

THE ROLE OF NF κ B IN THE INFLAMMATORY RESPONSE
ASSOCIATED WITH HEART FAILURE AND
CARDIOTHORACIC SURGERY

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by

Ginette Sarah Hoare

BSc

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Department of Cardiothoracic Surgery
National Heart and Lung Institute
Imperial College London
Heart Science Centre
Harefield Hospital
Harefield
Middlesex
UB9 6JH
United Kingdom



ABSTRACT

Increased circulating pro-inflammatory and pro-oxidant factors are considered as central components in the progression of heart failure and complications of open-heart surgery with the potential to activate inflammatory transcription factors in vascular cells. NF κ B is implicated in many inflammatory responses however, the net influence of the pro-inflammatory mediators associated with heart failure and cardiothoracic operations on endothelial cell responses with respect to NF κ B activation remains unknown since counteracting anti-inflammatory mediators might also be activated. In addition, the role of oxidative stress in cytokine-mediated NF κ B activation in human cells remains controversial. This thesis aims at clarifying some of these issues both clinically and in experimental models.

Deteriorating heart failure patients had increased plasma TNF α , IL-6 and IL-10 compared to patients with less severe heart failure, while sTNFR and IL-1RA concentrations were similar. Circulating TNF α and IL-1 β were increased in only a few patients following CABG utilising cardiopulmonary bypass and the predominant response to surgery was increased circulating IL-10, IL-1RA and sTNFR. TNF α and IL-1 β potently induced NF κ B activation and adhesion molecule expression in cultured human vascular cells however the increased TNF α in patient's plasma did not parallel capacity to stimulate NF κ B-driven gene induction *in vitro*. In addition, post-operative plasma from CABG patients inhibited cytokine-induced E-selectin expression. NF κ B activity, in the cell types studied, was not affected by pro-oxidants however, TNF α -induced, NF κ B-dependant E-selectin expression was inhibited by anti-oxidant treatment.

These findings demonstrate that increased circulating pro-inflammatory cytokines in patients with heart failure and in some CABG patients are not capable of inducing NF κ B-dependant inflammatory gene expression *in vitro*. Thus, there appears to be dissociation between single pro-inflammatory cytokines and net-inflammatory response, which may be explained by a predominant anti-inflammatory response.

These results may have implications to *in vivo* cellular responses involving various mechanisms of NF κ B activation and regulated gene transcription in heart failure and cardiothoracic operations.

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ABBREVIATIONS

ACE	Angiotensin Converting Enzyme
AP-1	Activator Protein 1
ASK1	Apoptosis Signal Regulating Kinase
ATP	Adenosine Triphosphate
CABG	Coronary Artery Bypass Grafting
cAMP	Cyclic Adenosine Monophosphate
ASK-1	Apoptosis Signal-Regulating Kinase 1
CBP	CREB-binding protein
C/EBP	CCAAT/Enhancer Binding Protein
CD	Cluster Designation
cGMP	Cyclic Guanosine Monophosphate
c-IAP	Cellular Inhibitors of Apoptosis
COX	Cyclo-Oxygenase
CPB	Cardiopulmonary Bypass
CRE	cAMP Response Element
CREB	cAMP Responsive Element Binding Protein
CRP	c-Reactive Protein
Cys	Cysteine
DAB	3,3 -diaminobenzidine
DCM	Dilated Cardiomyopathy
DD	Death Domain
DED	Death Effector Domain
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EC	Endothelial Cells
ECL	Enhanced Chemiluminescent
ECSIT	Evolutionary Conserved Signalling Intermediate in Toll Pathways
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbant Assay

ENA-78	Epithelial-Neutrophil Activating Peptide 78
ESL-1	E-selectin Ligand
FADD	Fas Associated Death Domain
FBS	Foetal Bovine Serum
FITC	Fluorescein Isothiocyanate
GCK	Germinal Centre Kinase
GM-CSF	Granulocyte/Macrophage- Colony Stimulating Factor
Grb2	growth Factor Receptor binding Protein
GRE	Glucocorticoid Response Element
GSH	Reduced Glutathione
GSK3 β	Glycogen Synthesis Kinase 3 β
GSSG	Oxidised Glutathione
GTP	Guanosine Triphosphate
H ₂ O ₂	Hydrogen Peroxide
HAT	Histone Acetyltransferase
HBSS	Hank's Balanced Salt Solution
HDAC	Histone Deacetalase
HF	Heart Failure
HMWK	High Molecular Weight Kininogen
HRP	Horse Radish Peroxidase
ICAM	Intercellular Adhesion Molecule
ICE	IL-1 β Converting Enzyme
IFN	Interferon
Ig	Immunoglobulin
IHD	Ischaemic Heart Disease
I κ B	Inhibitor of κ B
IKK	I κ B Kinase
IL	Interleukin
IL-1R	IL-1 Receptor
IL-1R AcP	IL-1R Accessory Protein
IL-1RA	IL-1 β Receptor Antagonist
IRAK	IL-1R Associated Kinase
iNOS	Inducible Nitric Oxide Synthase

JAK	Janus Kinase
JAM	Junctional Adhesion Molecule
JNK	c-Jun-N-terminal Kinase
LAD	Leucocyte Adhesion Deficiency
LPS	Lipopolysaccharide
LV	Left Ventricular
LVAD	Left Ventricular Assist Device
M	molar
MAPK	Mitogen Activated Protein Kinase
MAPKKK	MAP kinase kinase kinase
MCP-1	Monocyte Chemotactic Protein-1
MEKK	MAPK kinase kinase
mg	milligram
MHC	Major Histocompatibility Complex
MIP-1	Macrophage Inflammatory Protein
ml	millilitre
MLCK	Myosin Light Chain Kinase
mM	millimolar
μ M	micromolar
MMP	Matrix Metalloproteinase
M ϕ	Macrophage
MPO	Myeloperoxidase
mRNA	Messenger Ribonucleic Acid
MSK-1	Mitogen- and Stress- Activated Proteins Kinase 1
NAC	N-Acetyl L-Cysteine
NADPH	Nicotinamide Adenine dinucleotide Phosphate
NES	Nuclear Export Signal
NF-AT	Nuclear Factor of Activated T cells
NF κ B	Nuclear Factor κ B
NIK	NF κ B Inducing Kinase
NLS	Nuclear Localisation Signal/sequence
ng/ml	nanograms per ml
nm	nanometre

nM	nanomolar
NO	Nitric Oxide
NSAID	Non-Steroidal Anti-Inflammatory Drugs
NYHA	New York Heart Association
OD	Optical Density
OPCAB	Off Pump Coronary Artery Bypass Graft
PAF	Platelet Activating Factor
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PDEI	Phosphodiesterase Inhibitor
pg/ml	picograms per ml
PI3K	Phosphatidylinositol 3-Kinase
PG	Prostaglandin
PKA	Protein Kinase A
PKB	Protein kinase B
PKC	Protein Kinase C
PSGL-1	P-selectin Glycoprotein Ligand-1
RANTES	Regulated on Activation, Normal T-cell Expressed and Secreted
RAS	Renin Angiotensin System
RFU	Relative Fluorescence Units
RHD	Rel Homology Domain
RIP	Receptor-Interacting Kinase
RLA	Regulated and Late Accessibility
SDS	Sodium Docecyl Sulphate
SDS-PAGE	SDS Polyacrylamide Gel Electrophoresis
Ser	Serine
SH	Src Homology
SHP2	SH2-containing Phosphotyrosine Phosphatase
SOD	Superoxide Dismutase
SODD	Silencer of Death Domain protein
SOLVD	Studies on Ventricular Dysfunction
Sos	Son of Sevenless
SMC	Smooth Muscle Cells

STAT	Signal Transducer and Activator of Transcription
TAK1	TGF β Activated Kinase 1
TEMED	NNN'N-tetramethyl-1,2-diaminoethane
TF	Tissue Factor
TIMP	Tissue Inhibitor of MMP
TIR	Toll-IL-1 Receptor Domain
TLR	Toll-like Receptors
TNF α	Tumour Necrosis Factor
TNFR	TNF α Receptor
TRADD	TNF Receptor-Associated Death Domain
TRAF	TNF Receptor-Associating Factor
Tween 20	Polyoxyethylene (20) Sorbitan Monolaurate
Tx	Transplant
TYK2	Tyrosine Kinase 2
Tyr	Tyrosine
VCAM	Vascular Cell Adhesion Molecule

Chapter One: INTRODUCTION

1.1 INFLAMMATORY RESPONSE

1.1.1 INTRODUCTION

INFLAMMATION, originating from the Latin *Inflammati*o, *inflammare* meaning ‘to set on fire’ is a stereotyped response, at least in the first phase, to tissue injury produced by mechanical, chemical or microbial stimuli. Inflammation arises as a sequel of a complex series of events that follows tissue damage and is a major part of innate immunity that serves to initiate mechanisms to contain and eliminate the noxious agents and damaged tissue and set in process the mechanisms of tissue repair.

In most cases, the inflammatory response is successful and has the intrinsic capacity for complete resolution without tissue injury. Under such circumstances the inflammatory response is a type of self-limiting process that is under the control of strict endogenous mechanisms. However, if the local control is lost or the response is overtly activated, the inflammatory response can become self-perpetuating and damaging. Such prolonged inflammation can cease to be a beneficial event and can contribute to the pathogenesis of many disease states. Loss of function of tissues and organs is seen as a result of an inappropriate inflammatory response in a number of diseases such as ischaemia/reperfusion injury, rheumatoid arthritis, myocardial infarction and asthma. The continual activation of the immune system is the driving force behind the transition from acute to chronic inflammatory disease and it is crucial to identify the self-limiting mechanisms (stop signals) of the self-resolving inflammatory lesions.

1.1.2 MECHANISMS OF AN INFLAMMATORY RESPONSE

Inflammation collectively involves a series of vascular events that serve to bring fluid, protein and cells from the blood to the site of injury in order to cause the destruction and elimination of the injurious or infectious agent, limit the spreading of injuring factors, stimulate the immune response and aid in the tissue repair process.

The inflammatory response involves three major stages: vasodilation, increased microvascular permeability and cellular activation and adhesion. Vasodilation and increased microvascular permeability at the site of injury increase the local availability of oxygen and nutrients and produce heat, swelling and tissue oedema. These changes in local haemodynamics at the site of injury give rise to the classical signs of inflammation; redness (rudor), swelling (tumor), heat (calor) and pain (dolor).



Figure 1.1 Cartoon depicting five Greeks representing the cardinal signs of inflammation - heat, redness, swelling, pain and loss of function –(Lawrence et al., 2002). Figure was commissioned by Derek A. Willoughby and drawn by P. Cull for the Medical Illustration Department at St Bartholomew's Medical College.)

Activation of the vascular endothelium and inflammatory cells resident at the site of injury/inflammation facilitates the recruitment and subsequent tissue accumulation of activated neutrophils and monocytes/ macrophages that serve to destroy the inducing stimuli, clean up the damage and repair the tissue.

1.1.3 MAJOR CONTROLLING/DRIVING FACTORS OF THE INFLAMMATORY RESPONSE

The purpose of the inflammatory response is to eliminate invading pathogens or injurious substances and to repair the damage caused to the tissue. By its very nature the inflammatory response is required to be aggressive towards the invasion and rapid in its onset and thus demands exquisite control to efficiently achieve the desired outcome and avoid a prolonged response and tissue damage. Such specific requirements are fulfilled by the co-ordination of cellular and humoral factors. These may include the activation of blood elements and responses mediated by cytokines and chemokines.

1.1.3.1 Cytokines

The humoral factors responsible for controlling the inflammatory response can largely be attributed to a diverse group of low molecular weight polypeptides (generally 15 – 40kDa) termed cytokines. Cytokines act as humoral regulators at nano- to picomolar concentrations, which, either under normal or pathological conditions, modulate the

functional activities of cells and tissues. Cytokines have been implicated in diverse and conflicting functions such as precursor cell development and inhibition, clonal activation and tolerance, cellular apoptosis and enhanced survival, cellular differentiation, tumour rejection and metastasis, and pro-inflammatory and anti-inflammatory states.

Through the interaction with cellular receptors and the activation of intracellular signal transduction pathways and transcription factors cytokines can control the intensity and length of the inflammatory response by regulating protein expression and affecting the activation, proliferation and differentiating of various cells. The multiple agents involved in regulating the inflammatory response include pro-inflammatory cytokine tumour necrosis factor (TNF) and interleukin (IL)-1 and the anti-inflammatory T-helper (TH)₂ cytokines such as IL-4, IL-6 and IL-10.

1.1.3.1.1 TNF α

TNF α is generally considered to be a pro-inflammatory cytokines but is also an important mediator of necrotic and apoptotic cell death, haemorrhagic necrosis of tumours, cellular proliferation and differentiation and is known to be heavily involved in pathogenic disorders such as rheumatoid arthritis, asthma, septic shock, irritable bowel disorder, haemorrhagic fever and cachexia in cancer patients.

The 26kDa transmembrane precursor protein of TNF α is converted to the mature 17kDa protein by the proteolytic cleavage of the C-terminal end by a membrane-bound matrix metalloproteinase called TNF α converting enzyme (TACE, ADAM 17) (Black et al., 1997; Moss et al., 1997). The active form of TNF α is a homotrimer (Jones et al., 1989).

Regulation of human TNF α expression is quite complex and appears to involve controls at both transcriptional and post-transcriptional levels. The promoter region of the TNF α gene contains, in addition to a TATA box, consensus binding sites for various transcription factors including nuclear factor- κ B (NF κ B), ATF-2, c-jun/activator protein-1 (AP-1), AP-2, Sp-1, Egr-1 and NF-AT (nuclear factor-activated T cells). The relative importance of these sites and transcription factor binding and interactions depends on the stimuli and the cell type involved (Aggarwal et al., 2000).

The 3' untranslated region (UTR) of the TNF α mRNA contains an AU-rich element (ARE), which controls the posttranscriptional regulation of TNF α expression by

binding proteins that either stabilise or destabilise the mRNA transcript (Dean et al., 2001).

1.1.3.1.1.1 TNF α receptors

TNF α binds to two different high affinity cell surface receptors, the type I receptor (also known as TNFR1, p55 or CD120a) and the type II receptor (also known as TNFR2, p75 or CD120b). The extracellular domain of both receptors contains a pre-ligand-binding assembly domain (PLAD) that encourage receptor trimerization upon activation by TNF α (Chan et al., 2000).

The amino acid sequences of the intracellular domains of the two receptors are quite dissimilar suggesting delineation of their signalling functions (Grell et al., 1994). Both receptors lack any intrinsic enzymatic activity and must bind intracellular adaptor proteins to activate various kinase activities and control the signalling of the target cell's biochemistry (Darnay et al., 1994a, 1994b; MacEwan, 2002).

The intracellular domain of TNFR1 contains an 80 amino acid region, the so-called Death Domain (DD), named such because of its importance in mediating TNF α -induced cell death (Tartaglia et al., 1993). The DD is also present on a number of associating proteins and related molecules that are primarily involved in signalling for cell death. One such molecule, the silencer of DD protein (SODD), binds to the DD of the TNFR1 preventing the interaction of TNFR1 with other DD-interacting proteins (Jiang et al., 1999).

The type I receptor is expressed on all cell types examined to date. The type II receptor appears to be expressed by the cells of the immune system and haematopoietic cells such as macrophages, neutrophils, lymphocytes (B cells and T cells), thymocytes, and mast cells. Endothelial cells and cardiac myocytes also express the type II receptor.

1.1.3.1.1.2 Signalling through the TNF α type I receptors

Three major activities are associated with type I receptor activation – Nuclear Factor- κ B (NF κ B), c-Jun-N-terminal kinase (JNK) and apoptosis.

The interaction between TNF α and TNFR1 causes SODD to dissociate from the receptor complex and TRADD (TNF receptor-associated death domain) then binds directly to the now vacant TNFR1 DD through an interaction with its own DD sequence. Receptor-bound TRADD can then recruit TRAF2 (TNF receptor-associating

factor 2) and the DD-containing RIP (receptor-interacting kinase) into the TNFR1 signalling complex. Biochemical analyses of TRAF2 and RIP -deficient fibroblasts suggest that TRAF2 recruits the I κ B kinase (IKK) complex into the TNFR1 signalling complex and that RIP is responsible for the activation of the IKK complex, although this does not involve the kinase activity of RIP (Devin et al., 2000). TRAF2 has also been shown to recruit the serine/threonine kinase NIK (NF κ B inducing kinase) that binds to IKK α and IKK β inducing their activation and hence the activation of the NF κ B pathway. An alternative or co-operating pathway has also been described for TNFR1-induced activation of IKK whereby RIP binds to the adapter protein p62, which selectively interacts with the atypical protein kinase C (PKC ζ , PKC γ), in turn PKC ζ is able to phosphorylate and activate IKK β (Lallena et al., 1999).

The cytoplasmic protein TRADD has also been found to recruit the downstream signalling adaptor molecule Fas-associated death domain (FADD). FADD contains a death effector domain (DED) sequence, which interacts with the DED domain in caspase-8 and a number of other DED-containing molecules that regulate cell death.

TRAF2 has also been shown to be required for TNF α -induced activation of JNK and several responsible mechanisms have been suggested. Apoptosis signal-regulating kinase 1 (ASK1) is a mitogen activated protein kinase kinase kinase (MAPKKK) that activates JNK and p38 signalling cascades and recent evidence indicates that ASK1 interacts with TRAF2 to induce JNK activity (Nishitoh et al., 1998). Other studies suggested that an interaction between TRAF2 and germinal centre kinase (GCK) mediates TNF α -induced activation of JNK whilst an interaction between TRAF2 with RIP mediates TNF α -induced p38 activation by binding to up stream kinases (Yuasa et al., 1998).

TNFR1 also contains an intracellular sequence at Positions 309 319 which binds the FAN (factor associated with neutral Smase) adaptor protein, a key stimulatory element in the activation of neutral sphingomyelinase (SMase) (Adam et al., 1995). SMase catalyses the degradation of sphingolipids into smaller ceramide-containing molecules which are key signalling intermediates capable of activating ceramide-activated protein kinase (CAPK) which activates Raf kinase, an upstream activator of the MEK and MAPK serine/threonine kinase family (Mathias et al., 1991).

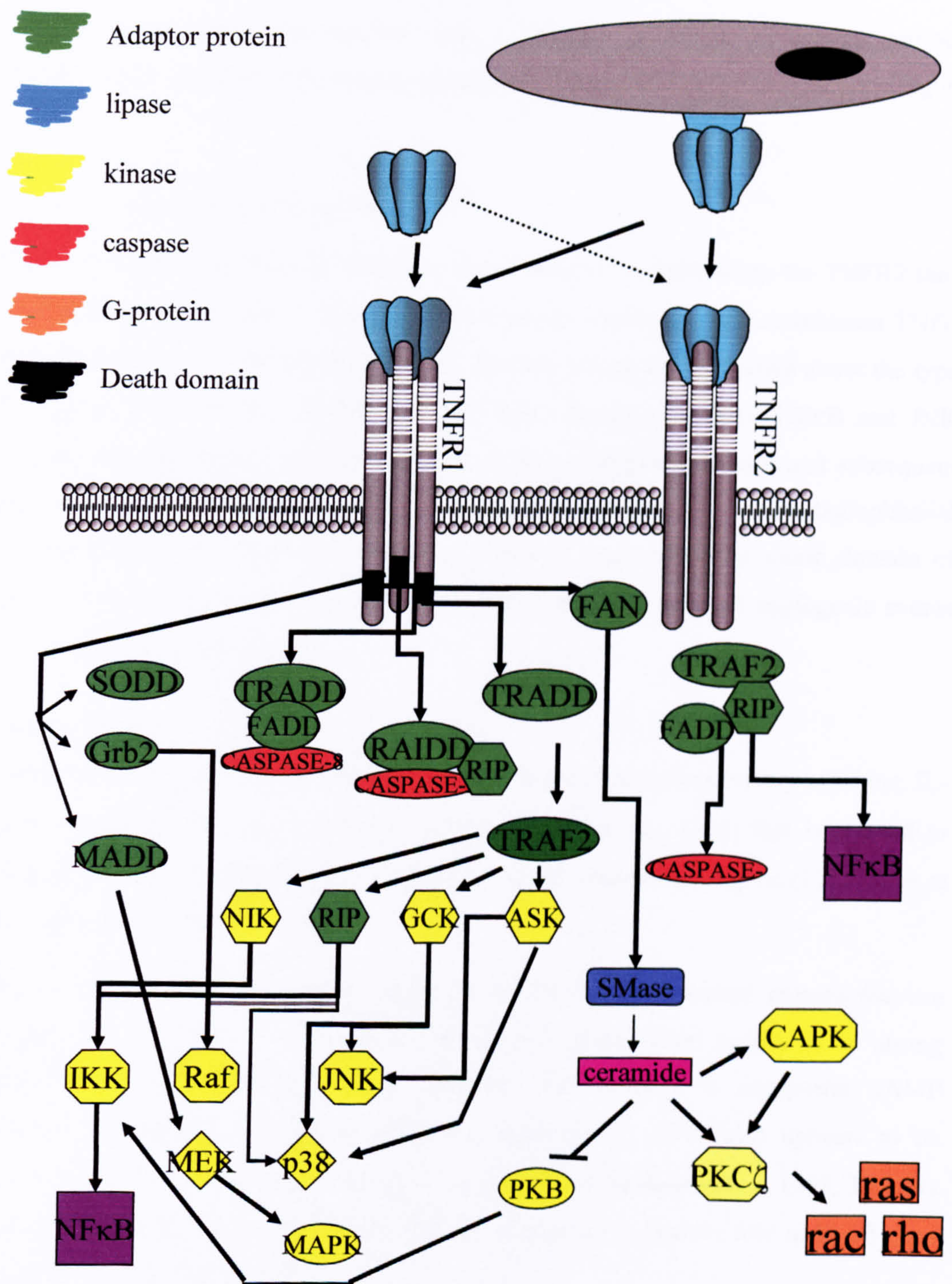


Figure 1.2. Intracellular signalling events induced through the binding of TNF α to cell associated TNF α receptors (MacEwan, 2002).

TNF α receptors have also been found to interact with the Grb2 (growth factor receptor binding protein) adaptor which constitutively associates with the Ras/GTP exchange factor son of sevenless (Sos). Activated Grb2 binds through its src Homology (SH)-3 domain to a motif within the TNFR1 allowing this activated complex to stimulate Raf kinase (Hildt and Oess, 1999).

In addition to these responses, the type 1 receptor is known to activate various phospholipases leading to the release of arachidonic acid and the prostaglandins (Figure 1.2).

1.1.3.1.1.3 Signalling through the TNFR2

The membrane bound form of TNF α is vastly superior at stimulating the TNFR2 than the soluble form and thus in laboratory experiments where soluble recombinant TNF α is used TNFR2 is not efficiently activated. For this reason less is known about the type 2 receptor. Like TNFR1, TNFR2 has also been shown to activate NF κ B and JNK possibly through direct binding of TRAF2 to its cytoplasmic domain and subsequent association with RIP (Haridas et al., 1998). More recently the endothelial/epithelial tyrosine kinase (Etk) has been shown to associate with the cytoplasmic domain of TNFR2 and is believed to be a critical mediator of TNF α -induced angiogenic events (Pan et al., 2002, Figure 1.2).

1.1.3.1.2 IL-1 β

Similar to TNF α , IL-1 β is generally considered to be a pro-inflammatory cytokine. IL-1 β is translated as a 31kDa precursor protein (Auron et al., 1984) that is cleaved to 17.3kDa mature IL-1 β by the cysteine protease IL-1 β -converting enzyme (ICE, Black et al., 1988), also known as caspase 1.

The IL-1 β gene promoter region contains a TATA box and several regions that are required for the inducible transcription of this gene. One region is a strong lipopolysaccharide (LPS)-responsive enhancer that contains a composite cAMP response element/NF-IL6 and a NF κ B-like binding site. AP-1 also appears to be important in LPS induction of IL-1 β gene transcription (Shirakawa et al., 1993; Tsukada et al., 1994). In monocytes Spi-1 also appears to play an important role in IL-1 β gene expression (Yang et al., 2000).

Despite the vigorous signal for the transcription of the IL-1 β gene by a number of agents, most of the IL-1 β mRNA is degraded and no significant translation into pro-IL-1 β takes place (Dinarello, 1997).

1.1.3.1.2.1 IL-1 β receptors

The effects of IL-1 β are mediated by the type 1 IL-1 receptor complex which is comprised of the type I receptor (IL-1RI) and its accessory protein (IL-1R AcP). IL-1R AcP is unable to bind IL-1 β but acts as the second chain in the receptor complex that is essential for signalling (Greenfeder et al., 1995).

IL-1R1 is a member of the IL-1 receptor/Toll-like receptor superfamily of which all members contain a conserved cytosolic region termed the TIR domain (Toll-IL-1 receptor domain). IL-1R AcP also contains a TIR domain.

A second IL-1 β receptor, type 2 (IL-1R2) has also been identified. The Extracellular domain of IL-1R2 is highly homologous to that of IL-1RI. However, the intracellular regions of the two receptors are very different with IL-1R2 having only a short cytoplasmic tail with no TIR domain. IL-1R2 is unable to signal, instead it is shed from the cell and acts as a decoy receptor, preventing IL-1 β from binding to IL-1RI (Colotta et al., 1993).

1.1.3.1.2.2 Signalling through the IL-1R1/IL-1R AcP complex.

Heterodimerization of the intracellular domains of the IL-1R1 and IL-1R AcP through their TIR domains is likely the trigger for IL-1 β signal transduction. The heteromeric IL-1 receptor complex then recruits the cytosolic adaptor proteins MyD88 and Tollip. MyD88 possesses an N-terminal DD that enables recruitment of a serine/threonine kinase, IL-1R-associated kinase (IRAK)-1, to the receptor complex through a homotypic interaction via its N-terminal DD. The region of MyD88 that lies between its DD and TIR domains is essential for MyD88 binding of IRAK-4, which, in turn, phosphorylates IRAK-1 (Burns et al., 2003). Once phosphorylated by IRAK4, IRAK-1 autophosphorylates a different site resulting in the recruitment of TRAF6. The complex of IRAK1 and TRAF6 dissociates from the receptor complex and interacts with a complex of TAK1 (TGF β activated kinase 1) and its regulators TAB1 and TAB2 (TAK1 binding protein) probably promoted by hyperphosphorylated IRAK1. TAK1 becomes activated by phosphorylation and then either directly or indirectly via NIK, activates IKK and MKK6, resulting in activation of NF κ B and JNK (Ninomiya-Tsuji et al., 1999a). Alternatively, TRAF6 may activate NF κ B through ECSIT (evolutionary conserved signalling intermediate in Toll pathways) an adapter protein that can bridge TRAF6 to MEKK1 (Kopp et al., 1999). MEKK1 can then activate IKK. Dominant

negative MyD88, IRAK-1, and TRAF-6 all inhibited IL-1 β -induced NF κ B activation (Jefferies et al., 2001).

