinely greater than 70% of the cells were positive suggesting a homogeneous culture.

4.2.1. FCS-mediated activation of c-Jun N-terminal kinase (JNK) and c-Jun in HVSMCs

Next the effect of FCS treatment on MAP kinase phosphorylation in HVSMCs was investigated. Cells were rendered quiescent overnight before stimulation with 10% FCS over a period of 120 min. In HVSMCs, JNK phosphorylation increased as early as 15 min, reaching maximum phosphorylation at 45 min with the response observed to be approximately 23 fold of basal values (fold stim. at 45 min = 22.52 ± 4.47, n=3) (Figure 4.2 **a**). The response rapidly declined thereafter and fell towards basal levels by 120 min. Cellular expression of JNK, as assessed by total JNK blotting was not changed, suggesting that the increase in density of the phosphorylated JNK bands was not due to any change in protein expression.

FCS also stimulated c-Jun phosphorylation. A peak response was observed after 15 min of FCS stimulation, approximately 8 fold of basal values and reached maximum phosphorylation by 45 min (fold stim. at 45 min = 21.40 ± 4.86, n=3) (Figure 4.2 **b**). The response rapidly declined towards basal level by 120 min.

4.2.2. PDGF-mediated activation of c-Jun N-terminal kinase (JNK) in HVSMCs

Having established the ability of FCS to induce JNK activation it was important to confirm a similar effect using another agent such as PDGF. Initially assay of JNK phosphorylation using specific antibodies was not successful, stimulation was low and hard to see over background. Therefore JNK activity was assessed by an *in vitro* kinase assay using GST-c-Jun as a substrate (see section 2.5). As illustrated in figure 4.3, JNK activity was induced by PDGF with similar kinetics to that observed using FCS. An increase in activity was observed by 15 min, reaching a peak by 45 min at approximately 16 fold of basal values (fold stim. at 45 min = 15.70 ± 3.03, n=3). Time constraints did not allow the analysis of c-Jun phosphorylation.

4.2.3. FCS- mediated activation of p38 MAP kinase and ERK in HVSMCs

Figures 4.4 shows p38 MAP kinase (panel a) and ERK (panel b) stimulated by FCS over a 120 min period. Phosphorylation of p38 MAP kinase peaked at 30 min (fold stim. at 30 min = 16.00 ± 1.57, n=3) and subsequently returning towards basal levels by 120 min. Phosphorylation of ERK was even more rapid giving a maximum response as early as 5 min (fold stim. at 15 min = 17.68 ± 4.07, n=3). After 15 min the response dropped off and returned to basal levels by 120 min. Total ERK was unchanged, indicating equal protein loading.

4.2.4. PDGF- mediated activation of p38 MAP kinase and ERK in HVSMCs

The effect of PDGF on p38 MAP kinase and ERK phosphorylation was also investigated. Figure 4.5 show p38 MAP kinase (panel **a**) and ERK (panel **b**) stimulated by PDGF over a 120 min period. Again a rapid phosphorylation of p38 MAP kinase was observed, approximately 12 fold over basal by 15 min (fold stim. at 15 min = 11.89 ± 2.13, n=3) and subsequently returning towards basal levels by 45 min. However, phosphorylation of ERK gave a response which reached a maximum after 5 min (fold stim. at 5 min = 20.96 ± 4.09, n=3). This response returned to basal levels by 120 min.

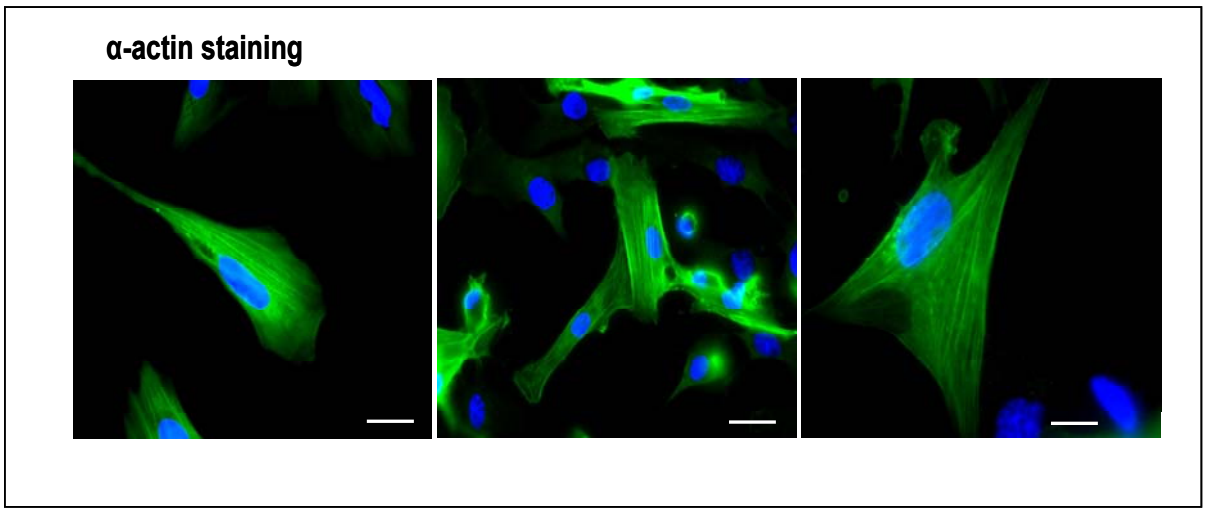


Figure 4.1: α-actin as marker for human vascular smooth muscle cells (HVSMCs). Cells were grown in the required media on cover-slips, and then rendered quiescent for 18 hr prior to stimulation with 10% FCS. Samples were stained for α-actin using α-actin antibody and analysed using fluorescent microscopy as outlined in section 2.10. All cells were viewed at x40 magnification, using a Nikon EP-1 fluorescent microscope. The results are representative of 4 independent experiments. α-actin stained with FITC (green) staining and the nucleus stained with DAPI (blue) staining (scale bar=10 μm).