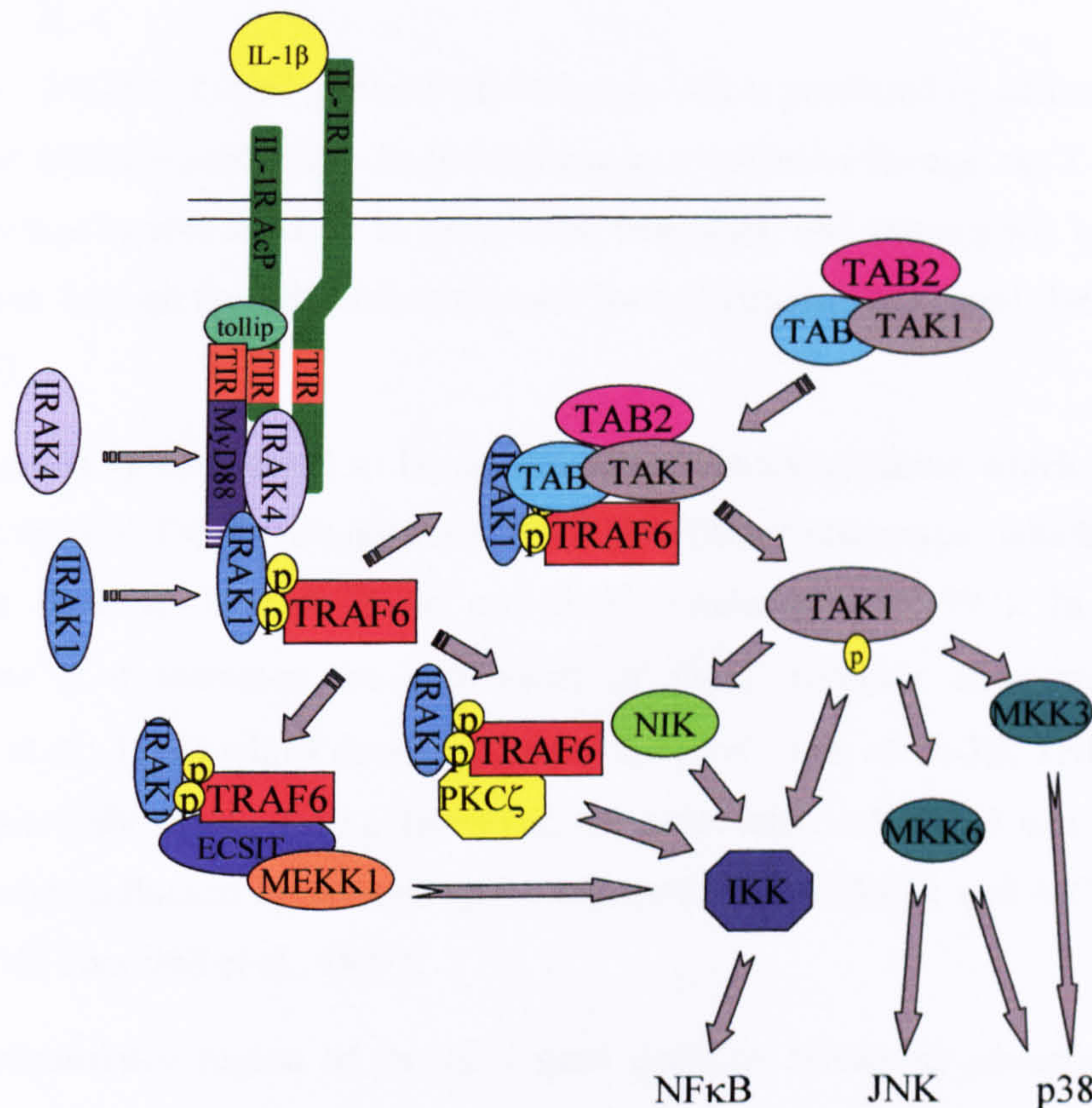


Figure 1.3. The principle intracellular signalling events induced through the binding of IL-1 β to cell associated IL-1R.

In addition, the low-molecular-weight G protein Rac1 has also been implicated in the IL-1 β signalling pathway regulating the transactivation of NF κ B p65-mediated gene expression (Jefferies and O'Neill, 2000). Rac1 has been shown to be involved in the regulation of phosphatidylinositol 3-kinase (PI3K) which has been shown to have a clear role in the phosphorylation and activation of p65 in response to IL-1 β (Sizemore et al., 1999). Such a role of Rac1 and PI3K has also been linked to the activation of NF κ B through the TLR2 activation (Arbibe et al., 2000).

Sanz et al. have shown that IL-1 β promotes the association of the atypical PKC-interacting protein p62 with TRAF6 and IRAK. In addition, the N-terminal part of TRAF6 is able to bind PKC ζ , which in turn may activate the IKK complex (Sanz et al., 2000).

IL-1 β signalling through the action of TRAF6 can also activate p38 mitogen activated protein kinase pathways (McDermott and O'Neill, 2002, Figure 1.3).

1.1.3.1.3 IL-4

IL-4 is a ~20kDa secreted globular glycoprotein that is produced by activated T cells in particular differentiated TH₂ cells in response to stimulation through the T cell receptor. IL-4 message is also induced in mast cells, basophils, and eosinophils in response to stimulation through the cell surface receptor for IgE (Brown and Hural, 1997; Woerly et al., 1999)

IL-4 is generally considered to be an anti-inflammatory cytokine which regulates the differentiation of T-cells driving them into a TH₂ helper phenotype, which secrete TH₂ cytokines IL-4, IL-5, IL-6, IL-10 and IL-13 (Seder et al., 1992). In addition, in monocytes IL-4 increases the expression of IL-1 β receptor antagonist (IL-1RA) (Ohmori et al., 1996) whilst down-regulating the production of IL-1 β , TNF α , IL-6 and IL-8 (Baumhofer et al., 1998). However, on endothelial cells IL-4 can exert a pro-inflammatory influence by enhancing the expression of VCAM-1 and MCP-1 (Colotta et al., 1992; Thornhill et al., 1991).

The 5'-untranslated region of the IL-4 gene contains numerous *cis*-acting sequence elements that can interact with *trans*-acting factors that work together to regulate IL-4 transcription. Essential for the transcription of the IL-4 gene is the transcription factor NF-AT for which there is five consensus sites in the IL-4 promoter. Two AP-1 binding sites lie adjacent to two of the NF-AT sites, the mutation of which reduces the IL-4 promoter activity by 50%. Multiple potential binding sites for the transcription factor GATA-3 are present throughout the whole IL-4 locus and STAT6 binding sites are also present in the promoter (Ranganath et al., 1998; Szabo et al., 1997).

1.1.3.1.3.1 IL-4 receptors

The IL-4 receptor consists of two separate chains (Russell et al., 1993): the 140 kDa IL-4-binding chain, also known as the IL-4R α chain and either the common gamma chain, γ c or the IL-13R α chain (Hilton et al., 1996). The complex containing the IL-4R α and the γ c is called the type I IL-4 receptor whilst that comprised of an IL-4R α and an IL-13R α is termed the type II receptor (Callard et al., 1996). The type 1 receptor will bind only IL-4 and the type 2 receptor complex will bind IL-4 and IL-13.

The cytoplasmic domain of the IL-4R α chain has no apparent serine/threonine or tyrosine kinase activity however Janus kinase (JAK)1 is constitutively associated with IL-4R α while JAK3 is coupled to the γ c and IL-13R associates with either JAK2 or tyrosine kinase (TYK)2 depending on the cell type (Murata et al., 1998; Nelms et al., 1999).

At least one of these receptor complexes is expressed on most cell types including cells of the brain, muscle, kidney, and placenta, and primary cell types including fibroblasts, epithelial cells, endothelial cells, and a number of different tumours.

1.1.3.1.3.2 IL-4 receptor signalling

The binding of IL-4 to the IL-4R α induces heterodimerization with the γ c or the IL-13R α . The dimerization activates the Janus kinases that initiate the phosphorylation cascade. Tyrosine residues in the cytoplasmic tail of the IL-4R α become phosphorylated and act as docking sites for STAT6. STAT6 then becomes tyrosine phosphorylated, dimerizes, migrates to the nucleus and binds to consensus sequences found within promoters of IL-4-regulated genes. IL-4-induced activation of STAT6 appears to require co-operative interactions with other transcription factors to induce efficient transcription of the target genes (Shen and Stavnezer, 1998).

A second signalling pathway activated by the IL-4 receptor is the insulin receptor substrate (IRS) family (IRS-1, IRS-2, IRS-3) pathway. The IRS proteins are recruited to the IL-4R α chain through an interaction with the receptor protein tyrosine binding (PTB) domain and become phosphorylated by the receptor associated JAKs. Tyrosine phosphorylated sites within the IRS proteins associate with cellular proteins that contain SH2 domains. All IRS proteins interact with the p85 subunit of PI3K that activates the catalytic subunit of the kinase. PI3K activity is important for growth, survival, and regulation of gene expression in response to IL-4. Several signalling molecules whose activities are downstream of PI3K activity have been implicated in IL-4 responses, including p70^{S6} kinase, the Akt kinase, and the non histone high-mobility group DNA-binding protein HMGI(Y) (Nelms et al., 1999). Phosphorylated IRS also associates with Grb2 resulting in subsequent ERK1/2 activation and regulation of cell cycle (Figure 1.4).

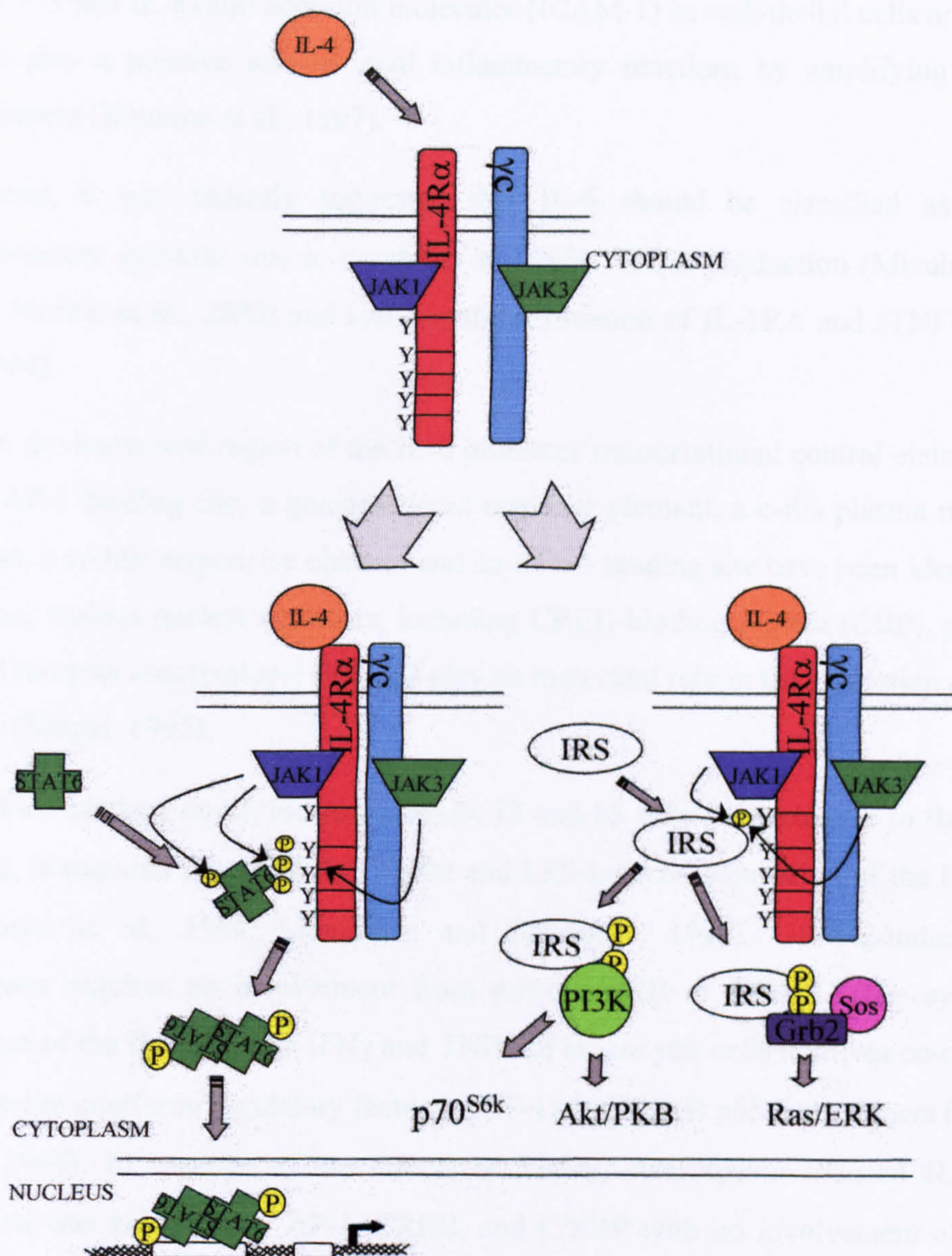


Figure 1.4. Intracellular signalling events induced through the binding of IL-4 to cell associated IL-4 type 1 receptor

1.1.3.1.4 IL-6

IL-6 is produced by a variety of cell types and although the main sources are macrophages, fibroblasts, and endothelial cells other cells such as T-cell, monocytes, eosinophils, mast cells, astrocytes, smooth muscle cells, islet- β cells and mesengial cells can also produce IL-6 (Van Snick, 1990).

IL-6 initially was classified as a pro-inflammatory cytokine, mainly because its expression increases in the early course of an infection and because of its key role in the induction of acute phase genes and proteins in the liver. In addition, IL-6, in combination with it's soluble receptor can induce the expression of chemokines (MCP-

1, MCP-3 and IL-8) and adhesion molecules (ICAM-1) in endothelial cells and thus IL-6 can play a positive role in local inflammatory reactions by amplifying leucocyte recruitment (Romano et al., 1997).

However, it was recently suggested that IL-6 should be classified as an anti-inflammatory cytokine due to its ability to inhibit TNF α production (Mizuhara et al., 1994; Starkie et al., 2003) and increase the expression of IL-1RA and sTNFR (Tilg et al., 1994).

Within the conserved region of the IL-6 promoter transcriptional control elements such as an AP-1 binding site, a glucocorticoid response element, a c-fos plasma responsive element, a cAMP responsive element and an NF κ B binding site have been identified. In addition, various nuclear cofactors, including CREB-binding protein (CBP), p300, and steroid receptor coactivator-1 (SRC-1) play an important role in the regulation of the IL-6 gene (Sehgal, 1992).

The NF κ B-binding motif, located between 73 and 63 base pairs relative to the mRNA cap site, is required for the IL-1 β , TNF α and LPS-induced expression of the IL-6 gene (Dendorfer et al., 1994; Libermann and Baltimore, 1990). TNF α -induced IL-6 expression requires no involvement from AP-1, CREB or C/EBP. The synergistic induction of the IL-6 gene by IFN γ and TNF α in monocytic cells involves co-operation between the interferon regulatory factor 1 (IRF-1) and NF κ B p65 homodimers (Sanceau et al., 1995). In contrast to the effects of TNF α , staurosporin induced IL-6 gene activation was mediated by AP-1, CREB, and C/EBP with no involvement of NF κ B. Thus the stimuli and the cell type will determine the relative importance of promoter binding sites, transcription factor activation and co-factor association in the transcription of the IL-6 gene (Sanceau et al., 1995; Vanden Berghe et al., 1999).

1.1.3.1.4.1 IL-6 receptors

The IL-6 receptor consists of two subunits; the α -chain (IL-6R), an 80kDa transmembrane glycoprotein which binds IL-6 with low affinity, and the β -chain (gp130), a 130kDa transmembrane glycoprotein which binds to the IL-6 IL-6R heterodimer to form the high-affinity signal transducing complex. gp130 also associates with and transmits signals from receptors for other members of the IL-6-related cytokine subfamily (LIF, CNTF, OSM, IL-11, and CT-1).

Binding of IL-6 to the IL-6R α chain triggers the association with gp130 leading to the formation of a hexamer composed of two of each of IL-6, IL-6R α and gp130 (Ward et al., 1994).

A soluble form of IL-6R exists which may be generated either by shedding of the membrane receptor (Mullberg et al., 1993) or by alternative splicing (Muller-Newen et al., 1996). The complex of IL-6 and sIL-6R has agonistic effects by associating with gp130, which is ubiquitously expressed (Hibi et al., 1990), activating the signal transduction pathway. In this manner IL-6, in association with the soluble receptor can exert an effect on cells that do not express the IL-6R α chain (Hirano et al., 1997).

1.1.3.1.4.2 Signal transduction through gp130

Binding of IL-6 to the IL-6R α chain causes dimerization to gp130 and initiates intracellular signalling through STAT proteins and through other SH2 domain-containing proteins, including SHP2. The Janus kinases, JAK1, JAK2, and TYK2 that constitutively associate with the intracellular domain of gp130 become tyrosine phosphorylated and activated upon gp130 dimerization, an absolute requirement for activation of STAT3 and SHP2/ERK signalling cascades. Activated JAKs subsequently tyrosine phosphorylate the cytoplasmic tail of gp130 resulting in the recruitment of SH2 domain-containing signal-transducing molecules. The cytoplasmic tail of gp130 contains six tyrosine residues which all have glutamine at the third position away (YXXQ, Stahl et al., 1995). Phosphorylation of the second tyrosine residue from the membrane provides a docking site for the protein tyrosine phosphatase SHP-2 whilst phosphorylation of any one of the four tyrosines (third to sixth from the membrane) in the carboxyl terminus provides docking sites for STAT proteins mainly STAT1 and 3. The STAT proteins are subsequently tyrosine and serine phosphorylated, dissociate from gp130, dimerize and translocate to the nucleus where they can regulate the transcription of target genes (Heinrich et al., 1998). Tyrosine phosphorylation of SHP-2 at the gp130 receptor by receptor-associated JAKs provides a docking site for Grb2 and the constitutively associated Ras/GTP exchange factor Sos. The GTP bound Ras activates Raf-1 and subsequent Erk activation (Li et al., 1994). In addition tyrosine phosphorylated SHP-2 also associates with Grb2-associated binder-1 (Gab1), a member of the Gab family of scaffolding/docking proteins and the p85 subunit of PI3K, which leads to the activation of Akt (Ernst and Jenkins, 2004; Hirano et al., 1997) Figure 1.5).

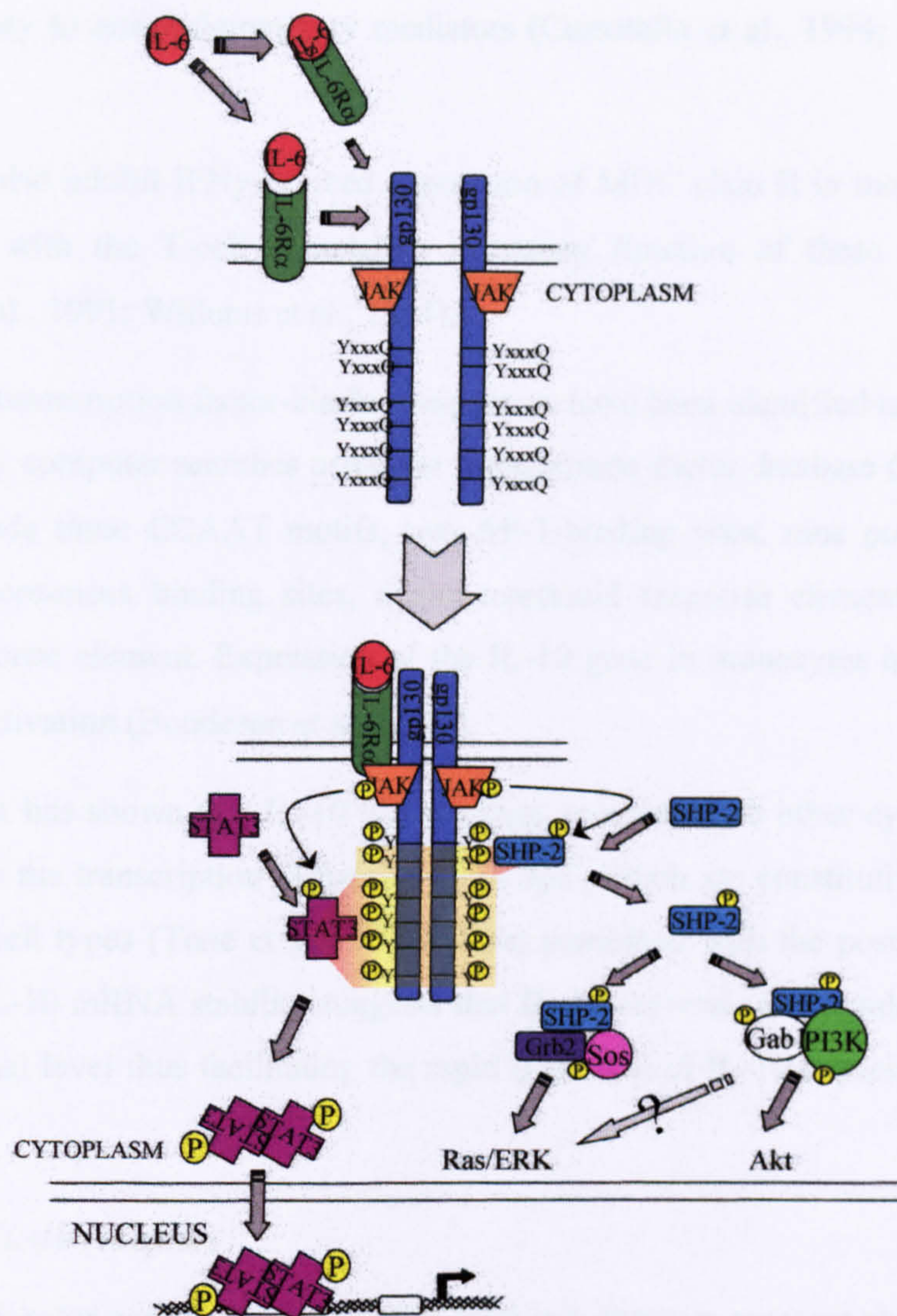


Figure 1.5. IL-6-induced signalling through gp130 receptor (Ernst and Jenkins, 2004)

1.1.3.1.5 IL-10

IL-10 is well recognised as a dominant immunoregulatory molecule with important immunostimulatory and immunosuppressive and anti-inflammatory activities. IL-10 significantly modulates expression of cytokines, soluble mediators and surface molecules on cells of myeloid origin. IL-10 strongly inhibits the production of $\text{TNF}\alpha$, IL-1 α , IL-1 β , IL-6, IL-8, IL-10 itself, IL-12, GM-CSF, G-CSF, M-CSF, MIP-1 α , MIP-1 β , MIP-2, RANTES and LIF by activated monocytes/macrophages (Moore et al., 2001a).

Furthermore, IL-10 can enhance LPS-induced expression of soluble $\text{TNF}\alpha$ receptors and the production of IL-1RA from monocytes, in part through the inhibition of mRNA degradation, indicating that IL-10 can mediate a shift from production of pro-

inflammatory to anti-inflammatory mediators (Cassatella et al., 1994; Dickensheets et al., 1997a).

IL-10 can also inhibit IFN γ -induced expression of MHC class II in monocytes thereby interfering with the T-cell stimulating accessory function of these cells (de Waal Malefyt et al., 1991; Willems et al., 1994).

Consensus transcription factor-binding sequences have been identified in the IL-10 gene promoter by computer searches using the transcription factor database (GenBank tfdb). These include three CCAAT motifs, two AP-1-binding sites, nine potential C/EBP β (NF-IL6) consensus binding sites, a glucocorticoid response element (GRE) and a cAMP response element. Expression of the IL-10 gene in monocytes is not dependent on NF κ B activation (Bondeson et al., 1999).

Recent work has shown that IL-10 transcription, in contrast to other cytokines, can be regulated by the transcription factors Sp1 and Sp3, which are constitutively active in a number of cell types (Tone et al., 2000). This, combined with the post transcriptional control of IL-10 mRNA stability suggests that IL-10 expression is regulated at the post transcriptional level thus facilitating the rapid induction of IL-10 expression (Powell et al., 2000).

1.1.3.1.5.1 IL-10 receptors

The IL-10 receptor complex consists of at least two separate receptor chains, a 90- 120 kDa , *N*- glycosylated ligand-binding IL-10R1 chain and a 40kDa IL-10R2 chain, which functions as an accessory subunit that is essential for the active IL-10 receptor complex to initiate IL-10-induced signal transduction events (Kotenko et al., 1997). Within the cytoplasmic region of IL-10R1 is a STAT3 docking site and a JAK1 association region. The cytoplasmic region of IL-10R2 is associated with TYK2 kinase.

IL-10/ IL-10R complex formation occurs in two steps: initially, IL-10 binds with high affinity to IL-10R1, and subsequently IL-10R2 is bound with lower affinity. A complex between the extracellular domain of the human IL-10R1 and IL-10 consists of two IL-10 homodimers and four receptors (Josephson et al., 2001; Syto et al., 1998).

IL-10R1 chain is expressed on T cells, B cells, monocytes, macrophages, dendritic cells, NK cells, mast cells, and various haematopoietic progenitors. IL-10R1 expression on monocytes is increased upon cellular activation (Moore et al., 2001a) and can be induced on human epidermal cells and keratinocytes by glucocorticoids (Michel et al.,

1997) and on fibroblasts by LPS (Weber-Nordt et al., 1994). In contrast to IL-10R1, IL-10R2 chain seems to be expressed ubiquitously.

1.1.3.1.5.2 IL-10 receptor signalling

Neither chain of the IL-10 receptor possess intrinsic kinase activity. Binding of IL-10 to the IL-10R1 chain causes the tyrosine phosphorylation of the IL-10R1-associated JAK1 and the IL-10R2-associated TYK2 kinases (Finbloom and Winestock, 1995). Phosphorylation of JAK1 and TYK2 activates their kinase activity and leads to phosphorylation of two tyrosine residues in the cytoplasmic tail of IL-10R1 thus creating a STAT3 docking site. Once recruited to the receptor, STAT3 is phosphorylated by the active JAK1 and TYK2. The phosphorylated STAT3 proteins form hetero- or homo-dimers, translocate to the nucleus and bind, with high affinity, to STAT-binding elements (SBE) in the promoters of IL-10-responsive genes (Figure 1.6, (Wehinger et al., 1996). Whilst STAT3 appears to be essential for the anti-inflammatory effects of IL-10, one or more additional pathways are also required. In addition, STAT3 transcriptional activity may be dispensable for some effects of IL-10 and under some circumstances acts solely as a docking molecule responsible for the recruitment of other proteins (Moore et al., 2001a). IL-10 also induces STAT1 and, in non-macrophage cells, STAT5 phosphorylation and activation. However, neither STAT1 nor STAT5 are directly recruited to the IL-10R1 chain and their activation likely occurs through other mechanisms (Weber-Nordt et al., 1996). The exact role of STAT1 and 5 conferring IL-10 functions is unclear.

1.1.3.2 Chemokines

Recruitment of circulating leucocytes requires a variety of soluble and cell-associated factors, which mediate their migration into injured or inflamed tissues. Chemokines are chemotaxis-inducing proteins of 70–130 amino acids that are produced by a variety of cell types in response to antigens, polyclonal stimulants, cell irritants and cytokines. Chemokines are characterized by their ability to induce not only directional migration of leucocytes but also their growth and activation and thus in such a manner play an important roles in controlling inflammatory and immune responses.

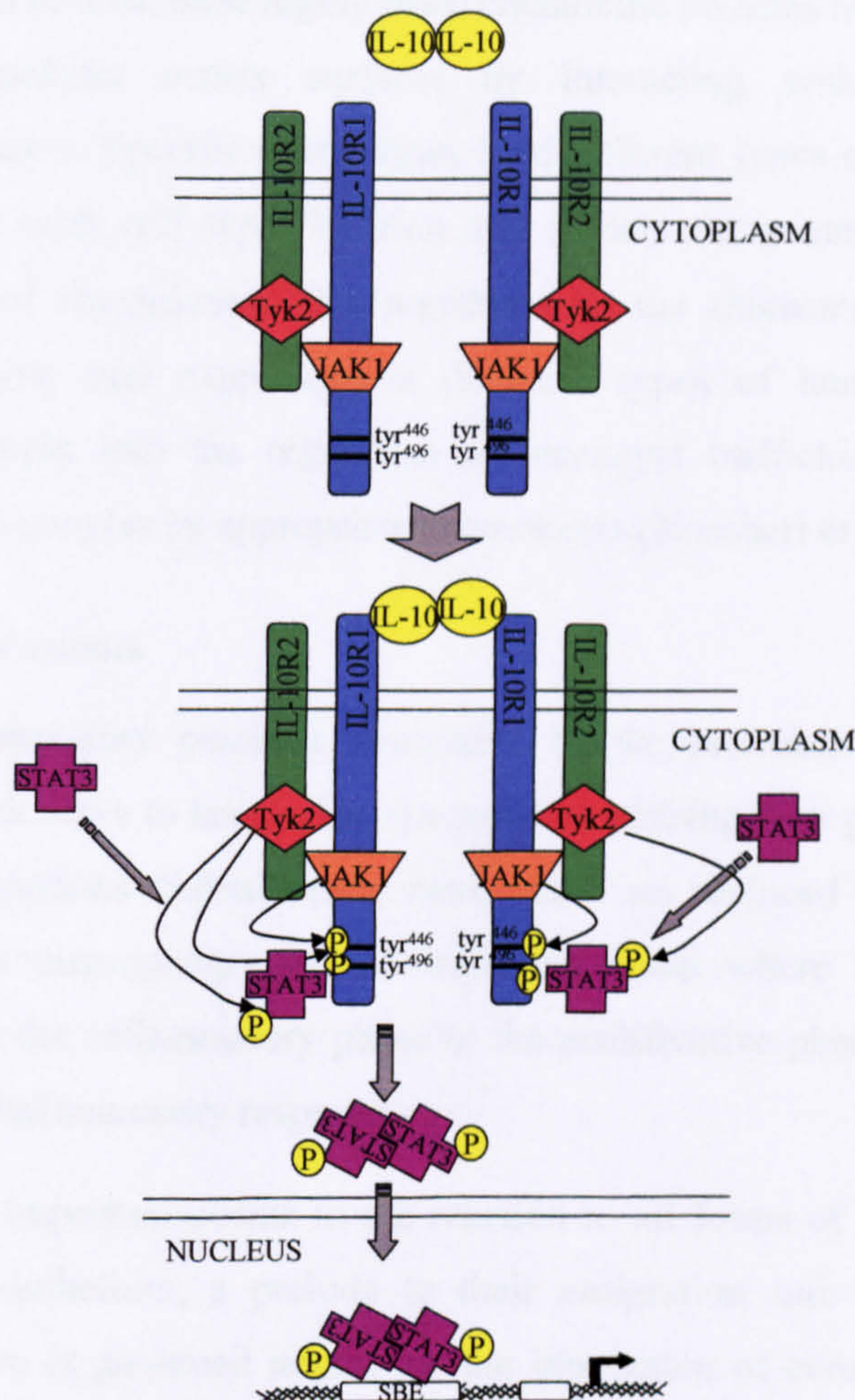


Figure 1.6. Signalling through the IL-10 receptor complex

Chemokines contain four conserved cysteine residues that are linked by disulfide bonds. Sequence motifs in the vicinity of the first two cysteine residues determine receptor recognition and triggering and form the basis of chemokine subfamily nomenclature (CXC, CC, XC, CX₃C). The disulfide bonds keep these functional domains in close proximity and are thus essential for biological activity

Chemokines mediate their activities by binding to G protein-coupled, seven transmembrane domain family of receptors of which there are four classes corresponding to their ligand usage (CXCR 1-6, CCR 1-10, XCR and CX₃CR). There is a certain amount of promiscuity among chemokine receptor usage with most receptors recognizing more than one chemokine and several chemokines binding to more than one receptor.

One effect brought about by all chemokines involves the chemotaxis of the cell expressing the receptor toward areas with higher concentrations of the chemokine. To

elicit chemotaxis *in vivo*, these highly basic chemokine proteins must be immobilized on cells or extracellular matrix surfaces by interacting with negatively charged glycosaminoglycans. Specific chemokines bind different types of glycosaminoglycans which can vary with cell type, location and inflammatory status allowing selective immobilization of chemokines. This together with the characterization of chemokine receptor selectivity and expression in different types of leucocytes has provided considerable insight into the regulation of leucocyte trafficking allowing selective recruitment of leucocytes by appropriate chemokines (Kuschert et al., 1999).

1.1.3.3 Cellular events

The initial inflammatory phase is dominated by the presence of large numbers of neutrophils, which serve to handle foreign material utilising their phagocytic, enzymatic and oxidative functions. Subsequently neutrophils are replaced by monocytes which differentiate into macrophages in the inflamed tissue where they co-ordinate the progression from the inflammatory phase to the proliferative phase and through to the resolution of the inflammatory response.

One of the most important events in the reaction to all forms of injury is adhesion of leucocytes to endothelium, a prelude to their emigration into tissues. Endothelial-leucocyte adhesion is governed largely by the interaction of complementary adhesion molecules on endothelia and leucocytes.

1.1.3.3.1 Endothelial cell activation

The vascular endothelium is a dynamic structure that regulates the peripheral blood flow and vasomotor tone through the production of a wide range of vasoactive factors and chemical signals. The general concept of vascular function is that the healthy endothelium controls the balance between several countervailing forces such as vasoconstriction and vasodilation, anti-coagulation and pro-coagulation, anti-inflammatory and pro-inflammatory, growth promotion and inhibition and oxidant and anti-oxidant. Under normal physiological circumstances the endothelium functions in an inhibitory mode to maintain relaxed vascular tone, inhibit smooth muscle cell proliferation, platelet aggregation, leucocyte activation and adhesion, and vascular extracellular matrix secretion. When this balance is disrupted, it predisposes the vasculature to vasoconstriction, leucocyte adherence, platelet activation, mitogenesis, pro-oxidation, thrombosis, impaired coagulation and vascular inflammation. In the early 1990s, the concept of endothelial activation was introduced to define these qualitative

changes in the function of the endothelium that cumulatively allow endothelial cells to perform new functions (Cotran and Pober, 1990).

Endothelial cell activation is accomplished by the signalling from cell surface receptors that recognise inflammatory or thrombotic stimuli or by traumatic stimuli. The phenotype displayed by the activated endothelial cell will depend principally on the activating stimuli, the time after stimulation, the concentration or magnitude of the stimulus, concomitant application of different stimuli, sequential stimulation after previous activation and other factors. In this manner the nature of the stimuli dictates the pattern of adhesion and signalling molecules expressed on the endothelial cells so as to ensure an appropriate response is mounted.

In the context of an inflammatory response, activated endothelial cells can express pro-inflammatory factors such as cytokines, chemokines, platelet activating factor, adhesion molecules and tissue factor that allow them to recruit and activate specific inflammatory cells and pathways in order to localize inflammation to sites of injury and effectively aid in the process of elimination of the injuring stimuli and tissue repair. In such situations cellular activation can occur as a direct result of the insult or in response to mediators released from cells in the affected tissue or from stimuli in the circulation.

1.1.3.3.2 Endothelial cell-leucocyte interactions

Endothelial cell-leucocyte interactions are early and critical events in acute and chronic inflammation. Defective adhesive and signalling interactions between activated endothelial cells and leucocytes, such as in leucocyte adhesion deficiency (LAD) syndromes, results in impaired cellular targeting to injured or infected tissue (Bunting et al., 2002). Conversely dysregulated adhesion and signalling can lead to excessive accumulation of activated leucocytes and tissue injury such as in severe acute and chronic inflammatory diseases.

The endothelial cell-leucocyte adhesion cascade involves a sequence of activation and adhesion events that culminates in the transmigration of leucocytes through the vascular endothelium into the site of injury. Leucocytes are usually free flowing in the central blood stream of post capillary venules. Initially circulating leucocytes tether to activated endothelial cells and roll along them. Localised signalling and subsequent activation-dependent firm adhesion occurs causing leucocyte arrest and emigration through the endothelium. Blocking any one of these events can severely impair leucocyte recruitment and the subsequent efficiency of the inflammatory response. A variety of



inducible endothelial cell molecules are used for these interactions with leucocytes, the specific expression pattern of which are determined in a stimuli- and time-dependant fashion and are recognised by different classes of leucocytes (Zimmerman et al., 1999).

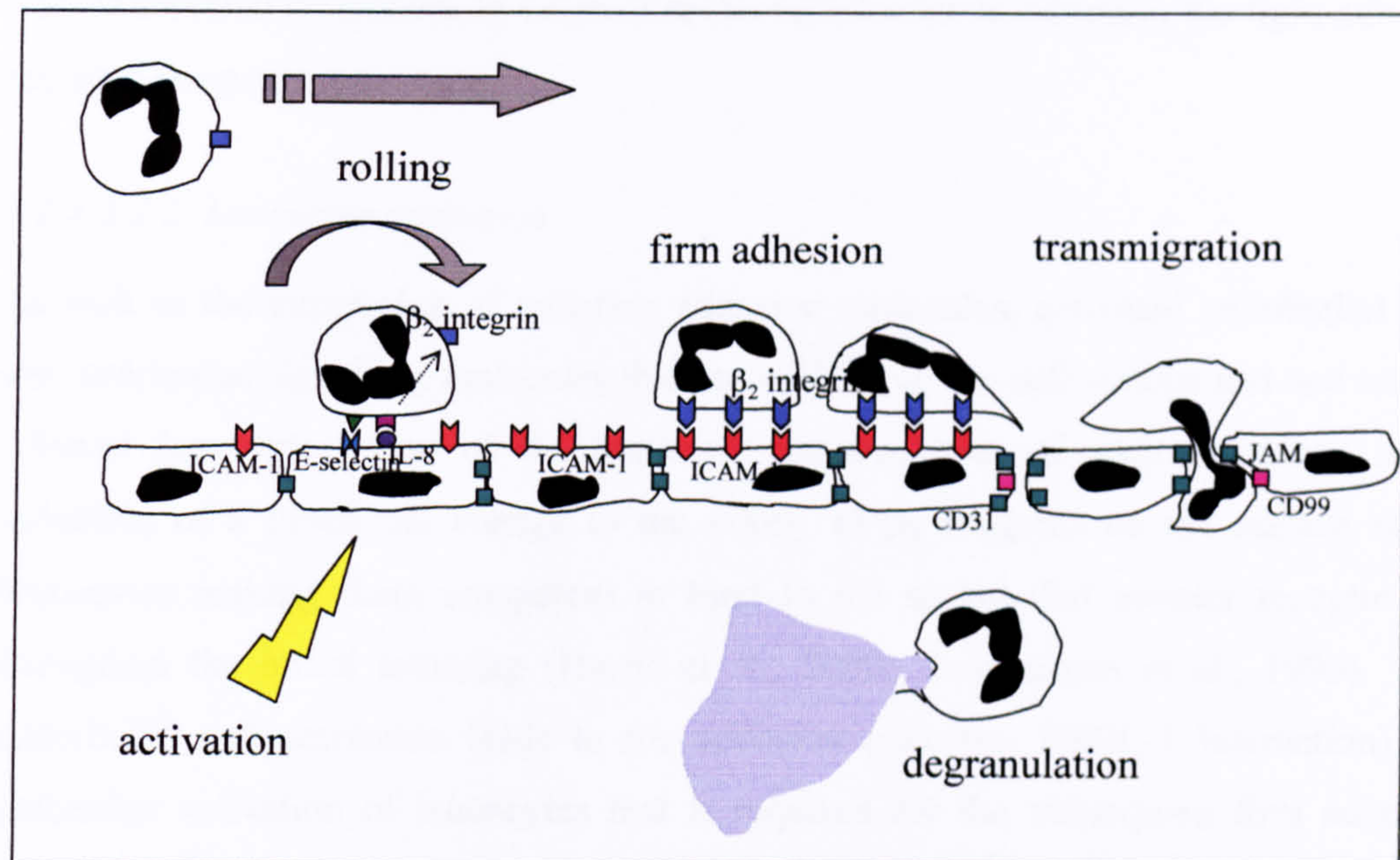


Figure 1.7. Key events and molecules in the transendothelial cell migration of leucocytes.

1.1.3.3.2.1 Leucocyte tethering and rolling

Leucocyte tethering or capture requires the induced expression of selectins on the surface of the activated endothelial cells, which bind to oligosaccharides with terminal sialylated and fucosylated structures on the leucocyte (McEver et al., 1995). The endothelial cell adhesion molecule P-selectin is primarily responsible for this initial process and is rapidly mobilised to the cell surface from the Weibel-Palade bodies following activation by histamine or thrombin. This rapid increase in surface expression of P-selectin makes the endothelium 'sticky' to the circulating leucocytes through interaction with its principle leucocyte ligand P-selectin glycoprotein ligand-1 (PSGL-1, (McEver and Cummings, 1997). Activation of human endothelial cells with agonists such as IL-1 β , TNF α or LPS induces the transcription of the E-selectin gene, the surface expression of which is evident within 2-6 hours (Bevilacqua, 1993). E-selectin interacts with the leucocyte E-selectin ligand (ESL-1) and also with PSGL-1 (McEver, 1997). The bonds between endothelial cell selectins and their leucocyte ligands, which do not require leucocyte activation, are rapidly established and then reversed (McEver et al., 1995). In this manner, E-selectin and P-selectin facilitate the capturing of

leucocytes by the activated endothelium and mediate their rolling along it. Once rolling along the endothelial surface tight adhesion between the endothelial cell and the leucocyte is required in order to arrest the movement to the leucocyte and allow transendothelial migration. In contrast to the initial selectin adhesion the tight adhesion requires leucocyte activation.

1.1.3.3.2 Leucocyte activation

As well as the expression of tethering adhesion molecules, activated endothelial cells also synthesise signalling molecules that immobilise on the cell surface and activate the tethered leucocytes. One of the important consequences of such activation is the induction of a functional change to the family of β_2 integrins on the surface of the leucocytes making them competent to bind to the endothelial counter receptor and strengthen the initial tethering (Harris et al., 2000; Zimmerman et al., 1996). Thus endothelial cell activation leads to the adhesion (selectin- PSGL-1 interaction) and juxtacrine activation of leucocytes that is required for the subsequent firm adhesion step. This localised activation of tethered leucocytes by signalling molecules generated by activated endothelial cells at the site of tissue injury confers significant biological advantages for spatial regulation of physiological inflammatory responses. The first such signalling molecule to be identified was platelet activating factor (PAF). Within minutes of activation with stimuli such as histamine or thrombin, PAF is co-expressed with P-selectin on the endothelial cell surface (Lorant et al., 1991; Prescott et al., 1984). PAF induces inside-out signalling of β_2 integrin on the neutrophils that are tethered to the endothelium via P-selectin interaction. Such co-ordinated action of adhesion and signalling is also observed in a more delayed adhesion response in which E-selectin and the CXC chemokine, interleukin-8 are upregulated in a transcription dependant manner following endothelial activation with $\text{TNF}\alpha$, $\text{IL-1}\beta$ or LPS (Huber et al., 1991; Zimmerman et al., 1996). Other chemokines such as MCP-1 are also synthesised by activated endothelial resulting in the selective activation and recruitment of monocytes (Ben Baruch et al., 1995). The synthesis of leucocyte activating molecules by the endothelial cells is, as with adhesion molecule expression, largely determined by the inflammatory agonist and the time after stimulation (Zimmerman et al., 1992). Thus the regulated expression of adhesion molecules and signalling molecules provides a mechanism for the differential activation of specific classes of leucocytes depending on the initiating stimuli.

1.1.3.3.2.3 Firm adhesion and transmigration.

Signal dependant alterations in integrin affinity are critical for the tight adhesion, arrest and emigration of leucocytes. Of importance in such events is the β_2 integrin family of leucocyte adhesion molecules that are comprised of a common β chain (β_2 , CD18) and one of three α chains (α^L , α^M and α^X - commonly referred to as CD11a, CD11b and CD11c respectively). β_2 integrins, which are constitutively expressed on leucocytes, are also stored in intracellular granules where they are rapidly translocated to the cell surface upon activation. The nature of the activation dependant changes in the integrin function, which increases their avidity for the endothelial counter receptors, remain largely elusive but appear to involve interactions between the cytoplasmic tails of the α and β chains (Johnston and Butcher, 2002; Liddington and Ginsberg, 2002). The best characterised endothelial cell ligands for the β_2 integrins are members of the immunoglobulin superfamily including ICAM-1 and ICAM-2. CD11b/CD18 interacts with the third Ig domain of ICAM-1 whilst CD11a/CD18 interacts with the N-terminal domain of both ICAM-1 and ICAM-2 (Diamond et al., 1991). On vascular endothelial cells ICAM-1 is constitutively expressed and is dramatically upregulated, in a transcription dependent fashion by inflammatory stimuli such as LPS, IL-1 β or TNF α (Bevilacqua et al., 1994). Such abundant expression of ICAM-1 on both endothelial cells and other cells is a common characteristic of an inflammatory response. ICAM-2 is also constitutively expressed on endothelial cells but its expression is not affected by endothelial cell activation.

The transmigration of leucocytes through the endothelium is a rapid process (taking about 90 seconds *in vitro* (Muller, 2003)) that appears to involve the modulation of selectin and integrin bonds leading to a regulated 'de-adhesion'. This is followed by homophilic interactions between endothelial and leucocyte CD99, CD31 and junctional adhesion molecule (JAM)-A or heterphilic interactions between leucocyte integrins and JAM molecules at the tight junctions in the endothelial monolayer (Schenkel et al., 2002). Signalling events initiated by binding of chemoattractants to leucocyte receptors and leucocyte-endothelial interactions trigger signalling cascades which result in cytoskeletal remodelling and both intracellular and cell surface protein interactions that modulate detachment at the rear of the migrating cell. Leucocyte transmigration through the endothelium is facilitated by endothelial retraction that occurs through an increase in intracellular free calcium in the endothelial cells adjacent to the transmigrating

leucocyte causing the activation of myosin light chain kinase and the unfolding of myosin II (Su et al., 2000). The increase in free calcium is triggered by two possible mechanisms; release of soluble factors from the activated, tightly adhered leucocytes such as heparin binding proteins released from the azurophil granules (Gautam et al., 2001) or the cross-linking of adhesion molecules on the activated endothelial cells (Lorenzon et al., 1998).

1.1.3.3.3 Neutrophils

Once an inflammatory response is initiated, neutrophils are the first cells to be recruited to sites of injury and are the major cell type involved in the acute inflammatory response. Once in the tissue neutrophils kill the infectious agent, remove the infectious and necrotic material and then aid in healing and scar formation. These functions are mediated by a combination of neutrophil granules that contain noxious anti-microbial products, which are released to the exterior, together with generation of oxygen radicals via the NADPH oxidase system and the phagocytic ability of these cells.

Neutrophil degranulation is a critical activation response. Human neutrophils contain secretory vesicles, gelatinase granules, specific or secondary granules, and azurophil or primary granules. These intracellular granules contain anti-microbial or cytotoxic substances, neutral proteinases, MMP, acid hydrolases and pools of membrane receptors. Mobilisation of the granular constituents appears to occur in a strict rank order that is dependent on the degree of neutrophil activation. The secretory vesicles are most readily translocated followed by the gelatinase granules, the secondary and then finally the primary granules (Topham et al., 1998).

Granular translocation and secretion are important for the interaction of neutrophils with the endothelium through the recruitment of integrins and matrix adhesion receptors to the cell surface. This will influence the binding of these cells to vascular structures and the release of granular proteases to facilitate the movement through the sub-cellular matrix. Among azurophil granule constituents, myeloperoxidase (MPO) is a critical enzyme in the conversion of hydrogen peroxide (H_2O_2) to hypochlorous acid forming the most effective microbicidal and cytotoxic mechanism of leucocytes. Activation of neutrophils causes the translocation of the cytosolic components of the neutrophil NADPH oxidase to the plasma membrane to assemble the active oxidase and neutrophil respiratory bursts result in the generation of large amounts of superoxide, the dismutation of which constitutes the primary source of H_2O_2 for MPO.

Activated neutrophils at the inflammatory loci also have the capacity to release pro-inflammatory cytokines and chemokines that are responsible for the activation and recruitment of other leucocytes to the affected tissue.

At the site of inflammation neutrophils undergo constitutive apoptosis, a process associated with the 'disabling' of their potentially injurious secretion responses and other effector functions through alterations of surface molecules including adhesion molecules and the FcγRIII (Whyte et al., 1993). In addition, apoptosis-associated surface markers, such as phosphatidylserine, changes in carbohydrate structures, the binding of C1q (the first component of the complement cascade) to blebs on the surface of apoptotic cells are thought to provide cues that lead to the rapid phagocytic clearance of apoptotic neutrophils by macrophages (Grimsley and Ravichandran, 2003; Maderna and Godson, 2003). This neutrophil apoptosis is believed to be the first step towards the resolution of inflammation and the initiation of tissue repair.

1.1.3.3.4 Monocytes/macrophages

Monocytes are the circulating precursors of tissue macrophages (Mφs). Normal monocytes express a number surface receptors for cytokines, chemokines, bacterial products and complement fragments. Activation of circulating monocytes via these receptors or via contact with non-endothelial surfaces results in the activation of transcription factor pathways, expression of tissue factor, the release large amounts of pro-inflammatory cytokines such as IL-1β, TNFα and IL-8 and the upregulation of adhesion molecule (CD11b/CD18) expression. Monocyte CD11b also serves as a receptor for iC3b (CR3), facilitating binding and phagocytosis of complement-opsonized particles (Sutterwala et al., 1996). Monocytes leave the circulation and enter the inflammatory site later than neutrophils. Once the monocyte leaves the circulation it changes its phenotype and differentiates into a Mφ (defined by its capacity to phagocytose). Monocytes are generally, therefore, considered to be immature macrophages. However, it can be argued that monocytes represent the circulating macrophage population and should be considered fully functional for their location, changing phenotype in response to factors encountered in specific tissue after migration. Tissue macrophages are the predominant cell type in tissue during chronic inflammation.

Tissue macrophages can function to activate the immune system by processing and presenting antigens to T-cells and also produce quantities of pro-inflammatory

cytokines, chemokines and growth factors to activate cells in the inflamed tissue that will participate in the remodelling and repair process. Mφs are professional scavengers and are responsible for the removal of bacterial and injured cell debris by phagocytosis. In addition, macrophages are responsible for the phagocytic clearance of apoptotic neutrophils, which importantly, unlike other phagocytic pathways, fails to promote release of pro-inflammatory cytokines by the macrophages (Meagher et al., 1992). Indeed ingestion of the apoptotic neutrophils by Mφs leads to a dampening down of their pro-inflammatory activities altering the Mφ to a cell that has the capacity to initiate the resolution of the inflammatory response (Maderna and Godson, 2003). Monocytes and macrophages, therefore, play a central role in the initiation and maintenance of inflammation and the subsequent tissue clean up and wound healing.

1.1.4 RESOLUTION OF THE INFLAMMATORY RESPONSE

The resolution of inflammation can be defined as the complete restoration of tissue to normal after an episode of inflammation. The likelihood of such an occurrence is determined by the extent of tissue damage, the tissue in which the inflammation occurred and its the ability to regenerate, the speed at which the initiating factor was destroyed or removed and the extent of tissue vascularisation to aid removal of debris and fluid. If inflammation does not resolve then chronic inflammation and wide spread tissue destruction can ensue.

Many of the defence mechanisms employed by neutrophils are potentially deleterious to host tissues; for example, excessive release of granule proteases or production of reactive oxygen species. Thus, it is important that granulocytes are effectively and rapidly destroyed concomitant with the removal of the inflammatory stimulus to avoid excessive tissue damage or chronic inflammation. One of the major events in the resolution of an inflammatory response is the clearing of neutrophil by apoptosis. Apoptotic cell death is advantageous over necrotic cell death in that it prevents the loss of membrane integrity and the release of the toxic intracellular content and thus prevents further tissue damage. Efficient recognition and removal of the apoptotic cells by phagocytes such as macrophages or, in certain situations, by cells with phagocytic potential such as fibroblasts is required in order to prevent the neutrophils from eventually undergoing 'secondary necrosis' releasing damaging intracellular contents and amplifying the inflammatory response. Phagocytosis of apoptotic neutrophils causes the suppression of pro-inflammatory mediator release from macrophages and increases

the release of anti-inflammatory factors (Voll et al., 1997), with the subsequent emigration of the macrophages from the inflamed tissue to draining lymph nodes (Bellingan et al., 1996). In contrast macrophage phagocytosis of post-apoptotic granulocytes leads to the production of pro-inflammatory mediators contributing to an exaggerated, prolonged inflammatory response (Stern et al., 1996). This process might underlie the development of chronic inflammatory conditions in which apoptotic cell load and phagocytic clearance mechanisms are mismatched. It is also possible that the presence of cytokines and growth factors at the site of inflammation provide pro-survival signals to the neutrophils that have the potential to override the granulocyte apoptotic pathway.