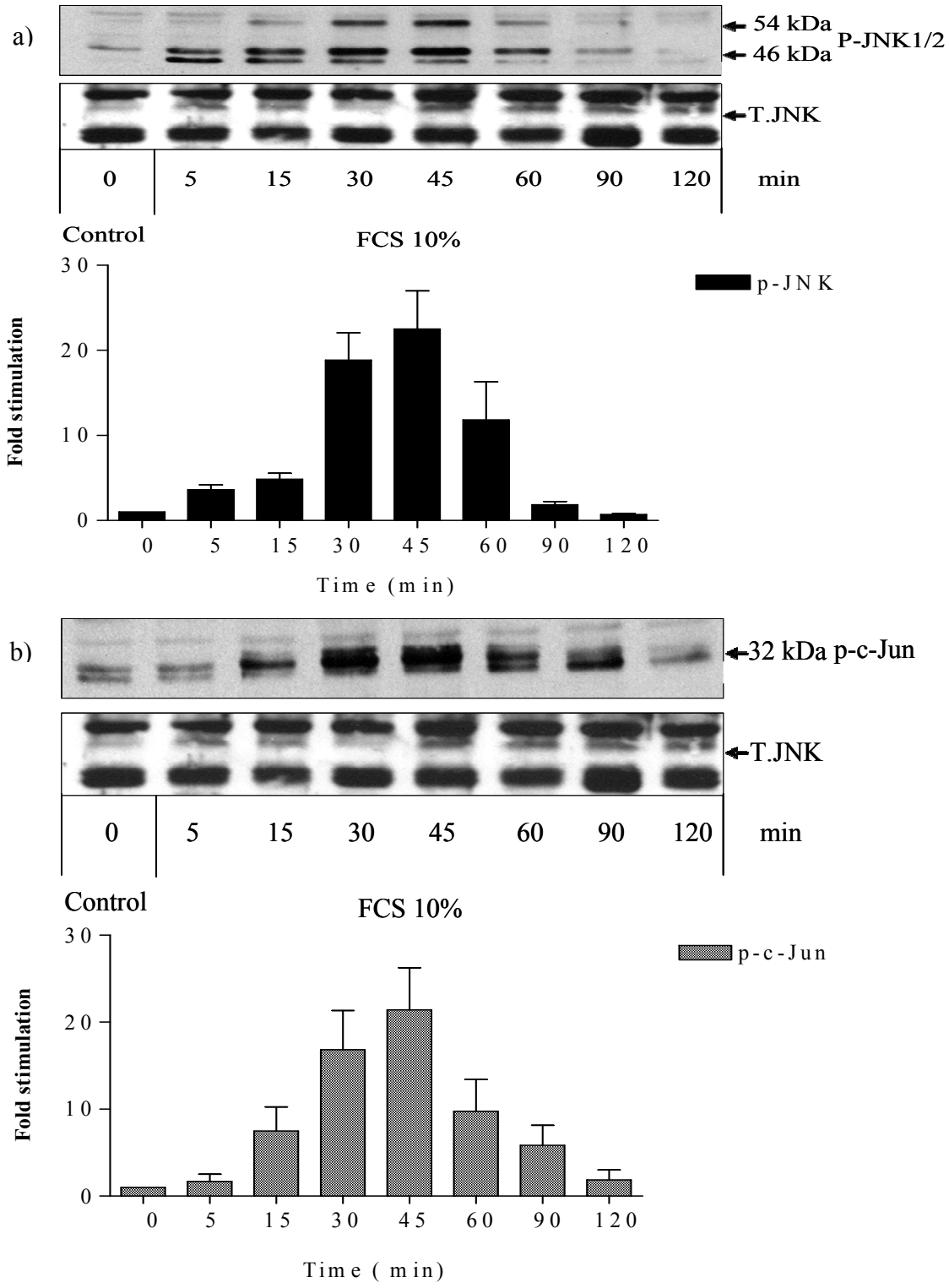


Figure 4.2: Time course of FCS –mediated JNK and c-Jun Phosphorylation in HUVSMCs.

Cells were rendered quiescent for 18 hr prior to stimulation with FCS (10%), for the times indicated. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for **a**) p-JNK (46/54 kDa) and T.JNK and for **b**) p-c-Jun (32 kDa) and T.JNK as outlined in section 2.3. Blots were quantified for fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. The results are representative of 3 independent experiment

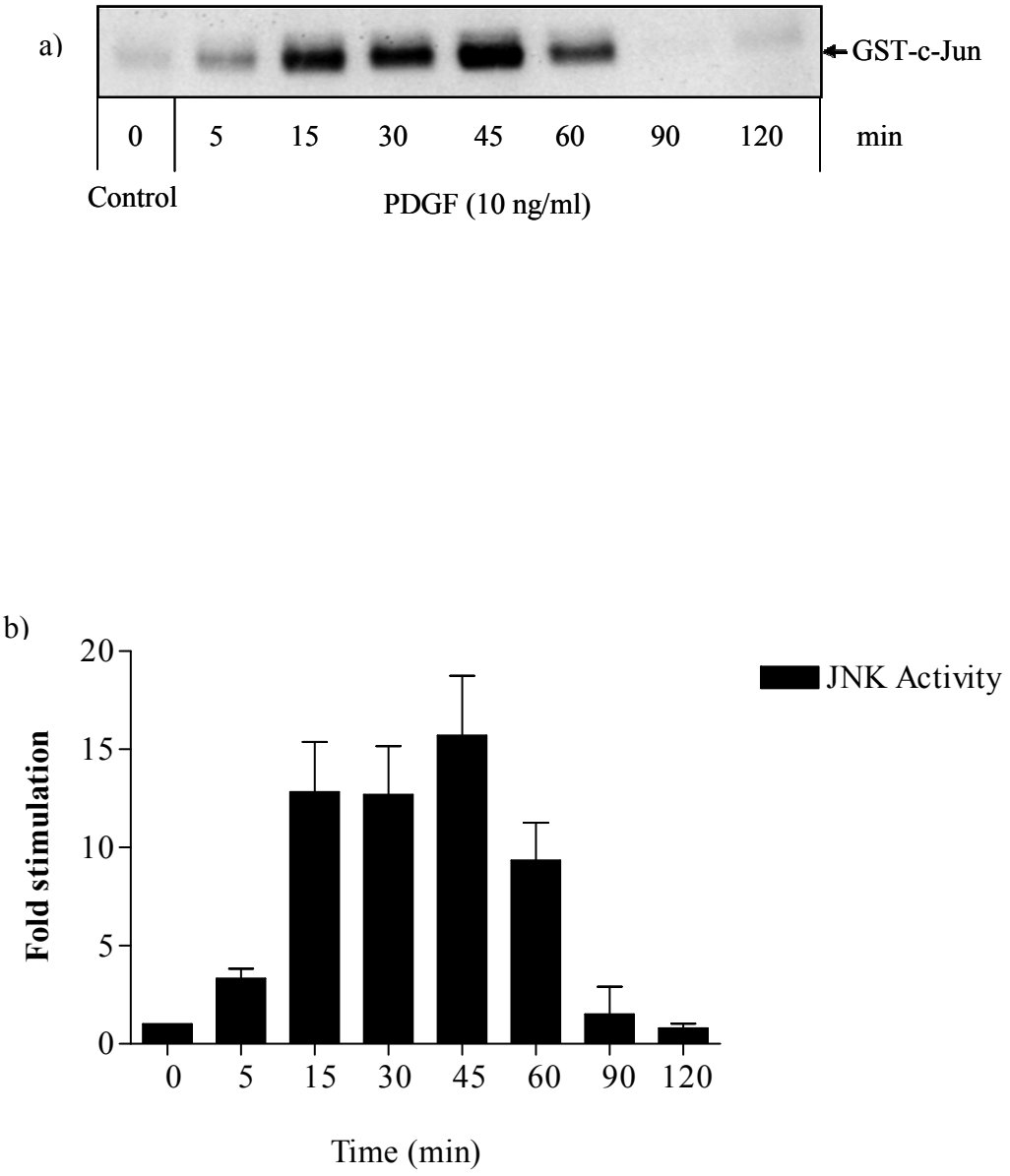


Figure 4.3: Time course of PDGF–mediated activation of JNK in HVSMCs.
Cells were rendered quiescent 18 hr prior to stimulation with PDGF (10 ng/ml), for the times indicated. Solid phase precipitates were analysed for JNK activity as outlined previously (section 2.5). Samples were assayed for **a**) JNK kinase activity. Blots were quantified for **b**) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. The results are representative of 3 independent experiments.