* * *

Dysregulated, excessive inflammation is associated with a number of acute and chronic conditions that contribute to the morbidity of the population. Such conditions even though driven by the same mediators often bare no clinical resemblance to each other even when affecting the same organ systems. Heart failure and complications following cardiac surgery are clinically very different disorders of the cardiovascular system in which excessive, dysregulated local and systemic inflammatory responses are believed to significantly contribute to their pathologies.

1.2 INFLAMMATORY RESPONSE ASSOCIATED WITH HEART FAILURE

1.2.1 BACKGROUND TO HEART FAILURE

The inability of the heart to pump enough blood to sustain normal bodily functions describes the syndrome of heart failure.

Heart failure is an immense public health problem in the United Kingdom and indeed throughout the developed world. The British Heart Foundation (BHF) estimate that there are over 878,000 heart failure patients in the UK and that the consequential direct cost to the National Health Service (NHS) is around £625 million a year. The true economic cost of heart failure is actually far greater when one considers the expenses due to illness and death in those of working age and the economic effects to their families and friends who care for them.

British Heart Foundation estimate that there are 63,500 new cases of heart failure each year in the UK and that in 2001, the true number of deaths from heart failure was at

least 24,000. This means that at least 4% of all deaths in the UK are due to heart failure (BHF Coronary heart disease statistics at www.heartstats.org).

1.2.2 PATHOGENESIS OF HEART FAILURE

The syndrome of heart failure is the summation of multiple anatomical, biological and functional changes to the heart and represents the last stage in the progression of cardiovascular disease, from the development of left ventricular (LV) hypertrophy and /or a myocardial infarction to LV dysfunction and, finally, to heart failure.

Heart failure can be viewed as a progressive disorder caused by either loss of myocyte function resulting from damage to the heart muscle or disruption to the ability of the myocardium to generate force thus preventing it from contracting normally. The initial decline in the pumping action of the heart (left ventricular dysfunction) is initiated by an ‘index’ event that may be either abrupt in onset (e.g. myocardial infarction), gradual or insidious in onset (e.g. haemodynamic pressure or volume overload) or it may be hereditary as in the case of many genetic cardiomyopathies.

Intriguingly, most patients will remain asymptomatic following this induction of LV dysfunction. A possible explanation for this asymptomatic state is the activation of a variety of compensatory mechanisms, triggered by the initial decline in LV function, that maintain normal cardiovascular function. However, with time the sustained activation of these systems leads to a series of changes within the myocardium collectively known as cardiac remodelling, the principle manifestation of the disease progression (Konstam et al., 2003; Pokharel et al., 2003). Patients may remain asymptomatic for a period of months or even years, however at some point in time a transition from asymptomatic to symptomatic heart failure occurs. How and why this transition occurs and whether it occurs in all patients with LV dysfunction is unknown. However, what is known is that the transition to symptomatic heart failure is accompanied by continued remodelling and the overexpression of biologically active molecules capable of exerting a ‘toxic’ effect on the myocardium and the circulation thus causing relentless disease progression.

1.2.2.1 LV remodelling

LV remodelling is a term used to describe changes in the structure and architecture of the left ventricular wall and cavity that impacts on the function of the ventricle. Remodelling is a normal feature of maturation and is an adaptive response to increased

demand. However in heart failure patients, regardless of the nature of the initiating cardiac insult, the progression from causative pathology to heart failure can be explained by an adverse LV remodelling process involving changes in structure, shape and topography at the global level and changes in myocytes and non-myocytes at a cellular and subcellular levels mediated by changes in gene expression. Collectively these changes lead to progressive LV dilation, decreased LV wall thickness and cardiac decompensation (Cohn et al., 2000).

A principle feature of remodelling in heart failure is an increase in myocardial mass associated with a change in ventricular shape. The major mechanism responsible for this change, which leads to impaired contraction and relaxation, is hypertrophy of individual cardiac myocytes with enlargement of their size being more pronounced in the long axis compared to the width. The dilated ventricle exhibits increased wall tension and stress because the thickness of the wall does not increase to compensate for the increase in chamber radius (Gerdes, 2002).

Intriguingly, although pathological remodelling is associated with increased myocardial mass there is also evidence that loss of viable myocytes through necrosis or apoptosis leading to progressive intrinsic contractile dysfunction (Anversa et al., 1996; Olivetti et al., 1997). Such cell death is followed by the deposition of excessive fibrillar collagen contributing to the development of excessive fibrosis in heart failure leading to myocardial stiffness (Weber et al., 1994, 1997).

The extracellular matrix is an important determinant of the structural characteristics of the myocardium. Evidence suggests that changes in the composition and the quantity of myocardial extracellular matrix are important processes in the remodelling of the ventricles in heart failure. Through the action of a family of zinc-dependant neutral endoprotease enzymes referred to as matrix metalloproteinases (MMPs) progressive degradation of the matrix that holds the individual myocytes together occurs leading to myocyte 'slippage', ventricular dilation and progressive ventricular dysfunction (D'Armiento, 2002). Indeed, overexpression of certain MMPs in ventricles undergoing progressive dilatation has been demonstrated. However the presence of tissue inhibitors of MMPs (TIMP) can inhibit the activity of MMPs and thus the control and regulation of matrix deposition and degradation is likely more complex than simply the presence or absence of specific MMPs (Barton et al., 2003; Spinale, 2002a).

In the failing heart changes to the cardiac myocytes designed to protect the cells from excessive sympathetic stimulation may collectively interact to produce contractile dysfunction. Decreased α -myosin heavy chain expression with a concomitant increase in β -heavy chain expression, loss of myofilaments, altered cytoskeletal and calcium handling proteins and desensitisation to β -adrenergic stimulation may all contribute to the phenotype of cellular contractile dysfunction associated with the failing heart (Mann, 1999).

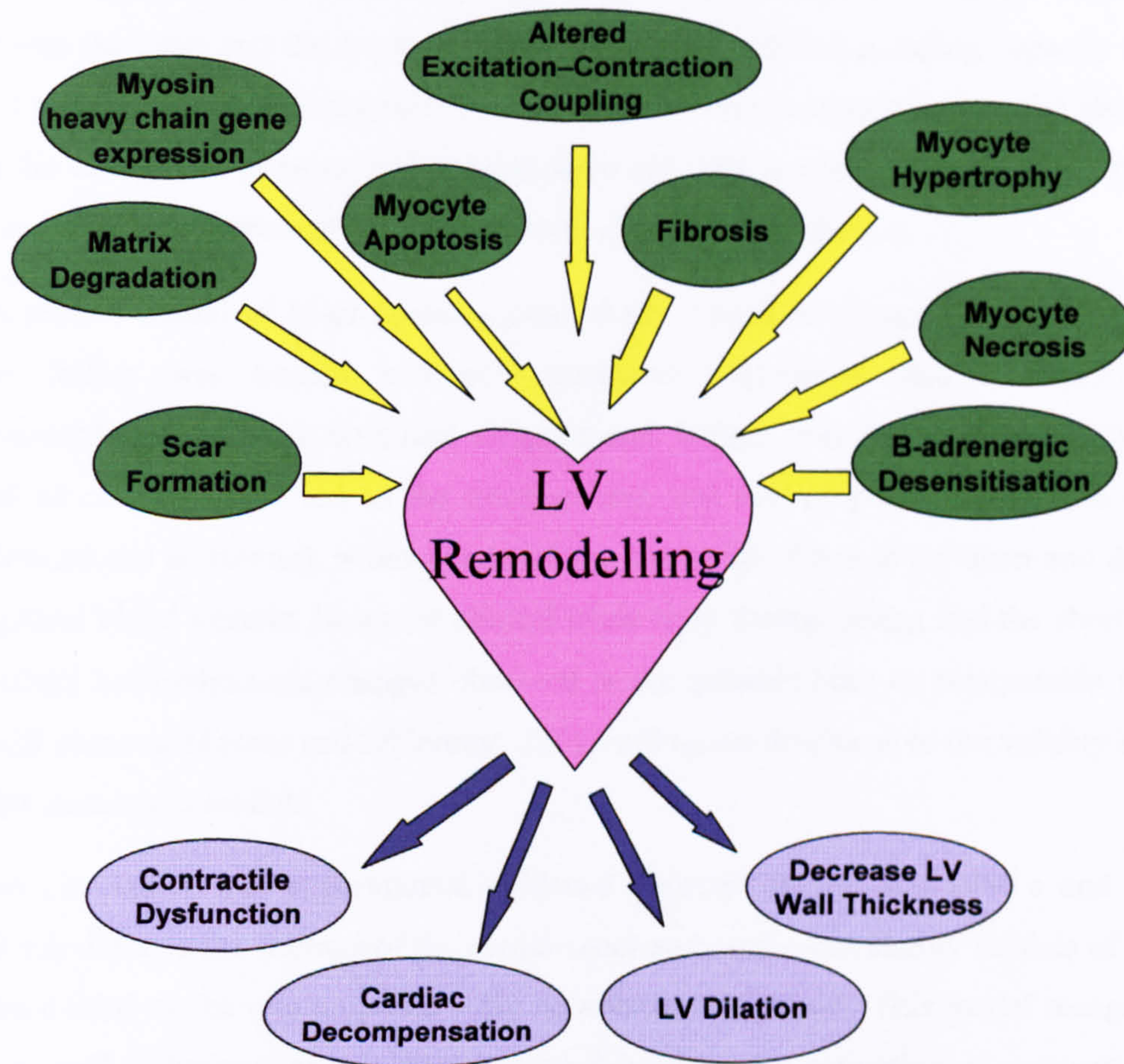


Figure 1.8. Processes and consequences of pathological left ventricular remodelling.

1.2.3 EVOLUTION OF OUR UNDERSTANDING OF HEART FAILURE

In an attempt to define, understand and treat the syndrome of heart failure, physicians and researcher developed conceptual models that formed the basis of clinical treatment and research hypotheses. Since the 1940s such models of heart failure developed, evolved and changed with increasing understanding of disease state (Packer, 1993).

In the 1940s and 1950s physicians viewed heart failure as an acute disorder, the major clinical manifestation of which was oedema due to excessive salt and water retention as a result of impaired renal blood flow. The concept of this cardio-renal model formed the basis of a rational approach to the treatment of heart failure; Digitalis increased the force of contraction of the heart and diuretics increased salt and water excretion to control the volume status of these patients.

Based on clinical and experimental observations in the 1960s, 70s and 80s heart failure became regarded as a chronic disorder caused by abnormalities in the interaction between the heart and the peripheral circulation with reduced pumping capacity of the heart and excess vasoconstriction. Importantly, this cardio-circulatory model also held that the changes in pressure and volume were not only a result of cardiac dysfunction but could also contribute to the development of cardiac dysfunction.

This revised model of heart failure prompted physicians to change the way in which heart failure was treated and new treatment regimes needed to relieve the vasoconstriction and the exertional dyspnea and fatigue that persisted even after the relief of oedema. This led to the development and use of positive inotropic drugs (milrinone and amrinone), which increased the contractile force of the heart and dilated peripheral blood vessels. However clinical trials were disappointing and the short-term beneficial haemodynamic changes observed in the patients bore no relationship to the clinical outcome (Felker and O'Connor, 2001) and raised doubts as to the validity of the cardio-circulatory models.

Based on clinical and experimental evidence obtained in the late 1980s and early 1990s and due to the failings of the cardio-renal and cardio-circulatory models of heart failure a third model was proposed – the neurohormonal model. This model recognised that, as well as haemodynamic disturbances there was also activation of compensatory neurohormonal systems as a result of the initial injury. Significantly the resulting overexpression of a portfolio of biologically active molecules contributed to the progression of heart failure independently of the haemodynamic status of the patients by a direct toxic effect on the heart and circulation (Packer, 1992). With the development of this model came the realisation that heart failure was a progressive disease and the concept of LV remodelling was born. This development in the thinking towards heart failure also indicated that treatment strategies had to do more than simply relieve the symptoms.

Clinical studies have shown that antagonising neurohormone systems, particularly the renin-angiotensin system (RAS) and the sympathetic nervous system leads to clinical improvement in heart failure patients (Miller, 2003, Tendera and Ochala, 2001). ACE inhibitors and β -blockers now form part of a recommended drug regimen for many patients with heart failure. However, despite the apparent ‘success’ of these two classes of drugs in impeding the progression of heart failure and reducing morbidity and mortality in these patients they do not ‘cure’ heart failure. In addition the lack of cumulative beneficial effects of multiple treatment strategies suggests that there are redundant signalling pathway in operation in the progression of heart failure that has led to the need to investigate further the molecular mechanisms involved in LV remodelling and the progression of heart failure as potential new therapeutic targets.

1.2.3.1 The cytokine hypothesis of heart failure

‘The cytokine hypothesis holds that the progression of heart failure begins because cytokine cascades exacerbate haemodynamic abnormalities and exert direct toxic effects on the heart. Cytokines do not cause heart failure per se but rather they are responsible for the progression of the disease’ (Seta et al., 1996).

The first publication proposing a role for pro-inflammatory cytokines in the setting of heart failure came from Levine and colleagues in 1990 (Levine et al., 1990a) who demonstrated that patients with heart failure had significantly higher serum levels of TNF α than age matched healthy volunteers and that those patients with high TNF α levels had more advanced heart failure. Subsequent studies have confirmed this initial finding and have consistently demonstrated a strong correlation between raised plasma cytokine levels and deterioration in NYHA functional class and LV ejection fraction (Torre-Amione et al., 1996a; Tsutamoto et al., 1998). In addition, a sub-study of the Studies on Ventricular Dysfunction (SOLVD) revealed the prognostic value of cytokines measurements, demonstrating that patients with circulating TNF α levels of less than 6.5pg/ml had a better prognosis than those with TNF α levels greater than 6.5 pg/ml. More recently circulating levels of TNF α , soluble TNF α receptors and IL-6 have all been shown to independently predict mortality in patients with heart failure (Deswal et al., 2001). Given this data it is not surprising that there has been growing interest in the potential role of inflammatory mechanisms and cytokines in the regulation of cardiac structure and function particularly with respect to their role in disease progression in heart failure.

Pro-inflammatory cytokines are not constitutively expressed in the heart (Kapadia et al., 1995, Torre-Amione et al., 1996c) but can be produced by myocytes as well as other nucleated cells within the myocardium in response to various forms of myocardial stress. Indeed, pro-inflammatory cytokines are consistently and rapidly expressed in response to a variety of different forms of myocardial injury (Table 1.1) suggesting that cytokine expression may form part of an intrinsic cardiac stress response system.

Pressure overload	(Kapadia et al., 1997)
Cardiopulmonary bypass	(Lahat et al., 1992; Wan et al., 1996b)
Unstable angina	(Matsumori et al., 1994)
Hypertrophic cardiomyopathy	(Matsumori et al., 1994)
Acute viral myocarditis/ auto immune myocarditis	(Smith and Allen, 1992 Matsumori et al., 1991)
Myocardial infarction	(Ikeda et al., 1992, Basaran et al., 1993, Maury and Teppo, 1989, Cruickshank et al., 1994)
Myocardial reperfusion injury	(Herskowitz et al., 1995)
Heart failure	(Matsumori et al., 1994, Levine et al., 1990a, Torre-Amione et al., 1996c)

Table 1.1. Cardiac pathologies associated with pro-inflammatory cytokines.

Increased pro-inflammatory cytokine expression is observed early in the course of LV dysfunction and may predict the occurrence of subsequent heart failure (Aukrust et al., 1999; Deswal et al., 2001; Raymond et al., 2001; Torre-Amione et al., 1996a; Vasan et al., 2003a) suggesting that these molecules represent an early warning system, similar to their role as effector molecules in the innate immune response, that allows the heart to rapidly respond to tissue injury and coordinate the homeostatic response that follows. In line with this theory there is a growing body of evidence to suggest that short-term expression of pro-inflammatory cytokines is beneficial. Both *in vivo* and *in vitro* studies have shown that pro-inflammatory cytokines can protect the heart from ischaemia/reperfusion injury (Brown et al., 1990; Eddy et al., 1992; Nogue et al., 1995; Yamashita et al., 2000) and can protect myocytes from hypoxic or ischaemic injury (Nakano et al., 1998). Through the activation of transcription factors, pro-inflammatory cytokines, such as TNF α can induce cardiac expression of cytoprotective proteins such as MnSOD (Wong and Goeddel, 1988), heat shock proteins (Nakano et al., 1996), cellular inhibitors of apoptosis 1 and 2 (c-IAP1 and c-IAP2, (Wang et al., 1998a) and matrix remodelling proteins that will serve to contribute to the adaptive responses to haemodynamic stress or myocardial injury.

However, at some point the beneficial adaptive response becomes maladaptive through the sustained overexpression of pro-inflammatory cytokines and heart failure progresses because the cytokine cascades exert a deleterious effect on the circulation.

In addition to the data demonstrating increased circulating cytokines, the current interest in understanding the role of stress activated cytokines in the progression of heart failure comes from the observation that when expressed in sufficiently high concentration such as those seen in heart failure, known biological effects of these molecules can explain many aspects of the heart failure phenotype.

1.2.3.1.1 Effect of pro-inflammatory cytokines on myocardial function

The first demonstration that pro-inflammatory cytokines could modulate cardiac function came from Tracey et al in 1986 who showed that the haemodynamic/cardiac responses seen during endotoxin-induced shock could be inhibited by injection of anti-TNF α antibodies and could be mimicked by TNF α (Tracey et al., 1986). Subsequent studies in animals demonstrated that single infusion of TNF α into dogs results in abnormalities in systolic function (Natanson et al., 1989; Pagani et al., 1992) and that TNF α , IL-1 β and IL-6 (Bozkurt et al., 1998; Hosenpud et al., 1989; Kinugawa et al., 1994) could produce negative inotropic effects, which appears to be mediated through nitric oxide dependant blunting of β -adrenergic signalling (Finkel et al., 1992; Kumar et al., 1999; Panas et al., 1998). In addition, high concentrations of IL-6 and TNF α have been shown to produce an immediate (within minutes) decrease in cardiac contractility believed to be the result of activation of the neutral sphingomyelinase pathway (Oral et al., 1997).

1.2.3.1.2 Effect of pro-inflammatory cytokines on LV structure (remodelling)

Evidence for a role of pro-inflammatory cytokines in the process of LV remodelling in heart failure comes mainly from experimental animal models. Infusion of TNF α into dogs or rats causes a change in LV dimension that is accompanied by progressive degradation of extracellular matrix (Pagani et al., 1992; Bozkurt et al., 1998). Similarly transgenic mice overexpressing myocardial TNF α develop progressive LV dilation and dysfunction (Kubota et al., 1997; Bryant et al., 1998; Sivasubramanian et al., 2001).

Studies suggest that pro-inflammatory cytokines such as TNF α can directly contribute to the observed LV remodelling by promoting myocyte hypertrophy (Yokoyama et al., 1997), altering foetal gene expression (Kubota et al., 1997; Thaik et al., 1995) and

calcium handling proteins (Thaik et al., 1995) and causing progressive myocyte loss through apoptosis (Krown et al., 1996). More recently data suggests that these cytokines can induce alterations in the extracellular matrix components of the myocardium through modulation of MMP and TIMP expression that fosters LV dilation (Li et al., 2000). Sustained pro-inflammatory signalling provokes a change in the balance between MMP and TIMP activity that results in excessive matrix synthesis and fibrosis (Sivasubramanian et al., 2001).

1.2.3.1.3 Anti-cytokine therapy for heart failure

To date several approaches have been used to inhibit the production or the action of pro-inflammatory cytokines in an attempt to treat heart failure.

The use of pentoxifylline, a phosphodiesterase inhibitor (PDEI), which increases intracellular levels of cAMP and blocks the transcriptional activation of TNF α , was one of the first successful attempts to use anti-cytokine approaches in the treatment of heart failure. Sliwa et al demonstrated a significant reduction in circulating TNF α levels in patients with severe heart failure (NYHA IV) following one month of pentoxifylline treatment, an effect that was accompanied by improvement in left ventricular function and NYHA functional class (Sliwa et al., 2002a). Other studies have demonstrated similar effects with milrinone (Liu and Zhao, 1999a).

Thalidomide, which inhibits TNF α production by enhancing mRNA degradation (Moreira et al., 1993a), has been reported to have a similar beneficial effect to the PDEI. In addition to inhibitory effects on TNF α production, thalidomide and its analogues have also been reported have other anti-inflammatory properties including inhibition of IL-6 and IL-8 production, inhibition of leucocyte trafficking possibly through the down regulation of adhesion molecule expression (Matthews and McCoy, 2003) and increase IL-10 production (Moreira et al., 1993b) all of which may contribute to its beneficial effects in the treatment of heart failure. The action of these compounds may in part be through the inhibition of IKK activity and NF κ B activation (Keifer et al., 2001). In two small studies thalidomide treatment tended to improve left ventricular function and quality of life and in some cases reduced circulating TNF α levels in patients with heart failure (Agoston et al., 2002; Gullestad et al., 2002).

Two different approaches have been used to selectively block the biological action of TNF α in patients with heart failure. The first used decoy receptors, etanercept, a

genetically engineered dimerised fusion protein composed of two TNF p75 receptors and an IgG₁:Fc portion. On the basis of the short term results showing a potential benefit of etanercept therapy to patients with moderate to advanced heart failure (Bozkurt et al., 2001; Deswal et al., 1999) two multi-centre clinical trials with etanercept were initiated in patients with NYHA class II to IV heart failure; The trial in North America called Randomised Etanercept North American Strategy to Study Antagonism of Cytokines (RENAISSANCE, n=900) and the trial in Europe and Australia called Research into Etanercept Cytokine Antagonism of Ventricular Dysfunction (RECOVER, n=900).

A third trial RENEWAL (Randomised Etanercept Worldwide Evaluation, n=1500) pooled data from RENAISSANCE and RECOVER.

These trials were stopped early after the Data Monitoring Safety Board deemed that the trials were unlikely to show a beneficial effect (HeartWire News and Wood, 2002).

The second targeted approach aimed at specific blockade of TNF α activity used Infliximab, a chimeric monoclonal antibody consisting of a genetically engineered murine Fab fragment fused to an FC portion of human IgG₁, which is successfully used in the treatment of rheumatoid arthritis and Crohn's disease. ATTACH (Anti TNF α Therapy Against CHF) was a phase II trial in 150 patients with moderate to advanced heart failure. This trial ran to completion and data demonstrated a dose related increase in death and hospitalisation in the treatment group compared to placebo (HeartWire News and Hughes, 2002).

On the basis of the disappointing results of these targeted approaches to TNF α inhibition prescribing information for etanercept and infliximab cautions against their use in patients with heart failure.

Given the wealth of pre-clinical data suggesting the important role of pro-inflammatory cytokines, particularly TNF α in the progression of heart failure the consistent observation that both infliximab and etanercept resulted in a worsening of heart failure and outcome was surprising. Several possible reasons for these observations have been suggested (Mann, 2002).

Firstly, infliximab is directly cytotoxic by fixing complement in cells that express TNF α on the membrane. Failing cardiac myocytes express TNF α on the cell membrane and

thus complement fixation in the heart would lead to myocyte lysis and sustained myocarditis.

Secondly, etanercept may act as a carrier protein, similar to endogenous soluble TNFR2, stabilizing TNF α and allowing accumulation of high concentrations of this cytokine in the peripheral circulation and thus increases the duration of bioactivity.

Thirdly, the targeted inhibition of TNF α bioactivity may result in the loss of one or more beneficial effects of TNF α as well as the deleterious ones.

These data also suggest that reduced levels of circulating TNF α observed during the treatment of heart failure may be as a consequence of improved clinical status rather than a cause of it.

Other strategies have suggested that modulation of the immune system in less specific ways than infliximab and etanercept may be of greater benefit in the treatment of heart failure. In a small study, intravenous immunoglobulin therapy has been shown to induce an increase in circulating anti-inflammatory factors (IL-10 and IL-1RA) in patients with heart failure which was accompanied by a significant improvement in LV function (Aukrust et al., 2003). Another small study using immune modulation therapy, has demonstrated a trend towards improved NYHA functional class (HeartWire News, 2002). Immune modulation therapy attempts to trigger the body's own immune system in a global manner. Blood is taken from the patient, exposed to oxidative stress, and then re-administered to the patient intramuscularly. Upon re-administration the blood cells undergo apoptosis, which, in a manner similar to the effects of neutrophil apoptosis in the resolution of the inflammatory response, causes the down regulation of pro-inflammatory factors (IL-1 β and TNF α) with a corresponding increase in anti-inflammatory factors (IL-10).

Taken together the current data suggests that the role of inflammatory cytokines in the progression of heart failure is somewhat more complex than simply viewing molecules in isolation and as being either good or bad.

1.3 INFLAMMATORY RESPONSE ASSOCIATED WITH CARDIOTHORACIC SURGERY

In the past surgery on the heart was limited for two principle reasons, 1) the notion that the heart was too fragile and important to operate on and 2) the inability to penetrate the outer walls. However on September 9th 1896 in Frankfurt in Germany the first

successful heart operation was carried out on a patient with a stab wound to the right ventricle. Dr. Ludwig Rehn placed three silk stitches through the heart wound (the suture was tied in diastole) and stopped the bleeding. The patient made a full recovery. From this first operation the development of heart surgery progressed slowly until probably one of the most important events in the development of current cardiac operations, the introduction in 1953 of cardio-pulmonary bypass (CPB). This revolutionised the disciplines of cardiology and cardiac surgery and made repair of intracardiac lesions possible. CPB overcame the major problem that previously confronted the surgeon; when the heart was stopped and opened, the patient died. To successfully redirect the circulation to bypass the heart and the lungs, one needs a basic understanding of physiology of the circulation, a method of preventing the blood from clotting, a pump to pump blood, and finally, a method to ventilate the blood. In 1937 Dr John Gibbon first demonstrated that life could be maintained with an artificial heart and lung and that the animals own heart and lungs could later resume function, if only for a few hours, when the machine was turned off. In 1953 Gibbon used the prosthetic heart/lung machine for a cardiac operation, opening the door to the routine application and success of extracorporeal circulation for cardiac surgery.

With advances in our understanding of the cardiovascular system and in technology, improvements in oxygenators, pumps, the use of hypothermia, anti-coagulation and the biocompatibility of the inner circuit of the system have been achieved and cardiopulmonary bypass has evolved into a routine procedure to achieve desirable conditions under which surgery on the heart can be successfully performed.

1.3.1 INFLAMMATORY RESPONSE FOLLOWING CARDIAC SURGERY UTILISING CPB

Despite the current technologies employed during cardiac surgery and the high level of success achieved, such procedures are associated with a significant inflammatory complication termed ‘systemic inflammatory response syndrome.’

1.3.1.1 Pathophysiology of the inflammatory response to cardio-thoracic surgery.

Although such a systemic inflammatory response can occur in response to many different types of clinical injury including major trauma, major non-cardiac surgery, infections or burns, it is particularly frequent in response to cardiac surgery. This may be related to the fact that several inflammatory insults take place simultaneously. These

insults include blood contact with the foreign surface of the CPB apparatus (Kirklin et al., 1983b), ischaemia and the subsequent reperfusion of the lungs and the myocardium as a result of aortic cross clamping (Sawa et al., 1996), presence of endotoxemia (Hill, 1998; Kharazmi et al., 1989) and the mechanical effects of intra-operative tissue manipulation. All of these initiating factors contribute concurrently to the humoral and cellular development of the response to injury or infection that can result in the systemic inflammatory response and organ dysfunction.

1.3.1.1.1 Humoral factors

The generation of humoral mediators in the inflammatory response associated with cardiac surgery utilising CPB is initiated by the activation of the contact protein cascade and the complement cascade upon exposure of blood to the wound and the foreign surface of the extracorporeal circuit.

1.3.1.1.1.1 Contact protein cascade

Blood contact with the negatively charged surface of the bypass circuit induces a conformational change in factor XII, which then binds to high molecular weight kininogen (HMWK). Factor XII is converted to its active form, factor XIIa. Factor XIIa subsequently activates factor XI thus propagating the intrinsic coagulation cascade via the activation of factor X resulting in the generation of thrombin. Factor XIIa also converts prekallikrein to kallikrein which functions to amplify the activation of XII and converts HMWK to bradykinin and plasminogen to plasmin. Kallikrein also augments the inflammatory response to CPB by cleaving C5 to form the anaphylatoxin C5a. Plasmin not only mediates clot lysis but also is able to activate the classical complement cascade, as is factor XIIa (Miller and Levy, 1997).

1.3.1.1.1.2 Tissue Factor / thrombin

Open heart surgery produces a large wound that directly stimulates thrombin formation by the extrinsic coagulation pathway. Tissue factor is an integral membrane glycoprotein that is normally constitutively expressed only in the extravascular space. Under conditions of oxidative and inflammatory stress tissue factor expression is induced on monocytes and endothelial cells. During cardiac surgery, tissue factor, which does not circulate, is expressed in the wound in the epicardium, vascular adventitia, and monocytes. Tissue factor expression is also induced in the microvascular endothelial cells following cardiac ischaemia-reperfusion (Erlich et al., 2000).

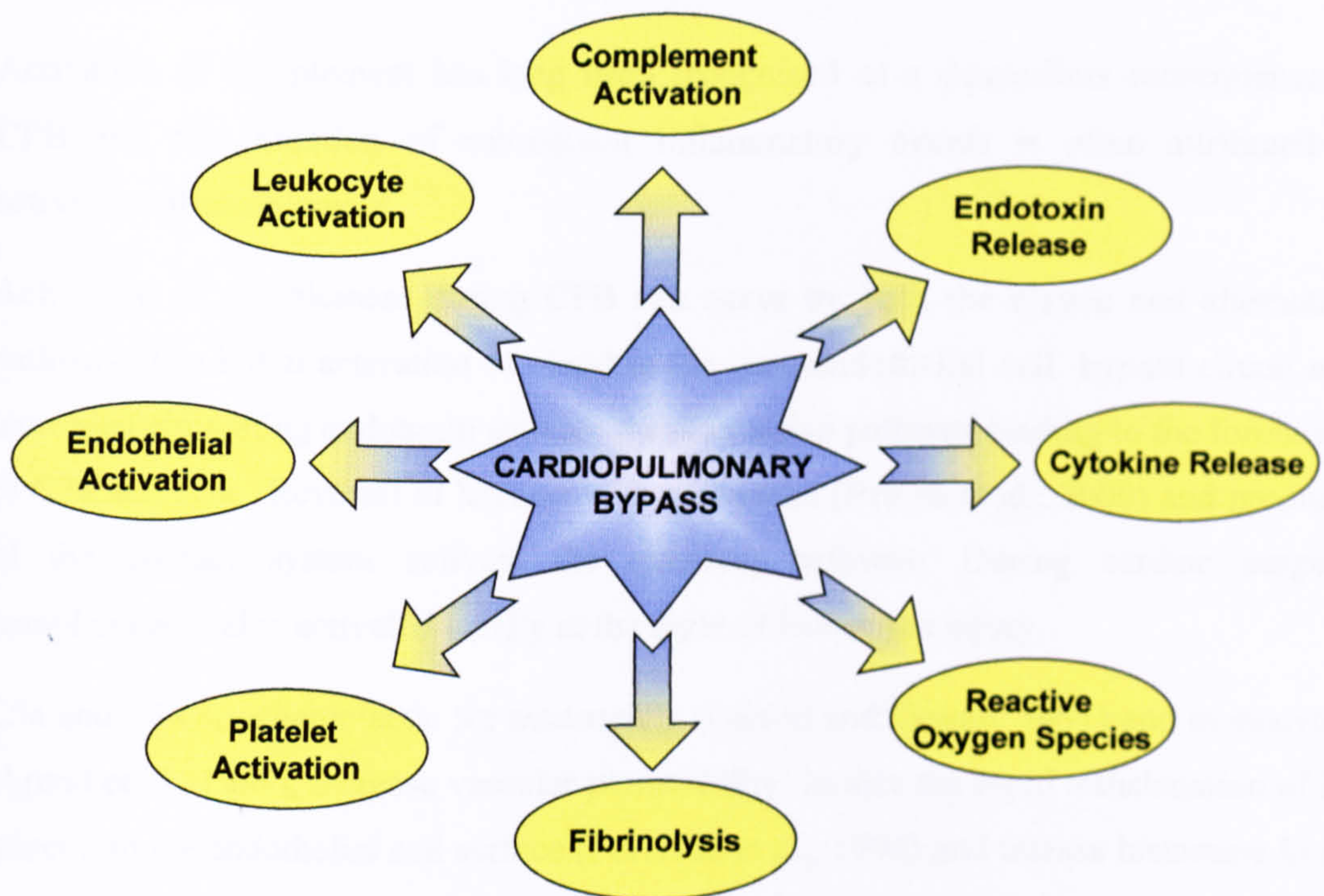


Figure 1.9. Inflammatory Response to Cardiopulmonary Bypass

Tissue factor rapidly binds to factors VII and VIIa to initiate the extrinsic pathway that proceeds to activate factor X and the subsequent generation of thrombin. Thrombin not only catalyzes the conversion of fibrinogen to insoluble fibrin but also acts as a pro-inflammatory agent further contributing to the local inflammation and tissue damage. Through the activation of a family of protease-activated receptors (Dery et al., 1998; Vu et al., 1991) thrombin can stimulate cells to express cytokines such as IL-1 β and IL-6; chemokines such as IL-8 and MCP-1 and adhesion molecules such as P-selectin, E-selectin and ICAM-1 (Johnson et al., 1998; Kaplanski et al., 1997; Sugama et al., 1992). In a rabbit model of myocardial ischaemia-reperfusion injury Erlick et al demonstrated that inhibition of TF with blocking antibodies or specific inhibition of thrombin with hirudin reduced the recruitment of neutrophils to the injured myocardium through the inhibition of IL-8 and MCP-1 expression (Erlich et al., 2000) demonstrating the contributing role of this system in the inflammatory response to cardiac surgery. However, other studies have failed to demonstrate increased monocyte tissue factor expression following cardiac surgery with CPB (Parratt and Hunt, 1998; Ernofsson et al., 1997) and suggest that, in addition to the surgical exposure of tissue factor in extravascular tissues, monocyte CD11b upregulation is the major contributor to the activation of coagulation by CPB through the direct activation of factor X and thrombin generation by the extrinsic pathway (Parratt and Hunt, 1998).

1.3.1.1.3 Complement

Activation of complement has long been recognised as a deleterious consequence of CPB and the initiation of subsequent inflammatory events is often attributed to activation of complement.

Activation of complement during CPB can occur by both the classic and alternative pathway. The initial activation of blood in the 'non-endothelial cell' bypass circuit and increased circulating endotoxin activate the alternative pathway leading to the formation of C3a and C5a. Reversal of heparin with protamine (Bruins et al., 2000) and products of the contact system activate the classical pathway. During cardiac surgery complement is also activated locally at the sight of ischaemic injury.

C5a and C3a are chemotactic for neutrophils (Gerard and Gerard, 1991) and monocytes (Arend et al., 1989); increase vascular permeability; induce the rapid translocation of P-selectin to the endothelial cell surface (Foreman et al., 1994) and release histamine from mast cells (Schulman et al., 1988) and as such are regarded as initiator of neutrophil activation and aggregation in response to CPB leading eventually to endothelial and parenchymal cell damage. In a simulated bypass circuit blockade of C3a, C5a and C5b-9 prevented neutrophil and monocyte activation (CD11b upregulation and elastase release, (Rinder et al., 1999). In addition, in an experimental porcine model of CPB blockade of C5a activity with anti-C5a monoclonal antibodies reduced neutrophil activation and vascular endothelial cell dysfunction (Tofukuji et al., 1998).

Increasing evidence suggests that inactive terminal complement complex (C5b-9) plays an integral role in the pathogenesis vascular injury after ischaemia-reperfusion and cardiopulmonary bypass. C5b-9 can amplify the local inflammatory response by inducing endothelial IL-8 and MCP-1 secretion (Kilgore et al., 1996) and endothelial surface expression of adhesion molecules (ICAM-1, VCAM-1 and E-selectin) and tissue factor activity (Tedesco et al., 1997).

A number of studies have correlated plasma levels of C3a with adverse outcomes following cardiac surgery including cardiac, pulmonary and renal dysfunction, bleeding and overall morbidity (Holmes et al., 2002). C3a plasma levels 3 hours after CPB have been shown to predict clinical outcome with higher plasma levels of C3a in patients requiring prolonged mechanical ventilation (Kirklin et al., 1983a). Mechanistically these events may represent neutrophil-mediated injury, especially the pulmonary dysfunction or a direct effect of C3a and C5a amplifying the inflammatory response through the

increased production of the inflammatory cytokines IL-1 β , TNF α , IL-6, IL-8, RANTES, MCP-1 and adhesion molecule expression in leucocytes and endothelial cells (Kilgore et al., 1996; Monsinjon et al., 2003; Tedesco et al., 1997). In addition, C3a has been shown to have negative inotropic effects (del Balzo et al., 1985) and thus may contributing directly to the myocardial dysfunction often observed following cardiac surgery.

Other groups however have suggested no correlation between complement activation and acute lung injury or adverse haemodynamic response (Steinberg et al., 1993; Tennenberg et al., 1990; Wakefield et al., 1991).

1.3.1.1.1.4 Cytokines

Serum cytokine levels have been extensively studied as markers of inflammation during CPB with elevated levels of IL-8 and IL-6 being most reliably elevated under such circumstances. Elevated levels of pro-inflammatory cytokines have been routinely associated with post CPB morbidity in animal models and serum levels of IL-8 and IL-6 have been shown to correlate with adverse clinical outcome in patients undergoing cardiac operations with CPB (Holmes et al., 2002; Ito et al., 1997; Rothenburger et al., 2001a). Liebold et al demonstrated that the vascular endothelium in the heart is potentially the major source of IL-6 following reperfusion of the ischaemic myocardium, supporting the critical role of the activated endothelium in the inflammatory response to CPB (Liebold et al., 1999). Plasma levels of IL-8 appear to correlate with the duration of CPB and the aortic cross clamp time (Holmes et al., 2002) (Whitten et al., 1998; Kawamura et al., 1993) and are also associated with increased plasma levels of elastase which have been associated with organ dysfunction (Tonz et al., 1995; Finn et al., 1993).

Elevated levels of other cytokines such as TNF α and IL-1 β following CPB have been less consistently demonstrated with as many studies reporting increased serum levels as those reporting no changes (Wan et al., 1997).

Roth-Isigkeit demonstrated a transient increased circulating levels of IL-1 β , TNF α , IL-6 and IL-10 following cardiac surgery with CPB with levels returning to pre-operative levels on the first post-operative day (Roth-Isigkeit et al., 1999a, 2001a). Frank et al also demonstrated increased circulating levels of IL-6 and TNF α and increased production of these cytokines from monocytes following cardiac surgery with CPB

(Franke et al., 2002b) however, no serious post operative complications were experienced by any of the patients in that study. Similar to other studies (El Azab et al., 2002; Misoph and Babin-Ebell, 1997), the study of Frank et al also demonstrated increased circulating IL-10 levels and production from monocytes in the same patients after CPB demonstrating a counteracting anti-inflammatory response. Other studies, however have failed to detect increased circulating pro-inflammatory cytokines in patients following CPB (Grundmann et al., 2000; Ito et al., 1997; Misoph and Babin-Ebell, 1997) even in patients requiring prolonged respirator therapy (Rothenburger et al., 2001b).

The use of corticosteroid administration before CPB has been shown to significantly reduce circulating levels of IL-6, IL-8 and TNF α and to increase levels of the anti-inflammatory cytokine IL-10 (Chaney, 2002). Such treatment has been associated with improved lung and myocardial function and fewer post-operative complication after CPB (El Azab et al., 2002; Giomarelli et al., 2003).

In contrast Schurr et al, demonstrated that pre-operative steroid treatment although inhibiting the production of TNF α , IL-6 and soluble E-selectin expression had no influence of post-operative recovery (Schurr et al., 2001) and some studies suggest that steroid treatment, although reducing certain manifestations of the systemic inflammation following cardiac surgery may in fact be detrimental in these patients by initiating postoperative hyperglycemia and pulmonary and haemodynamic effects that may hinder early postoperative tracheal extubation (Chaney et al., 2001).

These studies bring into question the importance of pro-inflammatory cytokines in the development of post-operative complications and cast doubt as to the beneficial nature of cytokine suppression in these patients.

1.3.1.1.1.5 Endotoxin

Endotoxin is a lipopolysaccharide associated with the cell membrane of Gram negative organisms. It has been hypothesised that the reduction in splanchnic blood flow observed during CPB results in the translocation of endotoxin across the ischaemic gut wall into the systemic circulation. Through the interaction with TLR4 on cells in the systemic vasculature endotoxin can activate a systemic inflammatory response.

The presence of endotoxin in patient blood after CPB has been demonstrated by a number of studies (Khabar et al., 1997; Rothenburger et al., 2001a; Rocke et al., 1987)

and systemic levels of endotoxin have, in some cases been suggested to be important contributor to post operative complications with levels correlating with the degree of haemodynamic compromise, aortic cross clamp time during CPB and myocardial dysfunction and prolonged requirement for mechanical ventilation after CPB (Hall et al., 1997).

The presence of endotoxin could be partially responsible for the activation of complement via the alternative pathway and for the increased release of cytokines via TLR4 mediated NF κ B activation in monocytes.

1.3.1.1.2 Cellular events

1.3.1.1.2.1 Neutrophils

Neutrophils are thought to play a significant role in the inflammatory response associated with cardiopulmonary bypass and have been shown to rapidly accumulate in the ischaemic-reperfused myocardium; the extent of which correlates with the severity of myocardial injury (Hansen, 1995; Zahler et al., 1999). There is also a significant increase in neutrophil count in the bronchial lavage (Jorens et al., 1992) and pulmonary artery, compared to the systemic arteries (Braude et al., 1986) after protamine administration suggesting these cells play a significant role in lung injury associated with CPB.

Neutrophil expression of the β 2 integrin CD11b/CD18 and plasma levels of myeloperoxidase and elastase have been shown to increase during CPB demonstrating neutrophil activation and promoting leucocyte-endothelial interactions and tissue damage (Asimakopoulos et al., 2000a; Defraigne et al., 2000; Larson et al., 1996). However, the importance of such enzymes in the development of post-operative complications or as clinically useful markers in predicting post-operative outcome remains a contentious issue. Plasma elastase has been reported to be a clinically useful marker of neutrophil activation following CPB with levels correlating with the degree of organ injury/ dysfunction (Faymonville et al., 1991; Finn et al., 1993; Tonz et al., 1995) and worse clinical outcome (Holmes et al., 2002). In contrast, Butler et al found no such association between elastase release and clinical outcome (Butler et al., 1993).

The importance of infiltrating neutrophil-led damage to the ischaemic-reperfused myocardium is demonstrated in animal models in which the use of a variety of monoclonal antibodies against endothelial cell-leucocyte adhesion molecules reduced

neutrophil infiltration and tissue damage (Byrne et al., 1992; Horgan et al., 1990; Lefer et al., 1996). In addition, CPB studies in animals in which CD11b/CD18 upregulation was prevented by the drug NPC15669 show a reduction in pulmonary damage and end organ damage (Gillinov et al., 1994a; Wilson et al., 1993).

1.3.1.1.2.2 Monocytes

Monocytes are also activated during cardiopulmonary bypass and open-heart surgery by the wound, the foreign surface of the bypass circuit, endotoxemia and complement resulting in the activation of NF κ B, production of cytokines and the expression of tissue factor with the subsequent activation of the extrinsic coagulation pathway. The role of activated monocytes in the complications of CPB thus likely includes both pro-inflammatory and pro-thrombotic effects.

Patient studies have shown that monocytes are activated early in the course of cardiac surgery with significantly greater expression of TF found in the cells in the pericardium than in the bypass circuit suggesting that the wound is a major activating stimuli (Chung et al., 1996). CPB can also contribute to the activation of monocytes during open heart surgery as demonstrated by the increased TF expression on monocytes when blood is circulated through a simulated extracorporeal circuit. However a 2-6 hour time period was required suggesting that this is only of significant concern during prolonged CPB (Kappelmayer et al., 1993).

1.3.1.1.2.3 Endothelial cells

The anatomical location of endothelial cells at the interface of the blood and tissue means that they are directly influenced by the increase in circulating mediators observed during cardiac operations. In addition, endothelial cell activation and the expression of adhesion molecules are essential prerequisites for the migration of activated leucocytes from the blood into tissue that is observed following cardiac surgery with CPB. Indeed many of the features of the inflammatory response associated with such operations can be attributed to a change in endothelial cell phenotype towards an activated state.

Studies have demonstrated that short-term hypoxia or anoxia induces increased adhesion of leucocytes to vascular endothelial cells mediated by the upregulation of endothelial adhesion molecules (Yoshida et al., 1992; Shreeniwas et al., 1992). Also cardiac expression of E-selectin and ICAM-1 mRNA are increase after CPB (Kilbridge et al., 1994) the inhibition of which can significantly inhibit neutrophil infiltration to the

lungs, liver, and heart in an animal model of systemic inflammation (Boyle, Jr. et al., 1998).

Since E-selectin is only expressed by activated endothelial cells, detection of soluble E-selectin in the blood can be taken as evidence of endothelial cell activation and/or damage. Paret et al demonstrated that increased plasma levels of soluble E-selectin following CPB were associated with the need for inotropic support, the use of anti-inflammatory drugs and the development of postoperative sepsis (Paret et al., 2000). Boldt et al, however, failed to demonstrate altered circulating levels of either soluble E-selectin or ICAM-1 following CPB, but none of the patients in that study suffered from organ dysfunction or the need for prolonged intensive care therapy (Boldt et al., 1995). In contrast another study demonstrated a sustained increase in plasma levels of soluble E-selectin and ICAM-1 after cardiac surgery with CPB that bore no relationship to post-operative clinical course (Grunenfelder et al., 2000). These studies suggest that endothelial cell activation is likely to occur following CPB, however it may not always develop into clinically significant events.

1.3.1.2 Clinical consequence of cardiac surgery-induced SIRS

Clinically the activation of molecular cascades and cell activation can manifest as coagulopathy, respiratory failure, myocardial dysfunction, renal insufficiencies and neurocognitive defects (Laffey et al., 2002). Indeed, impaired function of major organs such as kidney, myocardium and lungs following cardiac surgery are common phenomena and the systemic inflammatory response is regarded as a major contributing factor. However, the severity of the syndrome may vary widely. Most patients undergoing cardiac surgery with cardiopulmonary bypass will experience some degree of organ dysfunction as a result of activation of the inflammatory response but significant morbidity is rare, approximately 1-2% of cases experience long lasting cardiac, pulmonary, renal and neurological dysfunction.

1.3.1.2.1 Myocardial dysfunction

Major perioperative myocardial complications occur in about 10% of patients and can manifest as arrhythmia and contractile dysfunction (Mangano, 1998).

By its very nature, cardiac surgery causes the heart to undergo tremendous stress. In addition to the injury caused by direct surgical injury and handling, global ischaemia followed by reperfusion after removal of the aortic cross clamp is also an important

mechanism of potential damage. Myocardial ischaemia followed by reperfusion is associated with morphological, electrophysiological, metabolic, and functional changes along with alteration of enzyme release. In addition ischaemia and reperfusion have been shown to induce the production of pro-inflammatory factors from a number of affected organs including the heart. Indeed, the heart is believed to be the major source of TNF α and IL-6 during reperfusion as a result of cardiac surgery with CPB (Kukielka et al., 1995; Wan et al., 1996a). Such local production of pro-inflammatory cytokines can significantly contribute to myocardial injury, altered myocardial contractility and cause desensitisation to adrenergic stimulation all of which may contribute to the cardiovascular complications associated with CPB (Oddis and Finkel, 1997; Hennein et al., 1994). The importance of the inflammatory response in postoperative myocardial dysfunction is supported by several studies. Wan et al demonstrated a significant correlation between IL-8 and cardiac troponin-I (measure of myocardial injury) levels following coronary surgery suggesting that IL-8 is a critical mediator in inducing myocardial injury under such circumstances (Wan et al., 1999). In addition, left ventricular abnormalities correlate well with serum levels of IL-6 and IL-8 (Hennein et al., 1994; Wei et al., 2001). These studies imply a potentially important role of leucocyte activation in the development of cardiovascular complications of cardiac surgery. In further support of this notion, clinical studies have demonstrated that leucocyte depletion from the cardioplegic perfusate and at reperfusion can reduce both clinical and biochemical indices of myocardial reperfusion injury with reduced prevalence of low cardiac index and ventricular fibrillation and lower levels of creatinine kinase MB isoenzyme and troponin I in the early postoperative period (Hiramatsu et al., 2000; Palatianos et al., 2004).

1.3.1.2.2 Pulmonary dysfunction

Pulmonary dysfunction after cardiac surgery with cardiopulmonary bypass remains one of the major complications associated with such procedures and is evident by the presence of postoperative pulmonary functional, physiologic, biochemical, and histological changes. Severe acute lung injury, although relatively uncommon (1-3%) in patients following cardiac surgery with CPB, is associated with high mortality (30-70%). Previous coronary surgery, the use of bubble oxygenator compared to membrane oxygenator and poor pre-operative left ventricular function all appear to influence the severity of pulmonary dysfunction and in situations where these factors are avoided the incidence is considerable lower (Milot et al., 2001). With the use of sensitive markers

such as pulmonary protein accumulation index and bronchoalveolar lavage neutrophil and myeloperoxidase concentrations, lesser degrees of lung injury are detectable even in uncomplicated CPB (Sinclair et al., 1995). Pulmonary dysfunction occurs in the immediate post-operative period and although often attributed to cardiogenic pulmonary oedema and chest infections may have inflammatory origins with neutrophil/complement/monocyte based responses being important components in the development of post-operative lung injury.

Histological studies have demonstrated that bypass can cause pulmonary endothelial, epithelial and interstitial damage and significant accumulation of neutrophils in the lungs. In addition pro-inflammatory mediator concentrations in the circulation and in the lungs correlate with post-CPB lung dysfunction (Ito et al., 1997; Giomarelli et al., 2003) and mechanical removal of these mediators (Huang et al., 2003), or inhibition (Friedman et al., 1996; Gillinov et al., 1994b) or depletion (Gu et al., 1996) of leucocytes significantly attenuates lung dysfunction.

1.3.1.2.3 Renal dysfunction

Clinically detectable renal dysfunction following cardiac surgery has been shown to occur in 7.7-11.4% of cases with the incidence of dialysis-dependant acute renal failure being approximately 0.7-1.4% with an overall mortality of 28-63.7% (Mangano et al., 1998; Zanardo et al., 1994). Renal filtration of pro-inflammatory cytokines produced during the inflammatory response to cardiopulmonary bypass may contribute to proximal tubule damage and the renal dysfunction through the production of nitric oxide (Chatterjee et al., 1999). Indeed, the extent of renal dysfunction has been shown to correlate with the extent of the inflammatory response (Gormley et al., 2000). Recently, Tang et al demonstrated that leucocyte depletion in low risk patients undergoing cardiac surgery with CPB significantly reduce biochemical signs of renal dysfunction (Tang et al., 2002) further supporting the contribution of the inflammatory response to the renal damage observed during such procedures.

1.4 NUCLEAR FACTOR κ B

1.4.1 INTRODUCTION

NF κ B transcription factors can be activated by a variety of inflammatory stimuli including the pro-inflammatory cytokines IL-1 β and TNF α and as such are key regulators of immune, inflammatory and stress responses. Thus during pathological

conditions in which there is an increase in these pro-inflammatory cytokines, including those that have been described for heart failure and following cardiac surgery, NF κ B may influence or even determine the course of the inflammatory reaction and potentially clinical outcome. Therefore understanding the mechanisms that regulate the activity of NF κ B transcription factors and determining their role in specific inflammatory situations should provide a greater insight into mechanisms of such diseases and possible future therapeutic targets.

NF κ B was first identified in 1986 (Sen and Baltimore, 1986) as a nuclear factor bound to an enhancer region of the immunoglobulin (Ig) κ light-chain gene in mouse B-lymphocytes. Subsequent studies have demonstrated that NF κ B exists in most cell types and regulates the rapid, co-ordinated expression of many inducible early response genes encoding proteins with diverse functions that are critically involved in controlling the inflammatory response. Such genes encode a variety of cytokines, chemokines, adhesion molecules, acute phase proteins, inducible enzymes, immunoreceptors, stress response proteins, growth factors and regulators of apoptosis (Pahl, 1999).