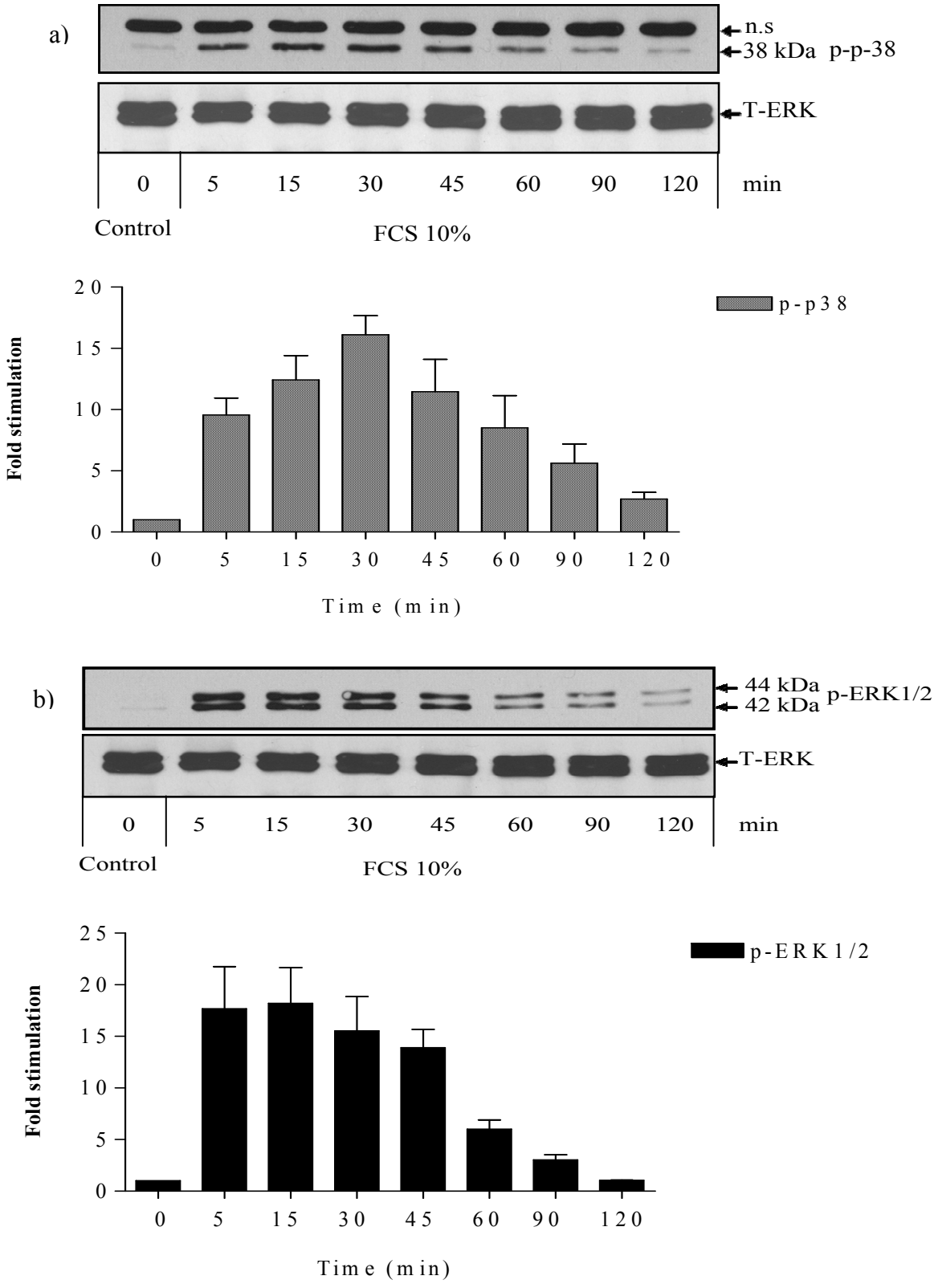


Figure 4.4: Time course of FCS-mediated p38 MAP kinase and ERK phosphorylation in HVSMCs. Cells were rendered quiescent for 18 hr prior to stimulation with FCS (10%) for the times indicated. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for **a**) p-p38 (38 kDa) and T-ERK and for **b**) p-ERK (42/44 kDa) and T-ERK as outlined in section 2.3. Blots were quantified for fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. The results are representative of 3 independent experiments.

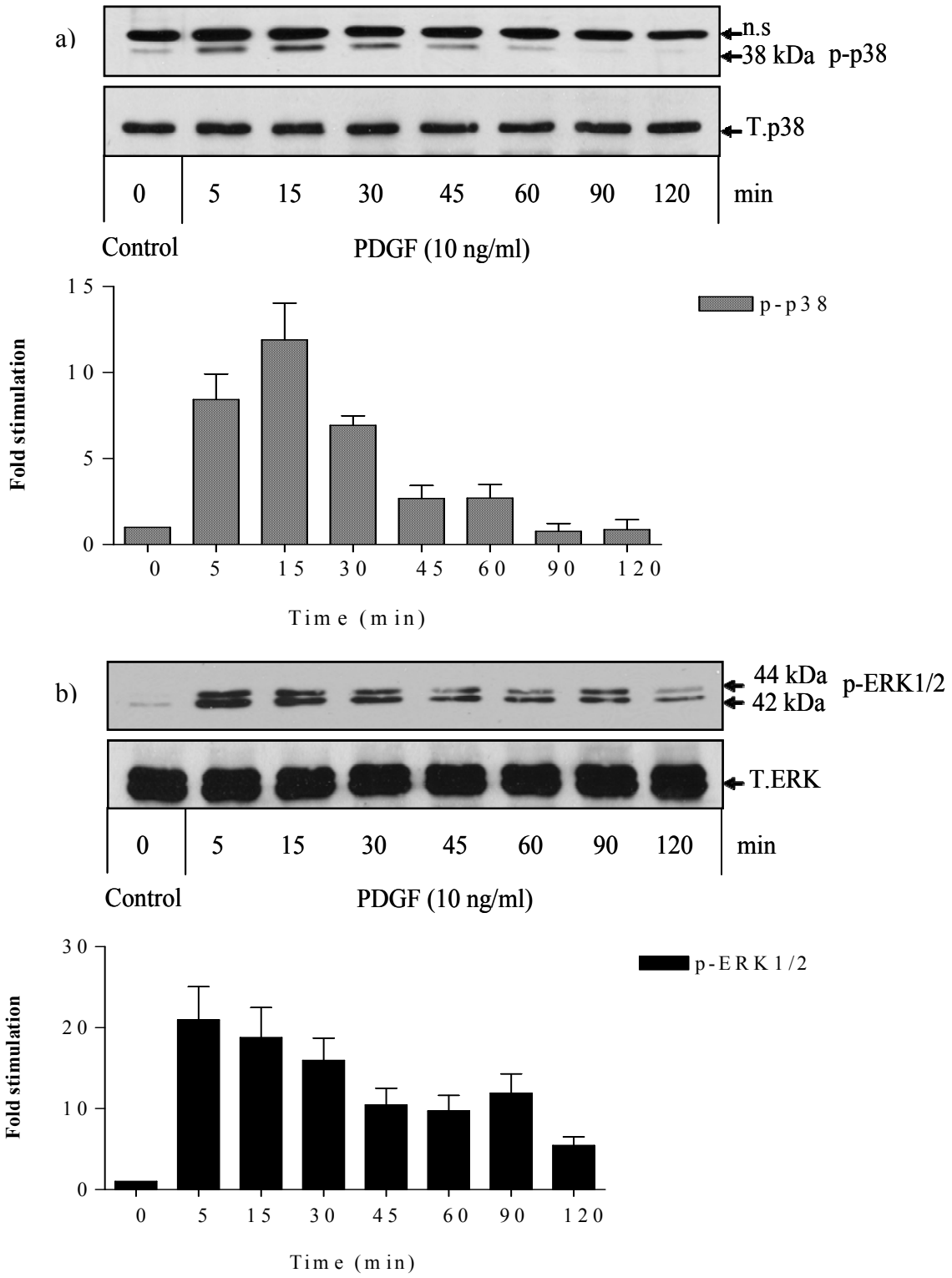


Figure 4.5: Time course of PDGF-mediated p38 MAP kinase and ERK phosphorylation in HVSMCs. Cells were rendered quiescent for 18 hr prior to stimulation with PDGF (10 ng/ml) for the times indicated. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for **a**) p-p38 (38 kDa) and T.p38 and for **b**) p-ERK1/2 (42/44 kDa) and T.ERK as outlined in section 2.3. Blots were quantified for fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. The results are representative of 3 independent experiments.

4.3. THE EFFECT OF ADV.MKP-2 ON MAP KINASE PHOSPHORYLATION STIMULATED BY FCS AND PDGF IN HVSMCS

4.3.1. The effect of Adv.MKP-2 on JNK activity mediated by FCS and PDGF in HVSMCs

Since over-expression of via infection with Adv.MKP-2 inhibited JNK phosphorylation and subsequently reduced the phosphorylation of c-Jun induced by TNF-α, H_2O_2 or serum deprivation in HUVECs (chapter 3), it was important to compare its effect on JNK activity mediated by FCS and PDGF in HVSMCs (Figure 4.6). Cells were incubated with Adv.MKP-2 (300 pfu/cell) for 40 hr, then the cells rendered quiescent for 18 hr prior to stimulation with FCS or PDGF for 30 min. JNK activity was examined as outlined previously by *in vitro* kinase assay. As figure 4.6 shows, in the presence of Adv.MKP-2, JNK activity induced by FCS was significantly inhibited (fold stim. at 30 min = 6.75 ± 4.26, n=3) by approximately 86% (% inhibition = 86.40 ± 7.50, $P<0.001$, n=3). Similarly a substantial increase in JNK activity (47 fold of basal values) was detected following PDGF stimulation and this again was significantly reduced in the presence of Adv.MKP-2 (% inhibition = 87.59 ± 10.58, $P<0.001$, n=3).

4.3.2. The effect of Adv.MKP-2 on c-Jun phosphorylation mediated by FCS and PDGF in HVSMCs

A similar effect was observed for c-Jun. Figure 4.7 illustrates that c-Jun phosphorylation, mediated by FCS, was almost completly inhibited in the presence of Adv.MKP-2 reducing from 45.64 ± 9.73 to 0.92 ± 0.75 fold of basal values (% inhibition = 98.37 ± 1.15, $P<0.001$, n=3). Moreover, the response to PDGF was also substantially reduced in the presence of Adv.MKP-2 (300 pfu/ml) (% inhibition = 97.96 ± 0.92, $P<0.001$, n=3).

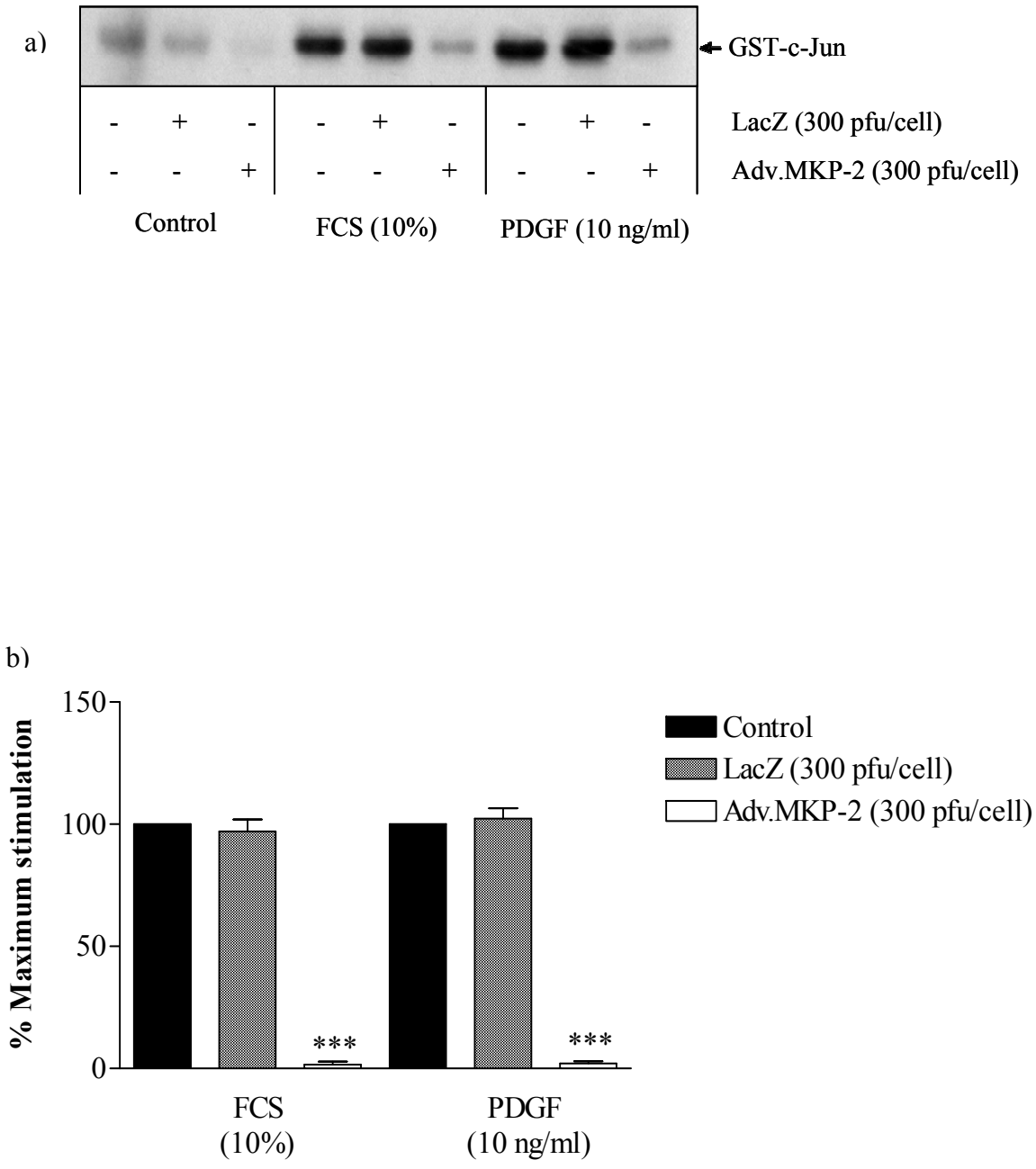


Figure 4.6: The effect of Adv.MKP-2 on JNK activity induced by FCS and PDGF in HVSMCs. Cells were rendered quiescent for 18 hr prior to stimulation with FCS (10%) or PDGF (10 ng/ml) for 30 min. Samples were assayed for **a**) kinase activity as outlined in section 2.5. Gels were quantified for **b**) % maximum stimulation by scanning densitometry; each value represents the mean ± s.e.m. The results are representative of 3 independent experiments. ***P<0.001 compared with agonist-stimulated control.

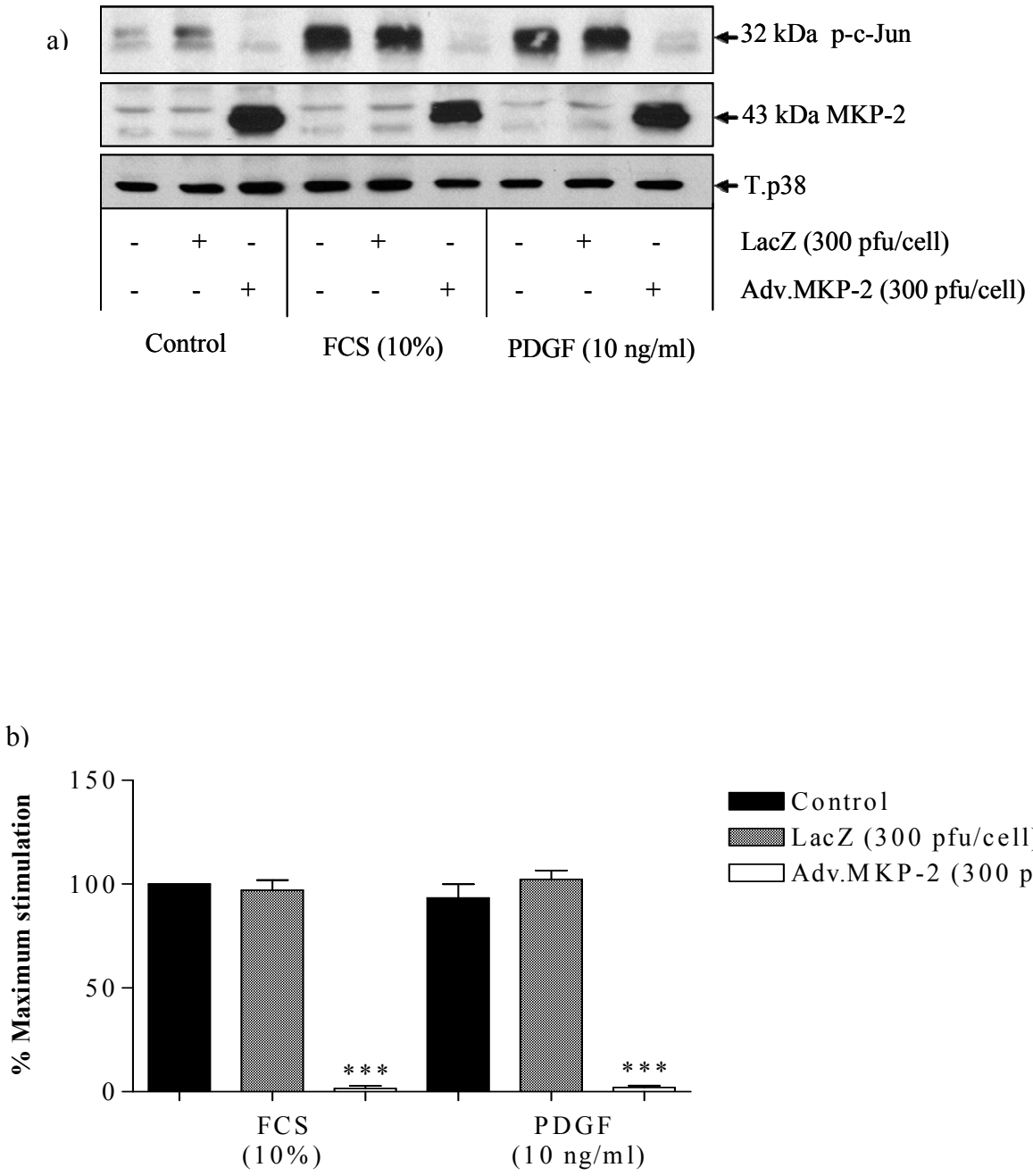


Figure 4.7: The effect of Adv.MKP-2 on c-Jun phosphorylation mediated by FCS and PDGF in HVSMCs: Cells were infected with Adv.MKP-2 for 40 hr then rendered quiescent for 18 hr prior to stimulation with FCS (10%) or PDGF (10 ng/ml) for 30 min. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for **a**) p-c-Jun (32 kDa), MKP-2 (43 kDa) and T.p38 as outlined in section 2.3. Blots were quantified for **b**) % maximum stimulation by scanning densitometry; each value represents the mean ± s.e.m. The results are representative of 3 independent experiments. ***P<0.001 compared with stimulated control.