In mammals, NF κ B transcription factor is a dimer comprised from a group of structurally related, evolutionary conserved proteins, which under resting conditions are bound to a member of the inhibitor of κ B (I κ B) family of proteins.

1.4.2 COMPONENTS OF THE NF κ B TRANSCRIPTION FACTOR PATHWAY

1.4.2.1 NF κ B proteins

To date five members of the NF κ B family of proteins, termed Rel proteins have been identified in mammalian cells (p65 (RelA), c-Rel, RelB, p50 and p52). All of the Rel proteins share an N-terminal 300 amino acid conserved region known as the Rel Homology Domain (RHD) that contains sequences that are essential for subunit dimerization, nuclear localization and DNA binding (Chen and Ghosh, 1999; Ghosh et al., 1995; Huxford et al., 1998; Jacobs and Harrison, 1998).

The Rel family of proteins can fall into two subclasses:

- RelB, p65 and c-Rel are all synthesized as transcriptionally active proteins that bind DNA weakly and contain one or more potent transcriptional transactivation domains.

- p50 and p52 are synthesised as larger precursor protein, p105 and p100 respectively, that require post-translational or co-translational processing. p105 processing is constitutive while processing of p100 is regulated (Chen et al., 1998). These processed proteins have strong DNA binding activity but only weak, if any, transactivation potential. Each of these precursor proteins contains a number of ankyrin repeats in their carboxy terminal that are characteristic of the I κ B proteins (see below), which may serve an inhibitory function as an I κ B-like molecule (Scheinman et al., 1993).

The first NF κ B molecule to be described was a heterodimer of p50 and p65 subunits (Kopp and Ghosh, 1995; Verma et al., 1995). This protein is still what is commonly referred to as NF κ B although many other homo- and heterodimer complexes can be generated through combinatorial mixing. Subunit composition of NF κ B allows differences in preference for certain κ B sites, transactivation potentials, kinetics of nuclear translocation, levels of expression in tissue and interaction with different I κ B proteins to confer differential regulation of this transcription factor and the numerous genes controlled by its activity.

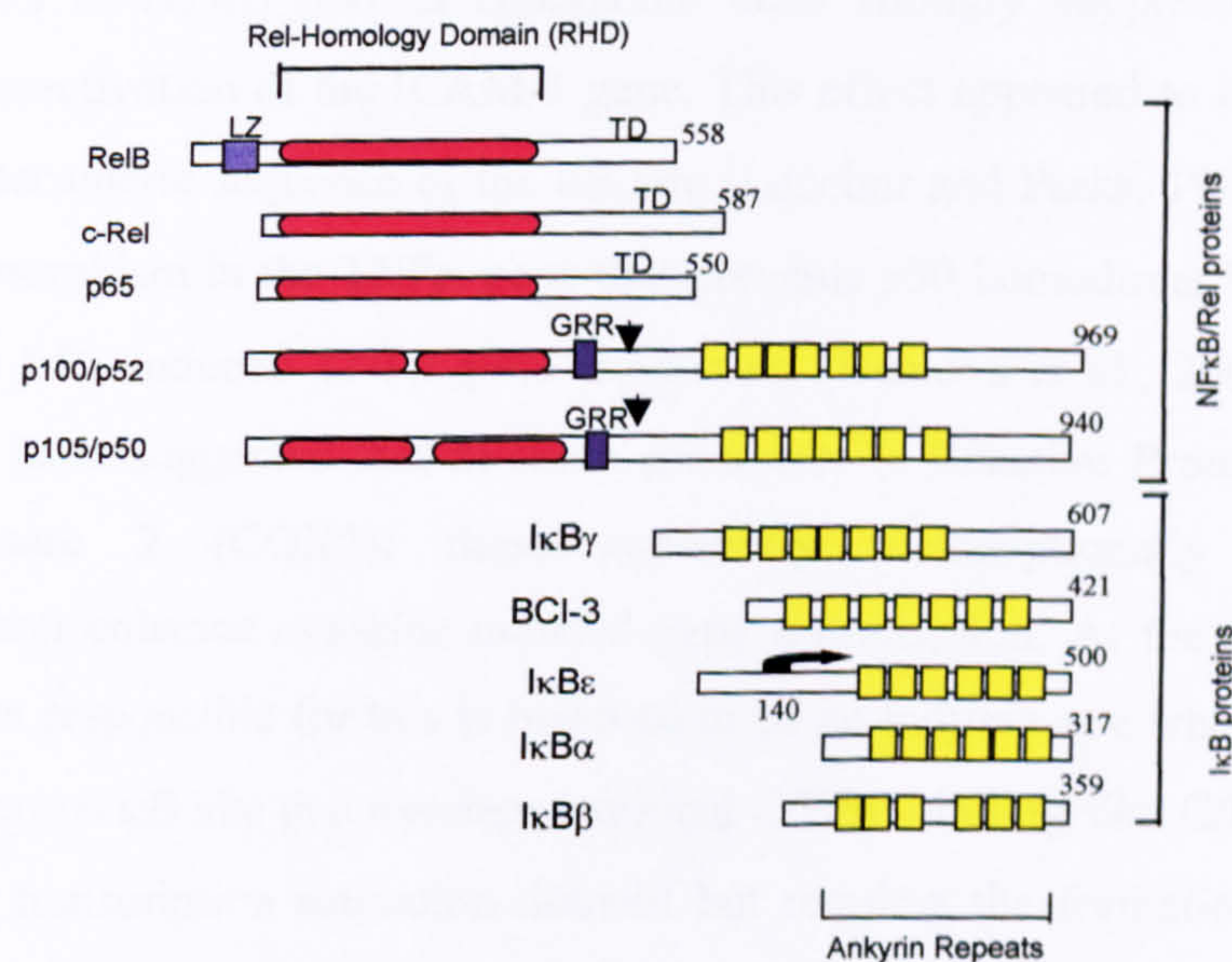


Figure 1.10. The Mammalian Rel/NF κ B/I κ B family of proteins. The number of amino acids in each protein is shown on the right. The *arrows* point to the endoproteolytic cleavage sites of p100/p52 and p105/p50: TD, transactivation domain; LZ, leucine zipper domain of RelB; GRR, glycine-rich region; I κ B ϵ -has been proposed to be translated from either the first methionine or from an internal methionine at position 140 (Ghosh et al., 1998).

The two Rel proteins making up the NFκB complex each contact one half of the DNA binding site on the target gene promoter. In such a manner, the slight variations possible in the 10 base pair consensus sequence, ⁵GGGGYNNCCY³, can exclude or recruit different homo- or heterodimeric combinations of NFκB providing a preference for selected Rel combinations (Kunsch et al., 1992). For example, an NFκB dimer consisting of p65/p50 NFκB preferentially binds to the sequence ⁵GGGRNNYYCC³, whereas p65/c-Rel dimers bind to a sequence ⁵HGGARNYYCC³; where H indicates A, C or T; R is purine and Y is pyrimidine (Parry and Mackman, 1994). This ability of different dimers to recognize slightly different DNA targets increases the flexibility and specificity of NFκB subunit utilisation to differentially regulate the expression of distinct genes.

Although most of the NFκB proteins are transcriptionally active some complexes that consist of solely of Rel subunits that lack a transactivation domain (p50/p50 and p52/p52) do not have the ability to initiate gene transactivation. However, because these proteins possess effective DNA binding regions they are believed to act to repress NFκB-mediated transcription. Evidence for this comes from studies in which the overexpression of NFκB p50 in endothelial cells strongly suppressed NFκB p65-mediated transactivation of the ICAM-1 gene. This effect appeared to be dependant on the specific decameric sequence of the κB site (Ledebur and Parks, 1995). In addition, genetic polymorphism in the TNFα gene that prevents p50 homodimer binding to a κB site increases LPS-induced TNFα gene expression (Udalova et al., 2000). In contrast other studies have suggested that at some promoters (c-Reactive Proteins (CRP) and Cyclo-Oxygenase 2 (COX2)) these apparently transcriptionally inactive p50 homodimers can enhance cytokine-induced gene transcription. At the CRP promoter, the mechanism responsible for this is believed to be an indirect one whereby p50 binds to a non-consensus κB site that overlaps a critical C/EBP binding site. C/EBPβ provides the necessary transcription activation domain but requires the formation of a ternary complexes between p50, C/EBPβ and the C/EBP binding site and also requires the binding of p50 to the overlapping κB site (Agrawal et al., 2001).

Similar mechanisms are also at work at the COX2 promoter where binding of p50/p50 forms an important interaction with members of the C/EBP family of transcription factors to induce transcription (Thomas et al., 2000).

The nearly ubiquitously expressed p50/p65 tends to be rapidly translocated to the nucleus in response to extracellular activating signals, whereas other complexes such as p50/c-Rel accumulate in the nuclei more slowly, exhibiting a delay of several hours before reaching peak levels (Molitor et al., 1990).

Certain dimers apparently do not exist; RelB dimerises only with p50 or p52 (Ryseck et al., 1995). In addition, RelB cannot bind to any known κ B sites on its own, therefore homodimeric complexes of RelB show no DNA binding activity. However, by binding as a heterodimer with p50 or p52, RelB can form transcriptionally competent transcription factors (Bours et al., 1994).

1.4.2.2 I κ B proteins

NF κ B activity is tightly controlled by its association with specific inhibitory proteins of the I κ B family (I κ B α , I κ B β , I κ B ϵ , I κ B γ or Bcl-3).

Inhibition of NF κ B activity is achieved by the presence of multiple copies of a thirty to thirty three amino acid sequence in the I κ B proteins called the ankyrin-repeat motif. The ankyrin repeats are the regions responsible for the interaction with the RHD of the NF κ B proteins. Such associations mask the NF κ B DNA binding sequence and/or nuclear localisation signal. In this manner transcriptional activity of NF κ B is only apparent once I κ B has been removed. The ankyrin repeats form a characteristic structure where each repeat consists of two closely packed helices followed by a loop and a tight hairpin turn (Jacobs and Harrison, 1998; Huxford et al., 1998, Figure 1.11). The multiple repeats stack on top of one another to form a slightly curved cylinder with the loops forming finger-like extensions. These loops contain the residues that specifically recognise and interact with the RHD of the NF κ B dimer. The specificity of which I κ B protein pairs with a particular NF κ B dimer appears to be governed by the number of ankyrin repeat sequences on the I κ B molecule.

I κ B α (Davis et al., 1991; Haskill et al., 1991) and I κ B β (Thompson et al., 1995) were the first of the I κ B proteins to be cloned and hence are the most extensively studied. These proteins are 37 and 45 kDa molecules, respectively, that are organised with an N-terminal domain that is phosphorylated in response to NF κ B activating signals and a central ankyrin repeat domain (Rothwarf and Karin, 1999). In addition, I κ B α contains a nuclear export signal (NES) located within the N-terminal domain at amino acids 45-55 (Johnson et al., 1999). Studies have indicated that the majority of p50/p65 and p50/c-

Rel complexes are regulated by I κ B α or I κ B β (Thompson et al., 1995; Whiteside et al., 1997). Binding of I κ B α to p50/p65 dimer changes the conformation of the p65 subunits causing the N-terminal IgG-like portion of the RHD to rotate 180° compared to the situation in the DNA bound complex. Such allosteric changes appear to occlude critical DNA binding residues (Huxford et al., 1998).



Figure 1.11. Crystal structure of the ankyrin repeat domain of Bcl-3

Following NF κ B-inducing stimuli, the inhibitory actions of I κ B α or I κ B β are rapidly removed by their targeted degradation following phosphorylation at specific N-terminal serine residues (section 1.4.3) thus exposing nuclear localisation signals and DNA binding regions of the NF κ B dimer.

I κ B ϵ is the most recently identified member of the I κ B family (Whiteside et al., 1997) and appears to associate exclusively with p65/p65 and p65/c-Rel complexes. Similar to I κ B α and I κ B β , I κ B ϵ degradation is induced by the majority of NF κ B inducing agents and is preceded by phosphorylation of two N-terminal serine residues. The specific inhibition of subsets of NF κ B complexes by I κ B ϵ suggests that it may regulate the expression of specific genes whose promoters preferentially bind p65 and c-Rel complexes.

Unlike I κ B α and I κ B β which bind to transcriptionally active NF κ B in the cytoplasm, Bcl-3 is unique in that it specifically binds to transcriptionally inactive p50/p50 and p52/p52 homodimers in the nucleus (Wulczyn et al., 1992) (Nolan et al., 1993). The precise role of Bcl-3 in NF κ B activation is unclear. Some reports suggest that the N- and C-terminal regions of Bcl-3 can act as transactivation domains (Bours et al., 1993)

possibly via recruitment of histone acetyl transferase (Dechend et al., 1999). Thus, by binding to NF κ B homodimers that can bind DNA but are transcriptionally inactive, Bcl-3 provides the ability to induce gene transactivation. In addition by interacting with p50/p50 and p52/p52 complexes, Bcl-3 may promote NF κ B-mediated gene transcription by removing their repressive activity from promoter regions (Franzoso et al., 1992; Franzoso et al., 1993). In contrast other studies suggest that Bcl-3 has negative effects on NF κ B transcription by increasing p50/p50 recruitment to certain gene promoters (Kuwata et al., 2003). The precise effects of Bcl-3 on p52 or p50 binding to DNA is likely to be determined by its phosphorylation state and/or its concentration (Bundy and McKeithan, 1997).

The sequence of I κ B γ is identical to the I κ B-like C-terminal domain of p105 and is the product of an alternative promoter usage that produces mRNA that encodes the C-terminal portion of p105 (Ghosh et al., 1998). I κ B γ is only found in mature B-cell lines from mice and is thought to specifically inhibit p50 and p52 homodimers suggesting that this protein serves a very specialized function (Inoue et al., 1992; Liou et al., 1992).

1.4.3 ACTIVATION OF NF κ B

In unstimulated cells, NF κ B generally resides in the cytoplasm. This sub-cellular localisation results from efficient masking of the nuclear localisation signal by I κ B β and I κ B ϵ (Malek et al., 2001; Tam and Sen, 2001). When bound by I κ B α the NF κ B NLS remains visible and the complexes continuously shuttle between the cytoplasm and the nucleus. Under such circumstances the highly efficient nuclear export of these complexes ensures that nuclear NF κ B in the un-induced state remains very low (Huang et al., 2000; Johnson et al., 1999).

The defining characteristic of NF κ B is its rapid inducibility that allows the cell to swiftly respond to stress situations. NF κ B activation can be induced by a diverse array of stimuli such as bacteria and viruses, pro-inflammatory cytokines, growth factors, physiological stresses such as adhesion, hyperoxia or sheer stress, environmental toxins, chemical agents and therapeutically used drugs (Pahl, 1999). NF κ B activation is achieved primarily through the removal of I κ B proteins by proteolytic degradation thus exposing the NLS and DNA binding domains of the NF κ B complex. NF κ B subsequently translocates to the nucleus, binds to a κ B site in the promoter region of target genes and induces transcription. Despite the diverse nature of activating stimuli

several common features appear to exist within the multistep pathway of NF κ B activation, the earliest of which appears to be the activation of an I κ B kinase (IKK). IKK is responsible for the site-specific serine phosphorylation of I κ B α (ser³² and ser³⁶) and I κ B β (ser¹⁹ and ser²³). Once phosphorylated the I κ B α is targeted for polyubiquitination on lysine 21 and 22, which in turn targets the I κ B protein for proteolytic degradation by the 26S proteasome. Recently it has become apparent that in addition to removal of the I κ B protein, post translational modification to NF κ B p65 are also essential to confer efficient gene transactivation.

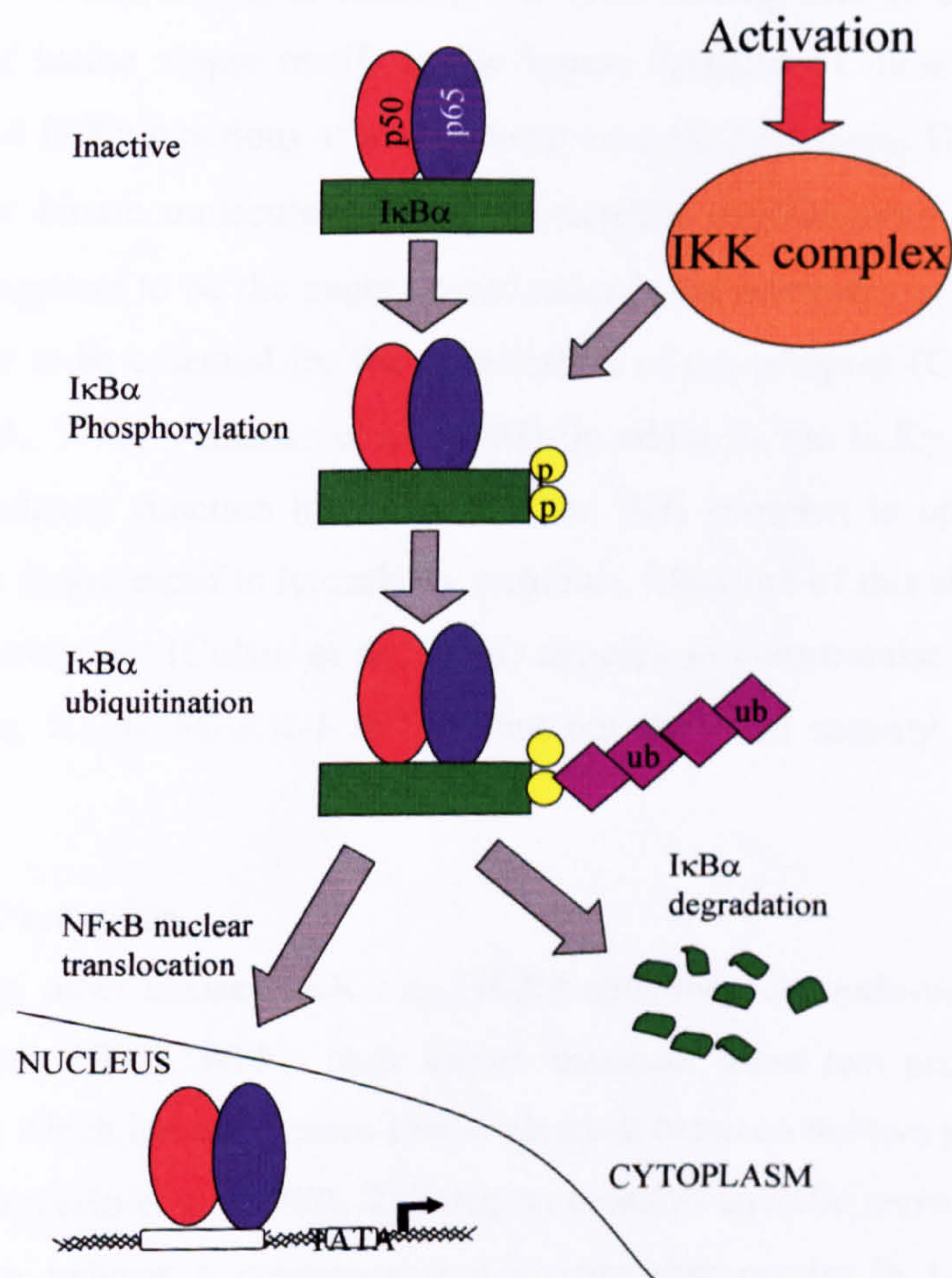


Figure 1.12. Key events in the activation of NF κ B.

1.4.3.1 I κ B kinase

Once it was firmly established that serine phosphorylation of I κ B was a critical step in the activation of NF κ B great effort was directed at finding the inducible kinase that matched the characteristics of I κ B α degradation (rapid, signal-induced activation, simultaneous phosphorylation of both serine residues and the preference for serine over

threonine). The kinase activity responsible for phosphorylation of I κ B proteins was characterized in 1997 and was named I κ B kinase (IKK, DiDonato et al., 1997; Mercurio et al., 1997; Regnier et al., 1997; Woronicz et al., 1997; Zandi et al., 1997).

1.4.3.1.1 Structure of IKK

IKK is a high molecular weight (~700kDa) complex containing several protein subunits of which proteins termed IKK α (or IKK1) and IKK β (or IKK2) possess the kinase activity responsible for I κ B phosphorylation. These two proteins are structurally similar and *in vitro* form either hetero- or homodimers, although only heterodimers have been found *in vivo*. Dimerisation is essential for IKK activity and is achieved by direct association of lucine zipper motifs in the kinase domains of these proteins. A third subunit termed IKK γ functions as a regulatory subunit. Tetrameric IKK γ is believed to sequester four kinase molecules, yielding a $\gamma_4(\alpha/\beta)_4$ stoichiometry (Tegethoff et al., 2003). IKK γ appears to be the major co-ordinator in the assembly of the IKK complex and is thought to be essential for the stabilization of the complex (Cohen et al., 1998; Rothwarf et al., 1998; Yamaoka et al., 1998). In addition, the IKK γ subunit serves an important regulatory function by connecting the IKK complex to upstream activators through a zinc finger motif in its carboxy terminus. Mutation of this site does not affect complex assembly but (Cohen et al., 1998) appears to compromise the activation of IKK by TNF α , IL-1 β , MEKK-1 or NIK but not the basal activity (Rothwarf et al., 1998).

1.4.3.1.2 IKK activation

Similar to many other kinases, IKK α and IKK β activation depends on phosphorylation (DiDonato et al., 1997). Within their kinase domains, these two proteins contain an activation loop which has a sequence that is identical between the two proteins (Delhase et al., 1999; Mercurio et al., 1997). This region contains specific serine residues whose phosphorylation induces a conformational change that results in kinase activation. Mutations of these serine residues interfere with IKK activity. For example, replacement of two conserved serine residues (ser¹⁷⁷ and ser¹⁸¹) within the activation loop of IKK β with alanine prevents IKK activation, whilst conversion of both sites to glutamate generates a constitutively active kinase (Delhase et al., 1999; Mercurio et al., 1997).

The identity of the upstream kinase or kinases responsible for the regulation of IKK α and IKK β activity is still unclear although a number of different candidates have been shown to possess the required activity. Candidate IKK kinases include NIK (Lin et al., 1998; Woronicz et al., 1997), TAK1 (Ninomiya-Tsuji et al., 1999b; Sakurai et al., 1999b), AKT/PKB (Ozes et al., 1999), MEKK-1, 2 and 3 (Lee et al., 1998; Nemoto et al., 1998; Zhao et al., 1999) and PKC ζ (Lallena et al., 1999; DiDonato et al., 1997). It is almost certainly the case that the vast array of stimuli that activate NF κ B do so via the activation of distinct kinases that are able to phosphorylate and activate IKK.

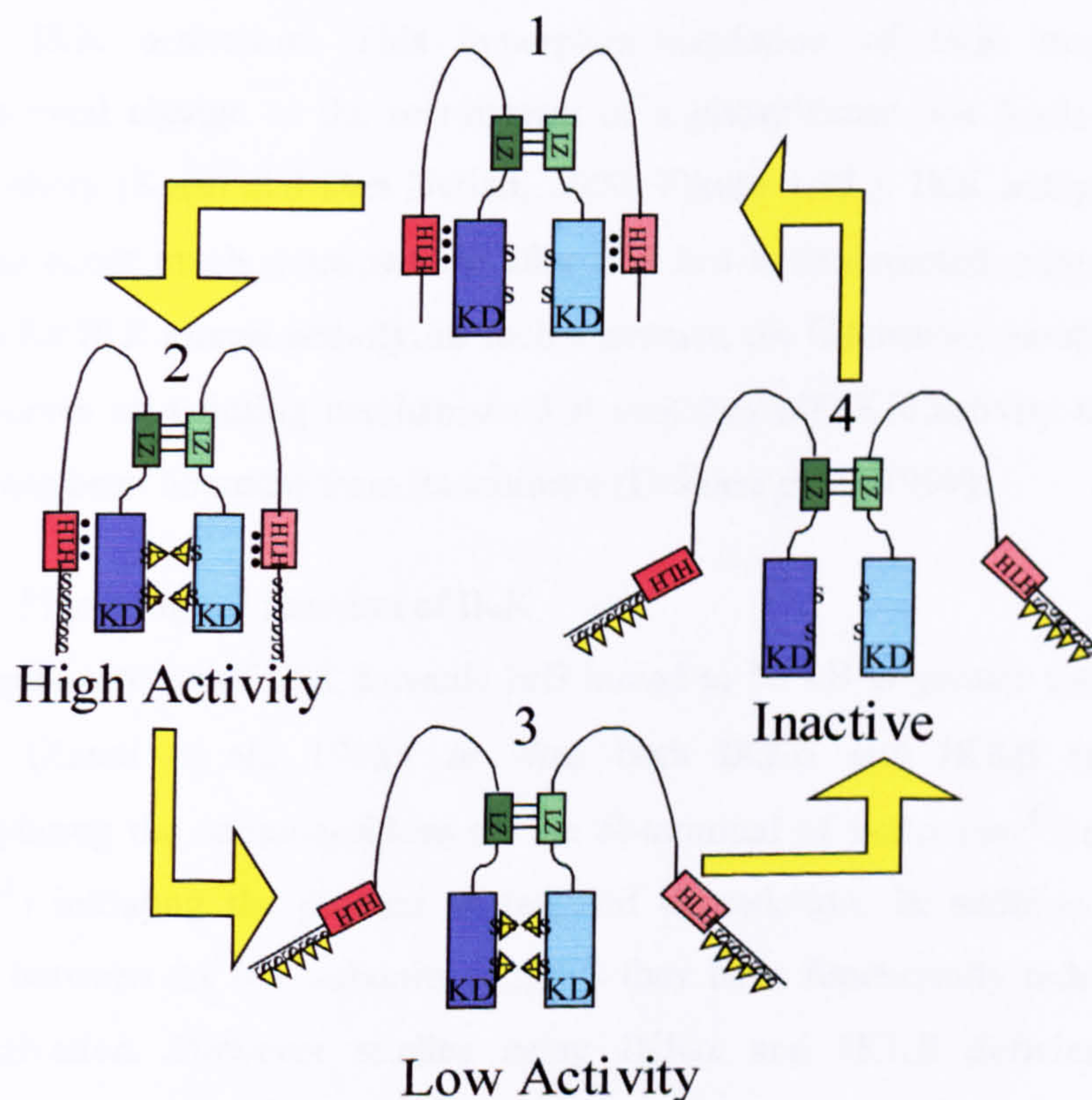


Figure 1.13. The four step model of regulation of IKK activity by phosphorylation. 1 - In unstimulated cells the two catalytic subunits of IKK are associated into a heterodimer via their leucine zippers. The helix-loop-helix domain (an intrinsic activator of the kinase) interacts with the kinase domain. The activation loops are unphosphorylated as are the C-terminal serine residues. 2 - Upon stimulation two critical serine residues in the activation loop of both IKK α and IKK β become phosphorylated resulting in high IKK activity. 3 - After phosphorylating I κ B and IKK undergoes autophosphorylation at the C-terminal serine cluster causing electrostatic repulsion altering the interaction between the HLH and the kinase domain such that the kinase reaches a low activity state. 4 - the conformational changes allows recruitment of phosphatases and kinase domains return to an unphosphorylated state (Karin and Delhase, 2000).