4.3.3. The effect of Adv.MKP-2 on p38 MAP kinase and ERK1/2 phosphorylation mediated by FCS and PDGF in HVSMCs

Figure 4.8 demonstrates the effect of Adv.MKP-2 on FCS induced p38 MAP kinase and ERK1/2 phosphorylation. Stimulation with FCS for 30 min resulted in a substantial increase in both p38 MAP kinase and ERK phosphorylation (fold stim. at 30 min = 15.05 ± 2.78, 17.64 ± 3.02, respectively, n=3). However, in response to a maximum concentration of Adv.MKP-2 (300 pfu/ml) no significant change in either of these responses was observed (300 pfu/cell fold stim. at 30 min = 18.54 ± 3.48 and 18.74 ± 1.92, n=3, respectively). Similarly, PDGF stimulated a substantial increase in p38 MAP kinase phosphorylation (fold stim. at 30 min = 18.97 ± 3.57, n=3) and a similar substantial increase in ERK1/2 phosphorylation (fold stim. at 30 min = 21.21 ± 3.64, n=3). Once again, in response to Adv.MKP-2, no significant change in either of these stimulations was observed (300 pfu/cell fold stim. at 30 min = 18.14 ± 4.55 and 20.39 ± 3.50, respectively, n=3). These data suggest that Adv.MKP-2 has no effect on neither p38 MAP kinase nor ERK in HVSMCs.

4.4. THE EFFECT OF MKP-2 OVER-EXPRESSION ON HUMAN VASCULAR SMOOTH MUSCLE CELL (HVSMCS) PROLIFERATION AND APOPTOSIS

Figure 4.9 demonstrates the effect of Adv.MKP-2 on FCS induced HVSMCs proliferation. Stimulation with FCS (10%) for 48 and 72 hr resulted in a substantial increase in HVSMCs proliferation using cell counting by hematoxylin staining which gave (fold stim. at 72 hr = 137.00 ± 27.51, n=3). However, when cell were incubated with Adv.MKP-2 (300 pfu/ml) for 40 hr prior to stimulation with FCS for 48 and 72 hr a significant inhibition was observed (300 pfu/cell fold stim. at 72 hr = 56.90 ± 9.05, $P<0.001$, n=3).

The effect of Adv.MKP-2 on HVSMCs apoptosis was also investigated. HVSMCs were infected with Adv.MKP-2 (300 pfu/cell) for 24 hr prior to stimulation with H_2O_2 (0.3 mM) (Brunt et al., 2006) for a further 24 hr in 10% FCS. Samples were then analysed for Annexin V-phycoerythin and 7-Amino-Actinomycin-D (AAD) staining as outlined in section 2.7 (Figure 4.10). Cells stimulated with H_2O_2 for 24 hr showed an increase in apoptosis (62%). However, infecting cells with Adv.MKP-2 (300 pfu/cell) prior to stimulation with H_2O_2 resulted in a decrease in apoptosis (30%).

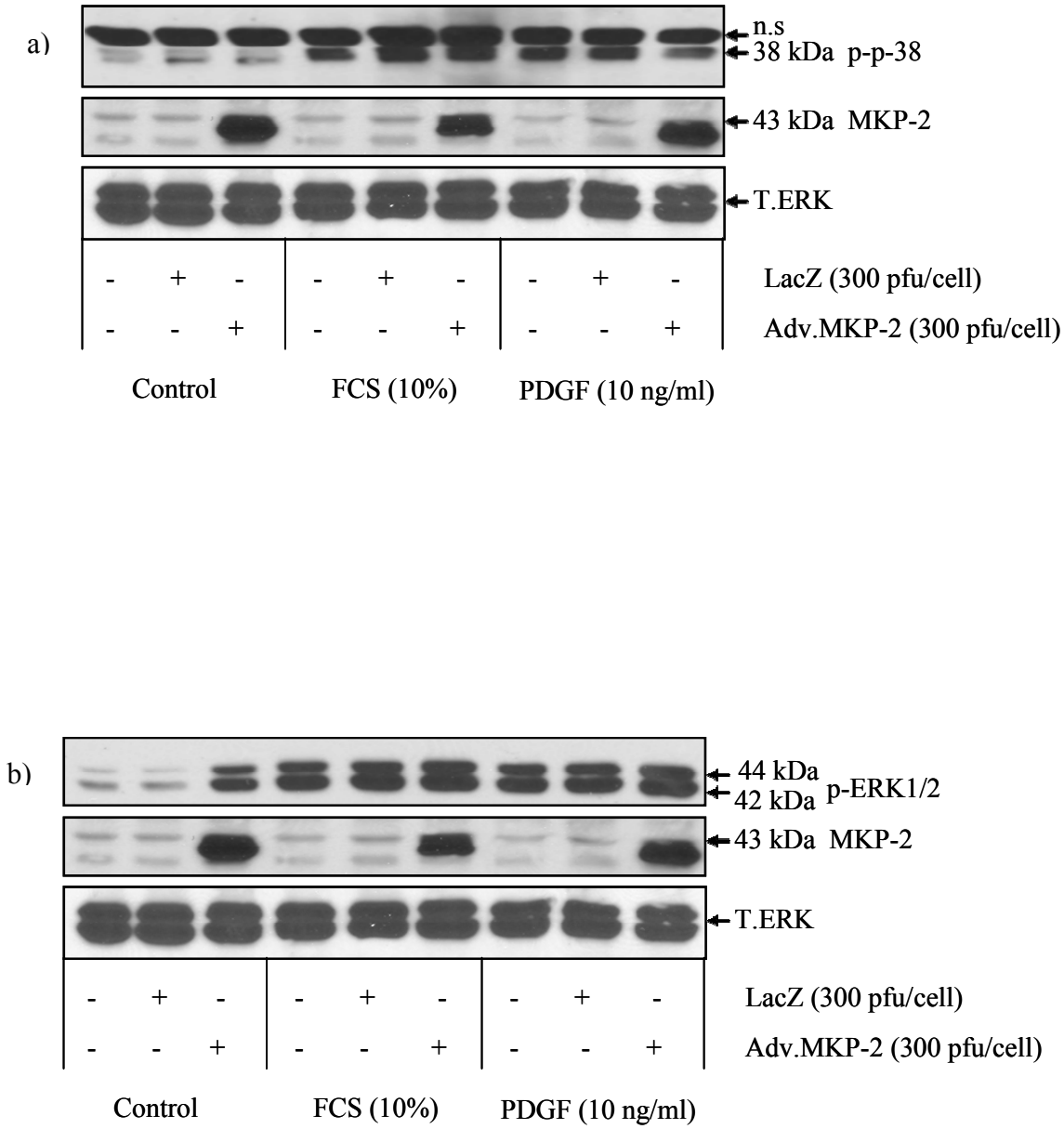
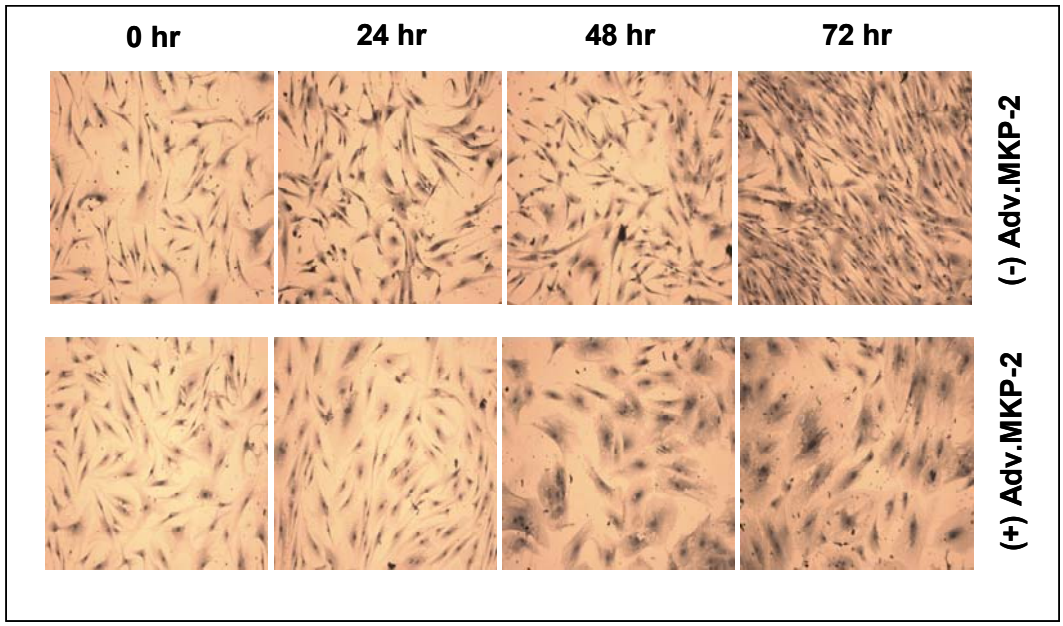