1.4.3.1.3 Inactivation of IKK

Because of the potential deleterious effects of persistent activation of NF κ B most stimuli cause only a transient activation of IKK. Consistent with the kinetics of TNF α and IL-1 β induced I κ B α degradation in most cells, IKK activity peaks within 5-15 minutes and by 30-60 minutes the activity has decreased to about 25% which appears to be adequate to cease I κ B α phosphorylation and subsequent NF κ B activation (Delhase et al., 1999; DiDonato et al., 1997; Zandi et al., 1997). The initial down regulation in IKK appears to be the result of IKK α and IKK β autophosphorylation at ten serine residues in their C-terminal regions (Delhase et al., 1999), the mutation of which prolongs IKK activation. This hyperphosphorylation of IKK may result in a conformational change or the recruitment of a phosphatase that leads to decrease in kinase activity (Karin and Ben Neriah, 2000, Figure 1.13.). IKK autophosphorylation appears to occur much more readily after I κ B has been depleted suggesting that I κ B competes for IKK kinase activity. In such a manner, the C terminal autophosphorylation of IKK serves as a timing mechanism that switches off IKK activity once the NF κ B complex has been liberated from its inhibitor (Delhase et al., 1999).

1.4.3.1.4 Physiological function of IKK

The catalytic activity of IKK towards I κ B bound to NF κ B is greater than that towards free I κ B (Zandi et al., 1998). *In vitro* both IKK α and IKK β are capable of phosphorylating the serine residues on the N-terminal of I κ B α (ser³²/ser³⁶) and I κ B β (ser¹⁹/ser²³) initiating the process of targeted degradation. In addition the sequence similarity between the two subunits suggests they have functionally redundant roles in NF κ B activation. However studies using IKK α and IKK β deficient mice have highlighted differences in the *in vivo* roles of these two kinases. Disruption of the IKK α gene in mice resulted in abnormal embryonic development and the animals died shortly after birth. These mice had a dramatically defective epidermis formation and organisation (5-10 fold thicker than normal) due to hyperproliferation of cells at the basal layer and a block in keratinocyte differentiation. However, IL-1 β and TNF α induced I κ B α depletion and NF κ B nuclear translocation in embryonic fibroblasts and thymocytes from these IKK α deficient mice remained unimpaired although there was a reduction in NF κ B binding activity. This data demonstrates that IKK α is not required for cytokine-induced activation of NF κ B but plays a major role in epidermis formation, a function that could not be replaced by IKK β (Hu et al., 1999; Takeda et al., 1999). In

subsequent studies Sizemore and colleagues demonstrated that IKK α activity was required for the generation of transcriptionally active NF κ B (Sizemore et al., 2002). IKK α also appears to be involved in the processing of p100 resulting in the activation of p52/RelB dimers (Senftleben et al., 2001).

In contrast, mice deficient in IKK β exhibited a phenotype similar to NF κ B p65 knockout mice and died between embryonic day 12.5 and 14 from extensive liver damage due to apoptosis. Embryonic fibroblasts from IKK β deficient mice showed marked reduction in NF κ B activation in response to TNF α and IL-1 β (Li et al., 1999a, 1999b). Since *in vitro* IKK α can effectively phosphorylate I κ B, it is interesting that IKK α was unable to substitute IKK β under these circumstances and possibly suggests that IKK α requires IKK β for full activity.

The physiological relevance of the non-catalytic IKK γ subunit of the IKK complex has also been investigated with the use of knockout mice. These animals exhibit a phenotype similar to NF κ B p65 and IKK β knockout mice and die between embryonic day 12.5-13 because of severe liver damage due to apoptosis (Rudolph et al., 2000). The essential nature of the IKK γ subunit is also demonstrated by the complete loss of IKK α and IKK β kinase activity and NF κ B activation in IKK γ deficient cells in response to a number of stimuli (Rudolph et al., 2000; Yamaoka et al., 1998).

1.4.3.2 Ubiquitination and degradation of I κ B.

NF κ B activation represents a striking example of the targeting of a regulatory protein, I κ B, for degradation by the ubiquitin-proteasome system. The process of site directed ubiquitination and degradation by the multi-catalytic ATP-dependant 26S proteasome was initially thought to be reserved for the turnover of misfolded or defective proteins. However it has subsequently been identified as the process responsible for signal-induced degradation of I κ B α .

Degradation of proteins via the ubiquitin-proteasome pathway involves three successive steps, covalent attachment of one or more ubiquitin polypeptides to the protein substrate, ubiquitin-ubiquitin conjugation and finally degradation of the ubiquitin-tagged protein by the 26S proteasome. Three components collaborate in the cascade of ubiquitin transfer reaction to attach the ubiquitin molecules to the ϵ group of lysine residues of the substrate protein. Firstly an ubiquitin activation enzyme (E1) activates

ubiquitin via an ATP-dependant mechanism. The activated ubiquitin is then transferred to cysteine in the active site of an ubiquitin-conjugating enzyme (E2) and finally an ubiquitin ligase (E3) transfers the activated ubiquitin to the target protein. A polyubiquitin chain is then created through the transfer of additional ubiquitin molecules to the previously conjugated ubiquitin molecule. The tagged substrate is proteolysed by a 26S proteasome complex with the release of free and reutilizable ubiquitin.

It is now accepted that a key element in the substrate recognition process is the E3 ubiquitin–ligase enzymes that recognises conserved sites on I κ B α and I κ B β target proteins (Yaron et al., 1997). The actual recognition of the N-terminally phosphorylated I κ B is carried out by an F-box /WD repeat containing protein called β TrCP (Kroll et al., 1999; Spencer et al., 1999). The recognition of I κ B by β TrCP appears to be due to the highly conserved region around the I κ B phosphoacceptor sites which also serves as a recognition site for IKK (Rothwarf and Karin, 1999).

Signal-induced phosphorylation of I κ B α at ser³² and ser³⁶ is believed to be prerequisite for conjugation of multiple ubiquitin molecules to neighbouring lysine residues (lys²¹ and lys²³). Indeed, putative E2 s have been identified that specifically conjugate ubiquitin molecules to ser³²/ser³⁶ phosphorylated I κ B α but do not recognize mutant I κ B α that cannot be phosphorylated at these positions (Gonen et al., 1999).

1.4.4 NF κ B TRANSCRIPTIONAL ACTIVITY

Induction of inflammatory gene expression by NF κ B relies on its binding to specific DNA sequences in the gene promoter region. In order for the transcription factor to gain access the DNA binding domains of most target genes the chromatin architecture must be remodelled to open up the DNA at the promoter region. This is achieved through the acetylation of specific lysine residues in the N-terminal tail of the core histone reducing the charge and resulting in the unwinding of the DNA from around the histone core (chromatin relaxation). This allows transcription factors and RNA polymerase II to bind more readily to the DNA and increase transcription. Some transcriptional co-activators have intrinsic histone acetyltransferase (HAT) activity, which is activated by transcription factor binding. Reversal of this process (histone deacetylation) is achieved by histone deacetalase (HDAC) and results in the increased winding of DNA around the histone and repression of gene transcription (Urnov and Wolffe, 2001).

At the promoter level NFκB can act in concert with other promoter bound transcription factors/activators, such as CREB and AP-1 to form the necessary platform to recruit non-DNA bound coactivators (proteins that bridge the transcriptional activators and the components of the basal transcriptional apparatus) in order to drive transcription. The entire associated structure of the promoter-defined, DNA bound transcription factors and the non DNA bound associated coactivators is called the enhanceosome.

Although the primary level of regulation of NFκB activity lies with the liberation from the inhibitory factor and translocation to the nucleus, the ability of NFκB to recruit the transcriptional apparatus and stimulate target gene expression (the transactivation potential) is ensured by additional modification of NFκB itself and the surrounding chromatin structure. It is now generally accepted that the ability of NFκB to drive transcription is a regulated event.

The first suggestion that NFκB subunits, in particular p65, are post transcriptionally regulated came by the demonstration that cellular stimulation caused the inducible phosphorylation of this NFκB protein (Naumann and Scheidereit, 1994). Subsequently, the critical importance of ser²⁷⁶ of NFκB p65 has been demonstrated by the complete loss of NFκB transcriptional potency when ser²⁷⁶ is mutated to render it resistant to phosphorylation (Zhong et al., 1998). Phosphorylation of this crucial serine residue is believed to serve two functions. Firstly it enhances DNA binding by p65 and secondly it provides a site for the interaction with the transcription co-activator, CBP (CREB-binding protein)/p300 thus contributing to the formation of a transcriptionally competent enhanceosome (Zhong et al., 1998). Before phosphorylation the C-terminal region of NFκB interacts with the RHD thereby interfering with the binding to DNA and CBP/p300. Phosphorylation at ser²⁷⁶ prevents this intra-molecular association thereby facilitating transcriptional activity. Co-activator molecules are believed to regulate the activity of inducible transcription factors by 1) providing a link between the enhancer-bound transcription factors and the general transcription machinery (Janknecht and Hunter, 1996) and 2) by directly acetylating histone structures and other protein within the promoter structure thus facilitating chromatin relaxation. CBP/p300 has been linked in such a way to the transcriptional regulation of NFκB and may also acetylate p65 (Gerritsen et al., 1997).

Several lines of evidence suggest that cAMP-dependant protein kinase (PKA) might be involved in NFκB phosphorylation and transcriptional activity. Most Rel proteins have

a conserved consensus recognition site for PKA within their RHD and phosphorylation of ser²⁷⁶ of p65 by PKA enhances the DNA binding activity of recombinant p65/p65 and p50/p65 dimers (Mosialos and Gilmore, 1993; Neumann et al., 1995). The catalytic subunit of PKA associates with the I κ B:NF κ B complex in the cytoplasm of resting cells with the catalytic activity inhibited by the masking of the N-terminal ATP-binding domain by I κ B α or I κ B β . PKA activation occurs in a cAMP independent manner as a result of signal-induced degradation of I κ B (Zhong et al., 1997).

The PKA-induced phosphorylation of NF κ B at ser²⁷⁶ is the best characterized of the post-translational modifications of this transcription factor although others have been reported.

In TNF α treated cells MSK1 (mitogen- and stress- activated protein kinase 1) has been shown to directly interact with NF κ B p65 at the IL-6 promoter and phosphorylate p65 at ser²⁷⁶ (Vermeulen et al., 2003). MSK1 is a nuclear kinase that is activated by ERK and p38 MAPK and has previously been described as a kinase that phosphorylates CREB and histone H3 within the nucleosome structure (Deak et al., 1998; Soloaga et al., 2003; Thomson et al., 1999). Long-standing models have suggested that histone modifications (phosphorylation, acetylation, ubiquitination and/or methylation) disrupt the electrostatic interaction between neighbouring histones or between the histone tails and the negatively charged DNA thus increasing the accessibility of the underlying genome to nuclear factors (Iizuka and Smith, 2003). In this respect MSK1 having been recruited to the inflammatory promoter region to phosphorylate p65 also phosphorylates surrounding chromatin environment thus contributing to the local chromatin relaxation and facilitating gene transcription. Indeed, the phosphorylation of H3 (a MSK1 substrate) has been linked to the expression of inflammatory type, NF κ B-dependant genes (Edelstein et al., 2003; Park et al., 2003; Vermeulen et al., 2003).

In addition to phosphorylation at ser²⁷⁶ other sites of p65 that undergo post translational modification have also been identified. Stimulation of mouse embryo fibroblasts with TNF α has been shown to induce phosphorylation of ser⁵²⁹ by casein kinase II (Wang et al., 2000) whilst another study has shown that p65 is phosphorylated on ser⁵³⁶ by IKK (Sakurai et al., 1999a). Indeed IKK α activity is essential for the generation of transcriptionally active NF κ B (Sizemore et al., 2002). The generation of knockout mice have highlighted the importance of other kinases in the transcriptional competence of nuclear NF κ B. For example, phosphorylation of the N-terminal domain of p65 in

response to IL-1 β or TNF α was inhibited in PKC ζ ^{-/-} embryonal fibroblast (Leitges et al., 2001) and in Glycogen Synthesis Kinase 3 β (GSK3 β)^{-/-} and Tbk/t2k/NAK^{-/-} mice NF κ B transcriptional activity is impaired despite showing normal I κ B depletion and NF κ B nuclear translocation and DNA binding (Bonnard et al., 2000; Hoeflich et al., 2000; Tojima et al., 2000). However, the targets for these kinases are still unclear and it remains to be established whether they are responsible for direct modification of NF κ B or function as upstream kinases in the activation pathway.

Recently Saccani and colleagues have proposed models whereby acute stimulation induces two distinct waves of NF κ B recruitment to target promoters. A fast recruitment to constitutively and immediately accessible (CIA) promoters, which are already heavily acetylated before stimulation and immediately accessible to NF κ B. Genes with such promoters are transcribed immediately after NF κ B nuclear translocation and recruitment. Such CIA genes include I κ B α , MIP-2, and MnSOD. The second wave recruitment of NF κ B to promoters occurs 90-120 minutes after stimulation and requires stimulus-dependent modification of the chromatin structure by the formation of an initial transcription factors complex that directs the hyperacetylation of the promoter to make the NF κ B binding sites accessible. These genes are termed regulated and late accessibility (RLA) promoters (Saccani et al., 2001). Similarly Ainbinder et al have demonstrated that the rapid activation of the NF κ B responsive gene, A20 occurs due to the constitutive association of the general transcription apparatus to the A20 promoter mediated by the transcription factor Sp1 (Ainbinder et al., 2002). The authors propose a model whereby the promoters of a subset of NF κ B-regulated genes are accessible to binding by the basal transcription apparatus through the action of a constitutively active transcription factor. This complex remains bound but essentially inert until certain extracellular signals promote the nuclear transport of NF κ B, which then associates with NF κ B-binding sites on the target genes resulting in high level of transcription.

1.4.5 INACTIVATION OF NF κ B

The inactivation of NF κ B is believed to occur as part of an auto-regulatory mechanism involving I κ B α (Arenzana-Seisdedos et al., 1995). The gene encoding I κ B α (*nfkb1a*) contains, within its promoter region, an NF κ B consensus sequence (Ito et al., 1994; Le Bail et al., 1993). Induction of successful NF κ B transactivating activity induces expression of the I κ B α gene and subsequent protein expression. NF κ B mediated I κ B α

gene induction is very rapid and newly synthesised I κ B α can appear in the cells within 60 minutes following inflammatory stimulation, supporting the notion that I κ B α gene induction by NF κ B requires only limited, if any, synergistic assistance from other factors on the promoter and enforcing the requirement for rapid activation of this gene (Saccani et al., 2001). Newly synthesised I κ B α is required to enter the nucleus to bind to NF κ B on the promoter and export it from the nucleus. Since I κ B α does not possess a classical nuclear localisation signal it is unclear how this is achieved. One study suggests the action of a non-classical NLS within the second ankyrin repeat (Sachdev et al., 1998), whereas another report suggests a 'piggy back' mechanism whereby I κ B α uses the NLS signal of an associated protein (Turpin et al., 1999). Once in the nucleus I κ B α associates with NF κ B on the promoter and transports the inactivated NF κ B back to the cytoplasm by virtue of its leucine rich nuclear export signal (Arenzana-Seisdedos et al., 1997; Rodriguez et al., 1999).

Interestingly, it has been suggested that NF κ B acetylation determines the efficiency of its interaction with I κ B α and that histone deacetylase plays a functional role in the inactivation of NF κ B. Given the studies suggesting a role for histone acetyl transferase recruitment to nuclear NF κ B and the implications for the role of CPB/p300 in NF κ B regulation (Gerritsen et al., 1997; Zhong et al., 1998) it is not surprising that recent studies have also suggested a role for HDAC. It has been shown that p65 can interact with HDAC isoforms and that these interactions can negatively regulate NF κ B gene expression (Ashburner et al., 2001; Ito et al., 2000; Lee et al., 2000; Zhong et al., 2002). It is, however, unclear which HDAC isoforms are involved. Immunoprecipitation studies have suggested that HDAC1 can associate with NF κ B p65 (Ashburner et al., 2001; Chen et al., 2001; Zhong et al., 2002) and TNF α -induced, p300 dependant acetylation of p65 can be reversed by HDAC3 (Chen et al., 2001). The authors of that study suggest a model whereby the acetylated NF κ B that enters the nucleus is refractory to inhibition by I κ B α . However, following recruitment of HDAC the deacetylated p65 associates with newly synthesised I κ B α which then transports the inactivated NF κ B back to the cytoplasm.

1.4.6 NF κ B AND INFLAMMATION

Inflammation is usually associated with the co-ordinated production and action of inflammatory cytokines, chemokines, adhesion molecules, growth factors, inducible

enzymes and regulators of apoptosis that play a key part in the initial recruitment of leucocytes to sites of inflammation. NF κ B has been shown to be essential for the initiation of the transcription of a wide variety of genes involved in the pathogenesis and progression of inflammation. For example NF κ B induces the expression of cytokines such as IL-1 β , TNF α IL-6, chemokine (IL-8, RANTES, MCP-1), adhesion molecules (VCAM-1, ICAM-1 and E-selectin), matrix metalloproteinases (MMP, 1, 3 and 9) and inducible enzymes such as nitric oxide synthase and COX 2. In addition to the induction of factors involved in the inflammatory response, NF κ B activity can be modulated by factors such as IL-1 β and TNF α observed to be increased during inflammation (Pahl, 1999). Animal models of inflammatory diseases and observations of NF κ B activity at the site of inflammation in human diseases all lend support for the critical importance of NF κ B activity in the regulation of the inflammatory response.

1.4.6.1 NF κ B activity in animal models in inflammation.

In animal models of arthritis, synovial NF κ B activity has been shown to be increased early in the development of the disease, before onset of clinical arthritis and continues to increase during the evolution of the disease (Han et al., 1998; Tsao et al., 1997). Inhibition of NF κ B nuclear translocation by intra-articular transfer of dominant negative IKK β or inhibition of NF κ B transcriptional activity by administration of lysosomal NF κ B decoy oligonucleotide inhibited the production of IL-1 β and TNF α within the joint and significantly reduced the severity of adjuvant, collagen or streptococcal cell wall-induced arthritis in rats (Miagkov et al., 1998; Tomita et al., 1999; Tak et al., 2001). In addition intra-articular transfer of a functional IKK β gene in normal rats, selectively activated NF κ B and led to joint inflammation and clinical signs of arthritis (Tak et al., 2001).

Similar NF κ B directed therapies have also been effective in the prevention of disease development in a murine models of inflammatory bowel disease, inhibiting histological signs of colitis and in preventing death in animal models of sepsis (Fujihara et al., 2000; MacMaster et al., 2003; Schlaak et al., 2001; Neurath et al., 1996). In a rat model of myocardial infarction NF κ B decoys inhibited the extent of ischaemia-reperfusion injury (Morishita et al., 1997) and in a murine model of auto-immune myocarditis NF κ B decoys reduced the expression and production of ICAM-1, IL-2, TNF α , and iNOS in the

heart, which was associated with reduced severity of myocarditis (Yokoseki et al., 2001).

High levels of NF κ B activity were found in bronchial cells recovered by bronchial brushing from heaves-affected horses (an animal model of asthma) during crisis (i.e., acute airway obstruction) and NF κ B activity paralleled ICAM-1 expression in these cells and the degree of lung dysfunction following crisis. This is in contrast to the very low levels of NF κ B activity found in bronchial cells from healthy horses (Bureau et al., 2000).

1.4.6.2 NF κ B activation in human inflammatory disease.

Inappropriate activation of NF κ B has been linked to inflammatory events associated with autoimmune arthritis, asthma, septic shock, lung fibrosis, glomerulonephritis, systemic inflammatory response syndrome and atherosclerosis.

In the inflamed synovium of patients suffering from rheumatoid arthritis both NF κ B p50 and p65 have been localised to the nuclei of synovial lining cells, endothelial cells and monocytes in the sublining fluid. In addition, whilst NF κ B was activated in the synovium of rheumatoid arthritis patients, in patients with osteoarthritis NF κ B was inactive, consistent with increased pro-inflammatory cytokine expression in the former and not the latter (Asahara et al., 1995; Han et al., 1998; Marok et al., 1996).

Human inflammatory airway diseases, such as asthma in which cytokine and adhesion molecule expression are increased are also associated with increased NF κ B activity in lung macrophages and bronchial epithelial cells (Hart et al., 1998, 2000) compared to that in control subjects. In addition, exaggerated NF κ B activity in PBMC appears to increase with increasing severity of disease and has been suggested to perpetuate the production of inflammatory mediators observed in severe asthma. Also alveolar macrophages from patients with acute respiratory distress syndrome, a disease characterised by increased TNF α and IL-8 levels in the lung, have considerably greater levels of activated NF κ B than alveolar macrophages from critically ill patients without signs of inflammation (Schwartz et al., 1996). Similarly, compared to healthy volunteers NF κ B DNA binding activity has been demonstrated in PBMC from critically ill patients with SIRS which increases further in patients who die (Paterson et al., 2000).

Activated NFκB has also been reported in biopsy specimens of patients with inflammatory bowel disease localized to the macrophages and epithelial cells from inflamed mucosa. The number of cells showing NFκB activation correlated with the degree of mucosal inflammation and was almost completely absent in uninflamed mucosa (Ardite et al., 1998; Rogler et al., 1998).

Atherosclerosis is now firmly established as an inflammatory disease. Consistent with such Brand *et al* have demonstrated the presence of activated NFκB in the fibrotic-thickened intima/media and atheromatous areas of the atherosclerotic lesion (Brand et al., 1996). Activation of NFκB was identified in smooth muscle cells, macrophages, and endothelial cells of diseased vessels whilst little or no activated NFκB was detected in vessels lacking atherosclerosis.

1.4.6.3 NFκB and the resolution of inflammation.

Recent studies using animal models have suggested that NFκB activation plays an important role in the resolution phase of an inflammatory response. In two, well characterised models of self-resolving inflammation (rat carrageenin pleurisy and mouse carrageenin air pouch) leucocyte NFκB DNA binding activity was observed to be biphasic, associated with the onset and the resolution of inflammation. However there were some striking differences between these two phases of the inflammatory process. During the onset of inflammation NFκB DNA binding activity was attributed to p65, c-Rel and p50 containing complexes with an increase in iNOS expression. During the resolution phase NFκB DNA binding complexes were predominantly p50/p50 with some p65/p50 complexes but no c-Rel and no iNOS expression. NFκB DNA binding activity during the resolution of inflammation appeared to induce the expression of COX2 protein and an increase in the anti-inflammatory cyclopentenone prostaglandin (PG) cy15-deoxy-Δ PGJ₂ (15dPGJ₂) and its precursor PGD₂ (Lawrence et al., 2001). p65/p50 and p50/p50 complexes bind to different κB sites within the COX2 promoter and p50/p50 homodimers have been shown to be of importance in the transcription of the COX2 gene through the association with C/EBP (Thomas et al., 2000). These PG have previously been shown to be important mediators during the resolution of inflammation (Gilroy et al., 1999) possibly through the inhibition of NFκB mediated production of pro-survival factors thus resulting in the induction of granulocyte apoptosis (Ward et al., 2002). 15dPGJ₂ can inhibit the activation of IKKβ

through acetylation of cys¹⁷⁹ in the activation loop (Rossi et al., 2000) and by the acetylation of critical cysteine residues in the DNA binding domains of NFκB p65 (cys³⁸) and p50 (cys⁶²) preventing DNA binding (Cernuda-Morollon et al., 2001; Straus et al., 2000). However in the study of Lawrence et al pharmacological inhibition of NFκB *in vivo* during the resolution phase of inflammation inhibited leucocyte apoptosis and prolonged the inflammatory response suggesting that NFκB plays an active role in the resolution of inflammation. The authors suggested that an alternative NFκB pathway promotes leucocyte apoptosis through the transactivation of pro-apoptotic genes through co-operative action of co-activating factors or through the inhibition of the repressive activity of p50/p50 homodimers (Lawrence et al., 2002).

* * *

1.5 AIMS AND OBJECTIVES

1.5.1 HYPOTHESIS

Heart failure and complications following cardiac surgery, although disparate conditions share a common theme of increased circulating pro-inflammatory cytokines that have been strongly associated with detrimental clinical status of these patients and poor prognosis. The increased circulating pro-inflammatory cytokines have the potential to activate NFκB in cells within the vasculature and in such a manner NFκB activity has the ability to drive the inflammatory process and influence clinical outcome.

1.5.2 AIMS

The primary aim of this thesis was to determine the inflammatory cytokine profile and inflammatory bioactivity to modulate NFκB activation in the circulation of patients presenting for cardiac surgery with varying severity of heart failure and to determine the changes that occur during the course of routine cardiac surgery.

In addition, many inflammatory conditions, including both heart failure and complications following cardiac surgery are associated with increased oxygen free radical production and oxidative stress. Such alterations in the redox environment of cells have been suggested to be of major importance in the regulation of gene expression induced by NFκB. It was therefore a second aim of this thesis to explore redox regulation and establish the role of oxidative stress in inflammatory cytokine-induced NFκB activation in cells relevant to systemic inflammation.