Figure 4.8: The effect of Adv.MKP-2 on p38 MAP kinase and ERK1/2 phosphorylation mediated FCS and PDGF in HVSMCs: Cells were infected with Adv.MKP-2 for 40 hr then rendered quiescent for 18 hr prior to stimulation with FCS (10%) for 30 min. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for **a**) p-p38 (38 kDa), MKP-2 (43 kDa) and T.ERK and for **b**) p-ERK1/2 (42/44 kDa), MKP-2 (43 kDa) and T.ERK as outlined in section 2.3. The results are representative of 3 independent experiments.

a)



b)

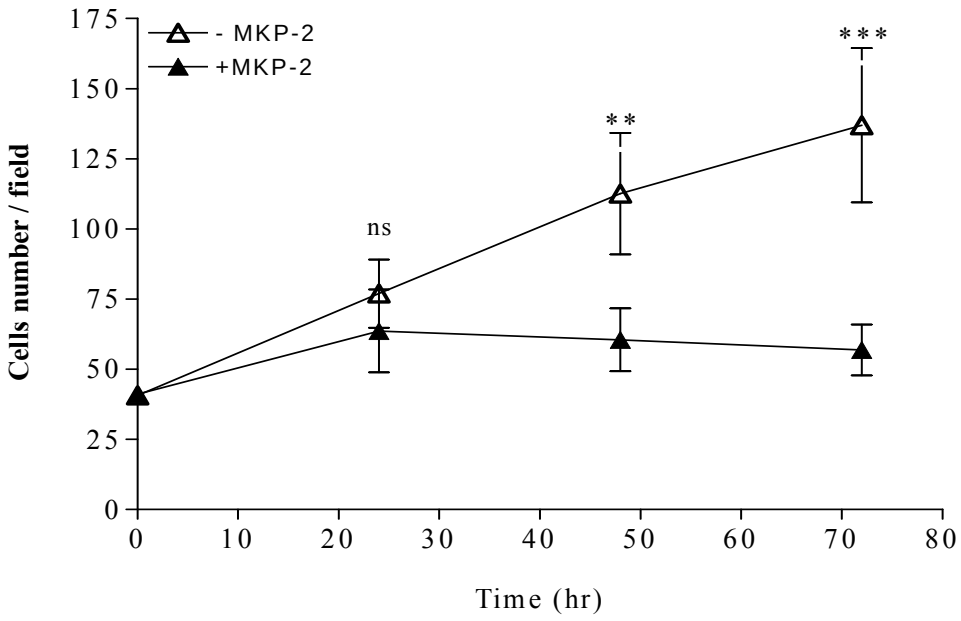


Figure 4.9: Effect of Adv.MKP-2 on HVSMCs proliferation

In panel (a) sub-confluent quiescent HVSMCs from either non-infected or infected cells with Adv.MKP-2 (300 pfu/cell). Cells were stimulated with 10% FCS for the times indicated and proliferation measured by cell counting over 72 hr as outlined in section 2.9 (Panels **a** and **b**), each value represents the mean ± s.e.m of at least 3 experiments.

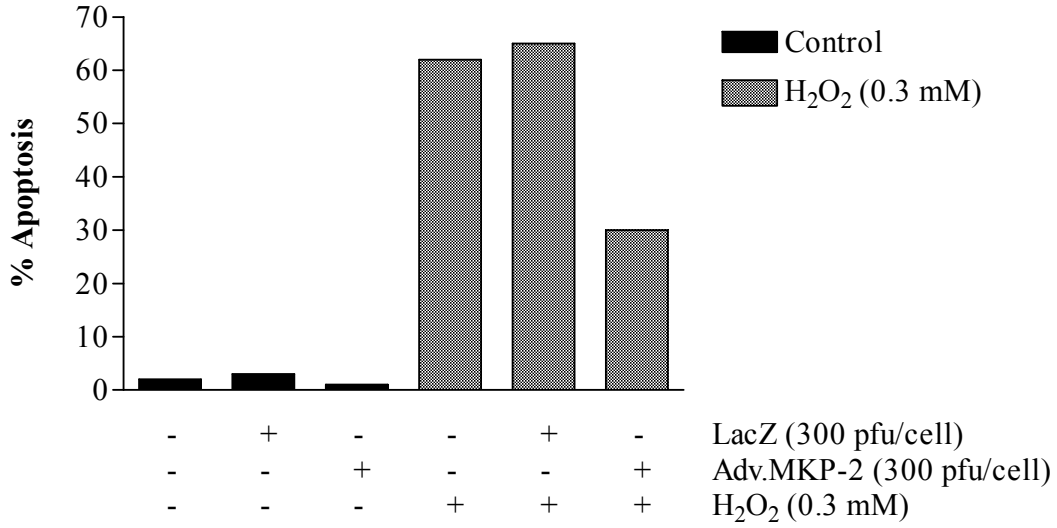


Figure 4.10: Effect of Adv.MKP-2 on H_2O_2- mediated apoptosis in HVSMCS. HVSMCs were infected with 300 pfu Lac Z or Adv.MKP-2 for 24 hr prior to stimulation with H_2O_2 (0.3 mM) for a further 24 hr. Samples were assessed for Annexin V and 7-AAD staining as outlined in section 2.7. The result is representative of a single experiment.

4.5. Discussion

The specific contribution of MKP-2 to the down-regulation of MAP kinases mediating the human vascular smooth muscle cell proliferation, and its contribution to the regulation of smooth muscle cells apoptosis, was addressed in this chapter. Here adenoviral mediated over-expression of MKP-2 was also used to reverse JNK-induced responses in human vascular smooth muscle cells (HVSMCs).

Initial experiments in HVSMCs illustrated that both FCS and PDGF activated ERK and p38 MAP kinase expression in a time dependent manner with similar kinetics. These results were consistent with previous findings in human aortic smooth muscle cells where FCS and PDGF induced DNA synthesis, proliferation and collagen synthesis (Dubey et al., 1999). However, the JNK pathway which is well known to be activated primarily by cellular stresses and implicated in mediating apoptosis has also been demonstrated to play a role in growth factor mediated cellular proliferation in other cell types (Potapova et al., 1997, Xia et al., 1995, Smeal et al., 1991). In this chapter, JNK was also phosphorylated in response to FCS and PDGF in HVSMCs. These results were consistent with other studies in other types of cells (Lin and Dibling, 2002, Weston and Davis, 2002, Manning and Davis, 2003). These results were also consistent with previous findings in vascular smooth muscle cells where growth factors and GPCR agonists such as ATP and AII were able to activate JNK (Zhan et al., 2003, Zhang et al., 2005, Robinson et al., 2006). Furthermore, inhibition of JNK activity using pharmacological or molecular inhibition of JNK inhibits SMC proliferation. Several studies have shown that SP600125 has a role in VSMC proliferation inhibition (Kavurma and Khachigian, 2003). Moreover, it has been demonstrated that SP600125 inhibited JNK activity and KB-3 human carcinoma cells proliferation with the same dose dependence, suggesting that inhibition of proliferation was a direct consequence of JNK inhibition (Du et al., 2004). In the same study, SP600125 also delayed transit time through S and G_2-M phases. In this chapter Adv.MKP-2, which has selectivity towards JNK, was able to inhbit HVSMCs proliferation. This finding was consistent with another study that showed MKP-1 expression inhibited VSMCs hypertrophy and proliferation in response to mechanical stress through AP1-DNA binding activity (Li and Xu, 2000).

Once again in agreement with studies on HUVECs in chapter 3, MKP-2 was not effective in inhibiting ERK and p38 MAP kinase phosphorylation. This may be due to the lack of nuclear translocation of both ERK and p38 MAP kinase. However, numerous studies have demonstrated translocation of ERK into the nucleus in a variety of cells (Lenormand et al., 1998, Brunet et al., 1999). This strongly suggests that the function of MKP-2, in terms of substrate specificity, may be cell type specific and agonist dependent rather than being defined by *in vitro* dephosphorylation studies.

Positive roles for JNK in cellular proliferation have been alluded to indirectly in many previous studies, principally in relation to cancer. Oncogenic Ha-Ras has been shown to activate the JNK and c-Jun pathway (Smeal et al., 1991). In addition, the transforming small GTP binding proteins Rac1 and Cdc42 have been reported to activate JNK (Coso et al., 1995, Hill et al., 1995). It was shown that the JNK pathway was activated in some tumor cell types, indicating that the JNK pathway might be essential for tumorigenesis (Zoumpourlis et al., 2000, Yang et al., 2003). In addition, JNK1 was shown to provide survival signals in transformed B lymphoblasts, contributing to leukemiogenesis (Hess et al., 2002).

In this chapter, over-expression of MKP-2 via infection with Adv.MKP-2 also inhibited c-Jun phosphorylation. This may be somewhat suprising given that in chapter 3, c-Jun was proposed to play a role in apoptosis however, recent evidence links c-Jun with proliferation. *In vivo* studies using cells from knockout and transgenic mice have indicated that c-Jun is essential for transition of the G1-S phase of the cell cycle, and cells lacking c-Jun have severe proliferation defects (Schreiber et al., 1999). In addition, it was demonstrated that c-Jun phosphorylation is important for efficient cellular proliferation (Behrens et al., 1999). Fibroblasts from mice carrying a mutant c-Jun allele with the JNK phosphoacceptor serine ^{63} and ^{73} changed to alanines (junAA) proliferate slower than wild-types cells, though this defect was not as severe as found with c-jun$^{-/-}$ fibroblasts (Behrens et al., 1999). Moreover, dominant negative c-Jun gene transfer inhibited vascular smooth muscle cells proliferation and neointimal hyperplasia in rats in response to balloon injury (Yasumoto et al., 2001, Izumi et al., 2001). In addition, c-Jun has shown to be required for both PDGF-induced VSMC migration and proliferation. In these studies, using recombinant adenovirus encoding a dominant negative c-Jun inhibits PDGF-

directed migration and proliferation by vascular smooth muscle cells (Ioroi et al., 2003, Zhan et al., 2002).

It was also found that Adv.MKP-2 partially reversed apoptosis in response to H_2O_2, presumbly due to an effect on JNK signalling. These different effects may be due to isoform specific functions. Whilst considerable evidence supports a role for JNK1 in apoptosis there is increasing evidence linking JNK2 to proliferation. JNK2 has been implicated in G_2/M-Phase transition in glioblastoma cells (Potapova et al., 2000; Yang et al., 2003), whilst another study in KB-3 carcinoma cells has demonstrated that antisense oligonucleotides to JNK2 but not JNK1 caused significant inhibition of cell proliferation (Du et al., 2004). Several studies have also demonstrated functional differences between the JNK isoforms despite the overall sequence and structural similarities. JNK isoform-selective binding partners have been identified, for example, JAMP, which act as a selective regulator of JNK1 signalling and as a scaffold for JNK3 but not JNK1 or JNK2 (Kadoya et al., 2005, McDonald et al., 2000). There is also a potential for JNK isoforms to interact with different transcription factors with differing affinities (Gupta et al., 1996). For JNK1, one such substrate includes the E3 ubiquitin ligase itch that links prolonged JNK signalling to apoptotic death mediated by FLIP ubiquitination (Chang et al., 2006, Gao et al., 2004, Venuprasad et al., 2006). For JNK2 these substrates include the microtubule-associated proteins DCX and Tau, as well as TIF-IA that regulate ribosomal RNA synthesis (Gdalyahu et al., 2004, Yoshida et al., 2004, Mayer et al., 2005). Such potential isoform-specific function has not been investigated in human smooth muscle cells, however, a study by Ricci et al.(2004) demonstrated that inactivation of JNK1 and JNK2 did not lead to a significant change in proliferation of VSMCs *in vitro* in response to PDGF (Ricci et al., 2004). Future studies assessing the expression and functional role of JNK isoforms in smooth muscle cells would be useful.

Taken together, simultaneous JNK inhibition in each cell type could be an effective therapeutic approach in cardiovascular conditions featuring both smooth muscle proliferation and endothelial cell apoptosis.

CHAPTER 5

GENERAL DISCUSSION

In this thesis I studied the effect of over-expression of MKP-2 on principally endothelial cell apoptosis but also growth factor stimulated proliferation. This was part of a longer term objective to explore MKP-2 as a possible tool for use in cardiovascular disease such as atherosclerosis or in conditions such as restenosis following interventions such as balloon angioplasty. In this work I demonstrated for the first time that Adv.MKP-2 could inhibit apoptosis in endothelial cells. However, the potential cross-talk between NF-κB and MKP-2 remained unconfirmed. Thirdly, I demonstrated that MKP-2 could inhibit proliferation in human vascular smooth muscle cells, further supporting the clinical potential of exogenously mediated MKP-2 over-expression.

In chapter 3, inhibition of the JNK pathway in HUVECs was found to attenuate pro-apoptotic protein activation and as a consequence reversed HUVECs apoptosis which appeared to be through inhibition of the JNK pathway, at least in response to the diverse agents that were tested. This included using UV-C, a non-physiological stimulus for endothelial cells (result not shown). Indeed a protective role for MKP-2 via inhibition of JNK has been demonstrated in HEK293 cells stimulated with UV-C or cisplatin (Cadalbert et al., 2005). This hypothesis was supported by another study in our laboratory which demonstrated that following MKP-2 deletion, MEFs proliferate poorly due to a defect upon G2/M phase transition and have an enhanced level of apoptosis (Lawan et al., 2010, submitted). In these cells, apoptosis is reversed by inhibiting JNK activity.

For TNF-α, in conditions such as NF-κB blockade, H_2O_2 or serum deprivation, apoptosis was enhanced and associated with nuclear effects such as c-Jun activation, PARP-1 cleavage and γ-H2AX, but also cytosolic effects such as caspase-3 and caspase-9 cleavage and mitochondrial cytochrome c release. Caspase-3 is a well defined effector caspase and γ-H2AX a histone protein (Kinner et al., 2008) which mediates DNA fragmentation and co-operates with the caspase-3/CAD pathway to mediate apoptosis (Lu et al., 2006). The results in this thesis indirectly give an insight as to the role of nuclear JNK in regulating apoptosis. Again in MKP-$2^{-/-}$ macrophages, LPS stimulated JNK phosphorylation and activity was enhanced (Almutairi at al.

2010, in press). Given that MKP-2 is a nuclear located enzyme and unlike MKP-1, which has been found in the mitochondria (Rosini et al., 2004), there is little evidence for discrete pools within other compartments, and this suggests that JNK mediated cell death is mediated in part by nuclear located JNK. Thus, it is surprising that in addition to changes in the phosphorylation of the nuclear substrate H2AX, the cleavage of caspase-3 and in particular caspase-9 is also enhanced. Cleaved caspase-3, whilst identified in the nucleus (Kamada et al., 2005), requires processing in the cytosol. This potentially argues a role for a JNK mediated mechanism, which involves targeting to the mitochondria, subsequent to phosphorylation within the nucleus. This possibility has not been properly examined and it is assumed the the presence of JNK in the mitochondria is due to translocation of JNK from the cytosol. Future studies could utilise flourescent tagged JNK isoforms to characterise the subcellular distribution of JNK following cellular activation.

The effect of over-expression of MKP-2 via infection with Adv.MKP-2 on cytosolic pro-apoptotic proteins through JNK inhibition in the nucleus would necessarily exclude the idea that Adv.MKP-2 over-expression results in non-specific effects due to the leakage of protein into the cytosol. Previous studies have shown MKP-2 to be strictly nuclear located due to not just one but two nuclear location sequences making cytosolic leakage unlikely (Cadalbert et al., 2005). Indeed, a single mutation of each NLS was not sufficient to cause cytosolic leakage whilst a new variant MKP-2 discovered in our laboratory was only found in the cytosol following high over-expression. However, preliminary studies using subcellular fractionation pointed to a pool within the cytosol, although the purity of the fractions was questioned.

The studies in this thesis implied that MKP-2 was selective for JNK over ERK. However, no proper correlation regarding the subcellular distribution of ERK versus MKP-2 was established. Previous studies have shown clear nuclear accumulation of ERK in response to serum in fibroblasts (Brunet et al., 1999, Lenormand et al., 1998), this cell type could perhaps be used to confirm MKP-2 activity towards ERK in a similar manner to the over-expression system used here. In addition, a cytosolic MKP-2 mutant could be used as a tool in analysis of cytosolic versus nuclear signalling of the MAP kinase pathways. Nishida et al. (2001) cloned MKP-7, which interestingly contained both NLS and NES. The NLS of MKP-7 was similar to the

NLS of MKP-2; the NES however was located on the C-terminal tail that is not present on MKP-2. This unfortunately makes it impossible to accurately add the NES of MKP-7 to MKP-2 in an attempt to create a cytosolic or shuttling protein (Tanoue et al., 2001).

One possibility which remained to be examined is the potential for long term down-regulation of JNK to regulate the expression of pro and anti-apoptotic genes which regulate initiator caspases. This includes intermediates such as c-FLIP (Albrecht et al., 2009, Suzuki et al., 2003), XIAP and A20 (Daniel et al., 2004). Preliminary studies however, did not reveal any changes in the expression of at least two of these (XIAP and c-FLIP). Future studies could utilise gene array technology to identify genes regulated by MKP-2 over-expression. This technology allows the evaluation of many genes at the same time either at the whole genome level or for specifically pro and anti-apoptotic genes. This could also help to determine if NF-κB dependent genes were involved. For example, several studies have reported that three NF-κB dependent *iap* gene family members (*xiap*, *hiap1*, *hiap2*) were strongly up-regulated in TNF-α stimulated HUVECs, which were resistant to TNF-α induced apoptosis. However, adenovirus mediated over-expression of IκBα, an inhibitor of NF-κB, rendered HUVECs sensitive to apoptosis and at the same time inhibited *iap* gene up-regulation (Stehlik et al., 1998). Furthermore, using a microarray assay it was also demonstrated that Angiopoietin (Ang-1) regulated many genes in HUVECs and one of these, the survivin gene, was found to have a role in survival (Papapetropoulos et al., 2000). A more recent study revealed that JNK positively regulated the expression of numerous pro-apoptotic genes in endothelial cells using microarray analysis (Chaudhury et al., 2009).

In human vascular smooth muscle cells, whilst MKP-2 was also able to reverse cell death through inhibition of JNK, the opposite phenomenon was observed assessing proliferation. Infection with over-expression of MKP-2 via infection with Adv.MKP-2 inhibited cell proliferation which correlated with abolition of growth factor-mediated JNK signalling. This implies that MKP-2 over-expression is negatively regulating processes relevant to cell cycle progression clearly in a manner opposite to the recent results using MKP-2 deficient fibroblasts, which implied a positive role for endogenous MKP-2 in cell cycle transition. Over-expression of the prototypic DUSP,

MKP-1, has also been shown to inhibit smooth muscle cell proliferation (Brondello et al., 1995, Noguchi et al., 1993) and cardiac myocte hypertrophy (Hiroi et al., 2001), whilst another study reported that transgenic mice constitutively expressing MKP-1 in the heart showed attenuated normal developmental hypertrophy (Bueno et al., 2001). These studies did not examine the potential for regulation of cell cycle protein intermediates although MKP-1 has been found to interact with p53 to regulate G1/S-phase transition (Bueno et al., 2001).

The dual effect of over-expression of MKP-2 in smooth muscle proliferation and apoptosis could be explained by the presence of different JNK isoforms which control different aspects of each pathway. Fibroblasts lacking JNK1 show reduced c-Jun phosphorylation and resistance to UV-mediated cell death, however JNK2 deficient cells show increased sensitivity to UV iradiation which correlates with elevated and sustained phosphorylation of JNK1 and c-Jun (Hochedlinger et al., 2002). Moreover, similar differences in the control of cellular proliferation in various cell types have also been noted. JNK2 has been implicated in G_2/M-Phase transition (Du et al., 2004, Potapova et al., 2000, Yang et al., 2003). Moreover, it was shown that c-Jun was required for proper hepatocyte proliferation after partial hepatectomy. In this study, $JNK2^{-/-}$ mice showed acceleration of hepatocyte proliferation (Behrens et al., 2002), suggesting that JNK2 works as a negative regulator of cellular proliferation (Sabapathy and Wagner, 2004).

Future studies could therefore focus on defining the roles of JNK isoforms in both endothelial and vascular smooth muscle cells. Several approaches can be used, for example using antisense oligonucleotides to target JNK isoforms *in vitro* (Tafolla et al., 2005) or by isoform-specific ablation using siRNA (Pawate and Bhat, 2006) which another study has shown may be utilised *in vivo* (Yang et al., 2003). In all cases, confirmation of specific knockdown would be essential, as in some instances isoform-specific targeting has not been clearly achieved (Nguyen et al., 2005). It is also of interest to determine whether small molecule inhibitors of JNK can show sufficient isoform specificity despite the general conservation of the JNK sequences and structure. To date, there appears to be only minimal discrimination possible with small molecule inhibitors of JNK, however recently a 6-anilinoindazole derivative has been shown to selectively inhibit JNK3 in an ATP-competitive manner despite a

highly conserved ATP- binding site within the three JNK isoforms (Swahn et al., 2005). With these developments, it should be possible to identify JNK isoform-specific functions.

Injury of the vascular endothelium represents the critical event for the initiation of a number of disease processes as discussed in section 1.3.3. In vascular diseases such as atherosclerosis, endothelium apoptosis is considered as an early marker in the pathogenesis of this disease and its progression (Rossig et al., 2001, Pober and Min, 2006, Pober et al., 2009). However, smooth muscle proliferation is also a key event in disease progression and a strategy which inhibits smooth muscle proliferation but enhances endothelial cell survival is required. Over-expressing MKP-2 has the potential to fit these criteria. In a preliminary study, human saphenous veins were incubated with Adv.MKP-2 in an attempt to prevent remodelling of the vessel following endothelial cell disruption, however viral expression in the vessels was poor and further studies are required. Nevertheless, MKP-2 remains potentially useful given that it was found to be a more effective JNK inhibitor than the SP600125. Its longer term effects may make it more useful than pharmacological inhibitors that lack kinase specificity and isoform selectivity.

Also in this thesis, whilst using over-expression of MKP-2 implied a possible role for this DUSP in endothelial cell and smooth muscle function, this waits to be established and indeed the progress in this and other cell systems is slow. Most progress has been made in studies relevant to cancer. MKP-2 is over-expressed in a number of human cancer tissues or cell lines (Shen et al., 2006, Sieben et al., 2005) and recently it has been shown that MKP-2 over-expression is essential for cellular senescence, suggesting a role in cell survival (Torres et al., 2003, Tresini et al., 2007). Work from our laboratory has demonstrated the presence of the second splice variant of MAP kinase phosphatase-2 (MKP-2-S) which has a different functional role from the full length MKP-2 protein and found to be expressed at high levels in some hormone dependent malignancies (Cadalbert et al., 2010). Thus, MKP-2 may also play an essential role in cancer development, but this remains to be established.

CHAPTER 6

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