1.5.3 PLAN OF INVESTIGATION

1. To compare the circulating levels of pro-inflammatory cytokines (IL-1 β and TNF α), anti-inflammatory cytokines (IL-4, IL-6, IL-10, IL-1RA) and soluble cytokine receptors (sTNFR1 and sTNFR2) in three groups of patients presenting for cardiac surgery. 1) Patients with coronary artery disease requiring CABG. 2) Patients with stable end stage heart failure requiring cardiac transplantation. 3) Patients with severe decompensated heart failure requiring LVAD implantation. In addition the balance between pro- and anti-inflammatory factors in the circulation of these patients will be determined.
2. To determine the inflammatory changes that occur in the circulation of patients during the course of cardiac surgery with respect to pro- and anti-inflammatory factors and the balance between them.
3. To establish whether the use cardiopulmonary bypass (CPB) is responsible for the observed changes during cardiac surgery by comparing the cytokine response to off-pump coronary artery bypass graft surgery with conventional CABG utilizing CPB.
4. To determine the effects of the pro-inflammatory cytokines IL-1 β and TNF α on NF κ B activation in cells relevant to the inflammatory response associated with heart failure and cardiac surgery.
5. To evaluate the net inflammatory bioactivity of simultaneously present multiple mediators by examining the influence of plasma from these patients on an *in vitro* bioassay system of endothelial cell inflammatory activation.
6. To determine whether modulation of the cellular redox environment can effect NF κ B activation in cells relevant to the systemic inflammatory response associated with heart failure and cardiac surgery.

* * *

It is hoped that this study will provide clarity as to true inflammatory situation in the circulation of heart failure patients and patients following cardiac surgery with respect to the ability to modulate endothelial cell activation and the activity of the inflammatory transcription factor NF κ B.

Chapter Two: METHODS

2.1 CHAPTER INFORMATION

This chapter describes the general techniques employed throughout this thesis. Any modifications, experimental design and specific details are outlined in the methods section of each chapter.

2.2 GENERAL CELL CULTURE TECHNIQUES

2.2.1 COLLECTION OF TISSUE FOR CELL CULTURE

Human tissue was collected during heart, lung and heart /lung transplantation from the trimmings of the donor organ. In the operating theatre the tissue was placed into a sterile pot containing Hank's balanced salt solution (HBSS, Sigma-Aldrich) containing penicillin (100 units/ml) and streptomycin (100µg/ml) solution (Sigma-Aldrich) and was transported to the laboratory. Where timing dictated tissue was often left at 4 °C for up to 24 hours before use.

2.2.2 SURFACE FOR CELL GROWTH IN CULTURES

Cell cultures were initiated and maintained in sterile, 'tissue culture treated' plastic vessels (TPP from Helena Biosciences) in which the growth surface was hydrostatic to aid cell adhesion.

2.2.3 MAINTENANCE, PASSAGE AND PROPAGATION OF CELLS IN CULTURE

Cells were maintained in a humidified atmosphere at 37°C in 5% CO₂. Cells were fed every 3 days by removal of all medium and replacement with fresh pre-warm complete medium. This feeding program ensured that cells remained viable and in a steady growth phase. Most of the media used contained phenol red for visual monitoring of pH for indications of adequate CO₂ environment and general metabolic activity of the cells. Cells were monitored visually with phase contrast microscopy.

Having reached confluence cell cultures were expanded by removal from the culture flask followed by re-seeding into culture vessels with larger surface area (passaging). Generally cells were removed from one 75cm² flask and re-seeded into vessels three times greater in surface area whether being multiple 75cm² flasks, 24 well plates, 10cm dishes or 6 well plates. The number of times cultures were expanded in this manner was closely monitored and noted (passage number) since cells in culture will start to deviate from their original phenotype and lose cell type specific characteristics with increasing cell divisions. Cells were used for experiments between passage 3 and 10.

2.2.3.1 Detachment of adherent cells with trypsin

Adherent cells were removed from the culture vessel with trypsin/EDTA solution (0.25% trypsin, 0.02% EDTA in 1L HBSS, Sigma-Aldrich). Trypsin is a strong proteolytic enzyme that breaks down extracellular matrix proteins that anchor the cells to each other and the culture surface. The presence of EDTA in this solution will chelate calcium and prevent calcium dependant adhesiveness of the cells thus avoiding cell clumping.

For detachment of cells from the plastic surface, the medium was first completely removed and the cells rinsed with sterile phosphate buffered saline (PBS, Sigma-Aldrich) to remove all trace of serum that can inhibit the proteolytic activity of trypsin. The PBS was removed and a small volume of trypsin/EDTA solution, sufficient to cover the cells was added. The cells were placed in the incubator for 2 minutes after which time the vessel was tapped hard to aid cell detachment. The cells were checked under the microscope to ensure all were detached before deactivating the trypsin by the addition of equal volumes of heat-inactivated fetal bovine serum (HI-FBS, Sigma-Aldrich) followed by dilution with 10-fold volume of PBS. The cells were pelleted by centrifugation at 365 x g for 5 minutes at room temperature. The supernatant was removed and discarded and the cell pellet resuspended in medium and used as required.

2.2.3.2 Freezing cells for long term storage

Freezing small vials of cells in suspension has become a widely used method of preserving cells for long-term storage in liquid nitrogen and depends on the use of dimethyl sulfoxide (DMSO, Sigma-Aldrich) as a cryopreservative. Generally when cells are subjected to low temperatures ice crystals will form which will disrupt the cell membrane leading to the death of the cells. The goal of DMSO as a cryopreservative is to replace some of the water thus reducing the water content and to protect the cells from mechanical and physical stress. The freezing medium must also contain a protein source to also protect the cells from the stresses associated with the freeze-thaw process.

Cells were detached from tissue culture plates with trypsin/EDTA solution as describes in section 2.2.3.1. For every 75cm² flask of cells trypsinised 2 ml of freezing medium (50% HI FBS, 40% Dulbecco's modified eagles medium (DMEM, Sigma-Aldrich), 10% DMSO) was used to resuspended the cell pellet. Cell suspensions were aseptically aliquoted in 1ml volumes into 2 ml cryovials (Greiner Bio-One, UK). Cells were placed in the liquid N₂ dependant slow freezing chamber (Planer KRY010 Series II, Planer

Select, Sunbury, UK) where the temperature was lowered stepwise, reaching -135°C over 40 minutes. On removal from the freezing chamber, cells were transferred, for long-term storage, to a liquid N_2 refrigerator (-196°C).

2.2.4 CHARACTERISATION OF CELLS BY IMMUNO-FLUORESCENCE

Cells were grown on 22mm x 22mm, 0.5% gelatine coated glass coverslips in 6 well plates until no more than 80% confluent. The medium was removed from the wells and the cells were washed in PBS. The cells were fixed and permeabilized by transferring the coverslips to a glass staining jar containing ice cold acetone for 5 minutes. Once removed from the 6 well plate it is important to maintain the same orientation of the coverslip to ensure antibodies are in contact with the cell covered face and not the reverse side. The cells were washed in PBS by transferring the coverslips to a glass staining jar containing PBS. After 5 minutes the PBS was replaced with fresh and the cells left for a further 5 minutes. 125 μl of working strength primary antibody in PBS was placed in the centre of a 50mm x 50mm piece of parafilm on a flat surface. The coverslips were removed from the staining jar one at a time and carefully lowered, cell surface downward, onto the correct antibody solution on the parafilm. The weight of the coverslip causes the antibody solution to spread under the slip so the whole surface is covered. Air bubbles were removed by lifting the coverslip removing the bubble with a pipette tip and replacing the coverslip as before. The cells were incubated with primary antibodies in this manner for 60 minutes. The cells were washed in PBS as before to remove all unbound antibody. Using the same procedure that was used for the primary antibody the cells were incubated for 20 minutes with a 4 $\mu\text{g/ml}$ solution of relevant secondary antibody conjugated to Alexa Fluor® 594 (Molecular Probes, Cambridge Bioscience, UK). Alexa Fluor® 594 is a fluorescent dye that emits in the red region of the spectrum and is efficiently excited by the 594 nm line of the orange HE-Ne laser. After the 20 minute incubation the cells were washed as before to remove unbound antibody. Where required the cell nuclei were counter stained with Sytox® Green nucleic acid stain (Molecular Probes, Cambridge Bioscience, UK). Bound dye fluoresces bright green upon excitation with the 488nm spectral. Cells on coverslips were transferred to a staining jar containing working strength Sytox solution (100nM in PBS) and incubated for 5 minutes. As before, the cells were washed in PBS. After the final PBS wash the coverslips were mounted onto pre labelled glass microscope slides with Permafluor aqueous mounting medium (Beckman Coulter) pre-warmed to room

temperature. Stained cells were visualised using a Zeiss Axioskop microscope and images were captured using Lucia F software.

2.3 PROTEIN ANALYSIS BY SDS-PAGE AND WESTERN BLOTTING

Recipes for all buffers used in this section are detailed in appendix I.

2.3.1 PREPARATION OF PROTEIN SAMPLES

2.3.1.1 Total cell lysis

This method utilises a Sodium dodecyl sulphate (SDS) based lysis buffer to destroy cell membranes and denature protein–protein interactions. This method was thus used to analyse levels of specific proteins in the cells regardless of cellular localisation or associations with other protein.

Following the experimental procedure the cells were washed in cold PBS to remove traces of cytokines and inhibitors and to slow the enzymatic activity in the cells. 1% SDS lysis buffer was added to each well and the cells were disrupted using the rubber plunger from a 1ml syringe. Cell homogenates from replicate wells were combined and aspirated ten times through a 25-gauge needle to shear DNA. Protein content of the resulting total cell protein preparation was determined by Bradford assay (Bradford, 1976) and the samples were stored at –20 °C until analysis.

2.3.1.2 Immunoprecipitation of NFκB p65 and associated proteins

In this method the cells are gently lysed using a non-denaturing lysis buffer to maintain protein-protein associations. Proteins of interest can be precipitated from this total cell protein preparation using specific antibodies and protein G-agarose (Roche Diagnostics). Protein G agarose is recombinant protein G from E-Coli covalently coupled to crosslinked agarose beads. Protein G binds to the Fc-part of most mammalian IgG with high affinity by non-immune mechanisms. This method can thus be used to isolate proteins of interest along with any associated proteins.

The whole procedure was carried out at 4°C. Following the experimental procedure cells were washed in cold PBS and incubated on ice with 50µl/well of immunoprecipitation lysis buffer for 10 minutes. Cells were disrupted using the rubber plunger of a 1ml syringe, triplicate wells combined and the samples were aspirated through a 25-gauge needle. 25µl from each sample was removed and the proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as total cells lysis

preparations. The remaining samples were centrifuged at 600 x g for 15 minutes to pellet the cell debris. The supernatant was transferred to a clean 1.5ml eppendorf and 0.5µg of anti-NFκB p65 polyclonal antibody (Santa Cruz) was added. Following 1 hour incubation, 10µl of protein-G agarose was added and the samples were incubated for a further 60 minutes on a rocker. The samples were then centrifuged at 420 x g for 5 minutes, supernatants were discarded and the pellet was washed 4 times in cold lysis buffer. The final pellet was resuspended in 150µl of Laemmli buffer containing 5.2% β-mercaptoethanol (VWR international) and the sample was divided in half and stored at -80°C. For analysis of proteins by SDS-PAGE and immune-blotting, thawed samples were heated to 100°C in a heating block, cooled and centrifuged to recover the samples to the bottom of the tube. SDS-PAGE, Western blotting and immunostaining were carried out as described in section 2.3.2, 2.3.3 and 2.3.4.

2.3.1.3 Isolation of nuclear proteins

This technique allows the separation of cytosolic and nuclear proteins. With the use of high salt solution and light detergents the cell membrane is disrupted whilst the integrity of the nuclear membrane is maintained. When centrifuged the cytosolic protein remain in solution in the supernatant whilst the heavier intact nuclei form a pellet. This nuclear pellet is then lysed and centrifugation allows the separation of nuclear proteins from cell debris.

Following the experimental procedure cells were scraped from 10cm dishes into ice cold PBS, duplicates were combined and the cells pelleted by centrifuged. The resulting cell pellet was resuspended in 1ml of Buffer A, aspirated ten times through a 25-gauge needle and incubated on ice for 15 minutes to lyse the cell membrane. The samples were centrifuged at 11,000 x g for 1 minute to pellet nuclei and cell debris. The supernatant containing cytosolic proteins was removed, assayed for protein content and stored at -80°C. The nuclei were washed twice in Buffer A, resuspended in 50µl of Buffer B and incubated (on a rocker at 4°C) for 30 minutes. The nuclear debris was removed by centrifugation and the resulting nuclear extract was assayed for protein content and stored at -80°C.

2.3.2 SDS-PAGE

2.3.2.1 Polyacrylamide gels

Gel cassette units consisted of a 10% polyacrylamide resolving gel (15 x 10 cm) cast between clean glass plates separated by 1.5mm thick spacers. Lanes were cast in a 3% polyacrylamide stacking gel above the resolving gel.

2.3.2.2 Samples preparation

Cell protein preparations were removed from freezer storage, thawed and vortex to ensure homogeneity. Equal quantities of protein (25µg whole cells lysis and 10µg of nuclear proteins) were transferred to clean, labelled eppendorf tubes and samples were made up to 40µl with relevant lysis buffer. For each gel one lane contained molecular weight rainbow markers (Amersham Biosciences, UK) consisting of 5 µl rainbow marker solution and 35µl of the same lysis buffer as the samples in that gel. Empty lanes contained 40µl of lysis buffer. 50µl of Laemmli buffer containing 5.2% β-mercaptoethanol was added to all tubes. The samples were heated for 2 minutes in a heating block set to 100°C. Samples were put on ice to cool and then briefly centrifuged to recover the entire sample to the bottom of the tube.

2.3.2.3 Electrophoresis

The entire sample (prepared according to section 2.3.2.2 or 2.3.1.2) was loaded into the relevant lanes of the gel. The entire gel cassette unit was inserted vertically, through a rubber gasket into the running tank filled with sufficient running buffer to cover the anode. Running buffer was pumped into the upper cathode chamber sufficient to cover the top of each gel. The gels were subjected to 60-100mA per gels at constant voltage until the bromophenol blue tracking dye front had migrated to the bottom of the gel. The gel cassette units were removed from the tank, disassembled and the stacking gel was discarded.

2.3.3 WESTERN BLOTTING

Following separation of proteins in the SDS-Polyacrylamide gels (section 2.3.2.3), proteins were transferred to Hybond™-C Super supported nitrocellulose membrane (Amersham Biosciences). The gels were first washed for 30 minutes in blotting buffer to remove all traces of SDS left by the running buffer. The gel was placed onto nitrocellulose membrane, sandwiched between four pieces of Whatman 3MM

chromatography paper (VWR International) and the cathode and anode electrodes. This blotting cassette was placed in the blotting tank full of blotting buffer. Transfer of proteins from the gel to the nitrocellulose took 60 minutes at constant voltage and 0.5 Amps per cassette. When transfer was complete the gel was discarded and the membrane carrying the transferred proteins was labelled, air dried and stored between two pieces of Whatman 3MM paper in a sealed plastic bag until proteins were analysed by immunostaining.

2.3.4 IMMUNOSTAINING

Hybond™ C nitrocellulose membranes are highly attractive to proteins and thus all unoccupied non-specific binding sites on the membrane were first blocked to prevent the non-specific adhesion of the antibodies to the membrane. This was achieved by incubating the nitrocellulose membrane in a sealed plastic bag with blocking solution consisting of 3% non-fat dried milk powder in PBS containing 0.05% polyoxyethylene (20) sorbitan monolaurate (Tween 20, VWR International) (PBS-T) for 30 minutes at room temperature or overnight at 4°C with constant rocking. The plastic bag was opened at one end, the blocking solution was discarded and replaced with primary antibody in the blocking solution. The bag was re-sealed using a heat sealer and the membrane incubated with primary antibody for 60 minutes on a rocker. The membrane was then removed from the bag using clean forceps and the antibody solution was discarded. The membrane was placed in a glass dish and washed by constant rocking with copious quantities of PBS-T for 30 minutes, changing the PBS-T three times. The membrane was then incubated in a sealed plastic bag on a rocker for 30 minutes with relevant horseradish peroxidase (HRP)-conjugated secondary antibody (DakoCytomation). After this time the membrane was washed as before. Immunoreactive protein bands were visualised using enhanced chemiluminescent (ECL™) Western blotting detection system (Amersham Biosciences). The membranes were drained of excess PBS-T and placed, protein side up in a clean glass dish. The ECL reagents were mixed in equal quantities and immediately poured over the membrane ensuring even covering of the whole membrane. The membrane was incubated for 1 minute with the ECL solution after which time excess ECL was drained from the membrane. The membrane was placed between two pieces of clear plastic and all air bubbles were removed by gentle smoothing with a cloth. The membrane, in the plastic, was taped by the edges into the top left hand corner of an autoradiography cassette. Chemiluminescence was visualised by exposing the membrane to Hyperfilm™

MP autoradiography film (Amersham Biosciences) that was then developed through an AGFA Curix 60 medical x-ray film processor.

2.3.5 STRIPPING THE ANTIBODIES FROM THE MEMBRANE

After staining the membranes for the first protein of interest the antibodies were striped from it by incubation with stripping solution at 50°C for 30 minutes with mixing every 10 minutes. The membrane was then washed in copious quantities of PBS-T before staining with antibodies exactly as before (section 2.3.4). Depending on the abundance of the protein of interest and the strength and specificity of the antibody, this procedure of stripping the membrane can be performed 2-4 times without adversely affecting the immunostaining results.

2.3.6 COUNTER STAINING

After immunostaining the membrane was stained with Ponceau S to visualise all the protein bands. Membranes were placed in a clean glass dish and rinsed with PBS-T. Ponceau S was added to the membrane and incubated on a rocker for 5 minutes. The Ponceau S dye was removed and the membrane washed with distilled water until proteins were seen as distinct pink bands and the washing water was clear.

2.3.7 LASER DENSITOMETRY AND DATA ANALYSIS

Developed autoradiograph films were scanned using a BIORAD GS-710 Calibrated Imaging Densitometer. Optical densities (OD) of individual bands were determined using Quantity One software, Version 4.1.1 003 (Bio-Rad Laboratories).

For each protein analysed the OD of each band was compared to the OD of that protein band in a control untreated cell sample run on the same gel. All values were then normalised to a counter stained protein (α -actin or β -tubulin). In this manner all data generated by Western blot analysis is expressed as a percent of that in control cells.

2.4 ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

EMSA were performed on nuclear proteins isolated by the method described in section 2.3.1.3. diluted five fold in Buffer C.

2.4.1 OLIGONUCLEOTIDE PROBES – SEQUENCES (SANTA CRUZ BIOTECHNOLOGY)

2.4.1.1 NF κ B binding element

5 -AGTTGAGGGGACTTTCCCAGGC-3

3 -TCAACTCCCCTGAAAGGGTCCG-5

2.4.1.2 NF κ B mutant

5 -AGTTGAGG□GACTTTCCCAGGC-3

3 -TCAACTCC□GCTGAAAGGGTCCG-5

2.4.2 ³²P LABELLING PROBE

Double stranded oligonucleotide probe was end-labelled using 0.74MBq [γ -³²P] ATP (Amersham Biosciences) and T4 polynucleotide kinase (T4PNK, New England Biolabs) to catalyses the transfer of the γ -phosphate from ATP to the 5'-terminus of the oligonucleotide. To 2 μ l of consensus oligonucleotide (1.75pmol/ μ l), 1 μ l of 10x T4PNK buffer, 1 μ l of [γ -³²P] ATP, 5 μ l nuclease free H₂O and 1 μ l of T4PNK were added. The mixture was left over night at room temperature and the reaction was stopped by the addition of 1 μ l 0.5M EDTA. 89ml of TE (10mM Tris-HCl (pH 8.0), 1mM EDTA) was added and the ³²P-labelled oligonucleotide was purified through PROBEQUANTTM G-50 micro-columns (Pharmacia Biotech).

2.4.3 PROTEIN DNA BINDING REACTION

4 μ g of nuclear proteins were incubated for 1 hour with 2 μ l (0.0175 picomoles) of ³²P labelled oligonucleotide, 2 μ l 10x buffer (100mM Tris (pH7.5), 200mM NaCl, 10mM EDTA, 50% glycerol, 2 μ l (2.5 μ g) poly (dI-dC) and water to give a final volume of 20 μ l. DNA binding specificity was determined by the addition of either 100-fold excess cold oligonucleotide, 100-fold excess of cold mutant κ B oligonucleotide, addition of 4 μ g of purified I κ B α protein corresponding to amino acids 1-317 or addition of 2 μ g of antibody directed against specific NF κ B protein subunits.

2.4.4 ELECTROPHORESIS AND BAND VISUALISATION

To each sample 2 μ l of loading buffer (250mM Tris-HCl (pH 7.5), 0.2% bromophenol blue, 40% glycerol) was add and the samples were loaded onto a 6% non-denaturing polyacrylamide gel. The gel was run in 0.5x TBE buffer at 120Volts until the dye front had migrated to the bottom of the gel. The gel was fixed in 10% methanol/ 10% acetone solution and transferred face up onto 3MM chromatograph paper and wrapped in saran wrap. The gel was dried using a Savant Speed gel SG 210D at 60°C and radioactivity

was visualised by exposure to autoradiography film and developed using an AGFA Curix 60 medical x-ray film processor.

2.5 IMMUNOCYTOCHEMISTRY

2.5.1 PREPARATION OF CELLS

Frozen samples were brought to room temperature before unwrapping and exposing to air.

Cells were fixed and permeabilised in ice-cold acetone for 5 minutes. The acetone was allowed to evaporate before rinsing the cells in 0.004M Tris Buffered Saline, pH 7.6 (TBS).

2.5.2 IMMUNOPEROXIDASE STAINING

Slides were placed in a humid chamber and the samples were incubated with primary antibody. After this incubation unbound primary antibody was removed by washing the slides in TBS for 30 minutes changing the washing solution three times. Samples were then incubated with an anti-mouse secondary antibody conjugated to an HRP-labelled polymer (EnVision™+, DakoCytomation) for 30 minutes at room temperature. Unbound secondary antibody was washed from the samples as before. After thoroughly washing, 3,3 -diaminobenzidine (SIGMAFAST™ DAB peroxidase tablet set dissolved in 0.1% sodium azide) solution was applied to the slides for exactly 5 minutes. Slides were then briefly washed in TBS followed by washing in cold running tap water before being counterstaining with Meyer's haematoxylin (VWR International) for 2 minutes. Slides were then washed in cold running tap water for 2 minutes, dehydrated through graded alcohol (70 - 100% methanol) and mounted in DPX (VWR International).

2.5.3 IMAGE ANALYSIS

Stained cells were examined under phase contrast light microscope. Positive staining appeared brown with cell nuclei being blue in colour. Images were captured using LUCIA Software (Nikon UK Ltd).

2.6 ANALYSIS OF ADHESION MOLECULE EXPRESSION IN CULTURED CELLS BY FLOW CYTOMETRY

2.6.1 ANTIBODY STAINING OF CELLS

Following the experimental procedure, cells were washed in PBS and removed from culture dishes by addition of trypsin/EDTA. Detached cells were transferred to polypropylene test tubes (12 x 75 mm) containing 5% FBS in PBS. Cells were pelleted by centrifugation at 365 x g for 5 minutes and all but 90µl of supernatant removed by aspiration. 10µl of primary antibody at 10 times concentration was added to the relevant tubes. For each individual experiment, cells in one tube were stained using an isotype specific negative control antibody and in another with positive control antibody. All other samples were stained using an antibody directed against the adhesion molecule of interest. Cells were vortexed and incubated on ice with primary antibody for 30 minutes. Cells were washed by dilution with 3ml PBS and centrifugation at 365 x g for 5 minutes. All but 90µl of supernatant was removed by aspiration and 10µl of 10-times concentrated fluorescein isothiocyanate (FITC) conjugated secondary antibody added. Samples were vortexed and incubated on ice for 30 minutes. Cells were washed as before and resuspended in 500µl 0.5% formaldehyde solution. Cells were stored at 4°C in the dark until analysis by flow cytometry.

2.6.2 ANALYSIS OF FLUORESCENCE

The fluorescence intensity of individual cells was analysed using Coulter EPICS®-XL flow cytometer using EXPO cytometer software (version 2, Beckman Coulter) recording forward and side scatter characteristics and Log fluorescence intensity using Coulter XL fluorescence channel FL1.

The Coulter EPICS XL was set up using cells stained with the irrelevant isotype control antibody. Forward and side scatter voltages were adjusted to display the cell population in the centre of the plot and a gate was drawn around the cell population. The fluorescence voltage was adjusted to give a histogram showing low fluorescence intensity and a gate was set to show 2% of these events having positive fluorescence. These settings were used for all samples in a given experiment. Data was acquired for 10,000-20,000 cells per sample using a medium-high flow rate.

2.6.3 DATA ANALYSIS

Data is expressed as the percent of cells demonstrating positive fluorescence when stained with a specific antibody with 2% of cells stained using the irrelevant isotype control antibody always being positive.

Chapter Three: PRO AND ANTI-INFLAMMATORY CYTOKINE BALANCE IN PATIENT PLASMA

3.1 INTRODUCTION

It is now widely appreciated that the ancient aspect of host immune response and inflammation is largely governed by cytokines, a limited number of which orchestrate the course of many disease states that scarcely resemble one another. Under normal physiological conditions pro-inflammatory cytokines are generally not found in the peripheral circulation. Because their activity is exerted over a short distance the systemic detection of cytokines generally implies excessive production.

Pro-inflammatory cytokines have been implicated in the initiation, progression and termination of a number of pathological states of the cardiovascular system including heart failure and the systemic inflammatory response to cardiac surgery. In particular IL-1 β and TNF α are believed to be of critical importance due to the ability of many cells to secrete these molecules in response to a number of different stimuli. In addition the number of different cell types that express receptors for these cytokines allows a broad spectrum of effects to be stimulated by their presence.

Both specific and non-specific endogenous cytokine inhibitors have been described that provide mechanisms whereby the biological effects of pro-inflammatory cytokines are limited. Such discoveries have increased our appreciation of the complexity of cytokine signalling pathways and inflammatory cascades. To date much of the focus of inflammation research in the setting of heart failure and in response to cardiac surgery has been on the production of pro-inflammatory cytokines, in particular TNF α and IL-1 β . Whilst this gives some indication as to the extent of activation of the inflammatory response it does not consider the potential for the presence of the counter-acting, anti-inflammatory factors.

The expression of anti-inflammatory cytokines and specific endogenous inhibitors (soluble receptors and receptor antagonists) have the exquisite capability of reducing the cellular effects and molecular consequences of increased IL-1 β and TNF α and therefore their presence may somewhat invalidate the information gained from considering individual factors in isolation. Indeed the balance between pro- and anti-inflammatory mediators has been shown to be of greater physiological and clinical relevance than individual cytokine concentrations in a number of inflammatory diseases (Casini-Raggi et al., 1995; Park et al., 2001b).

This chapter will investigate the balance between pro-inflammatory cytokines, IL-1 β and TNF α and endogenous anti-inflammatory factors in the circulation of patients with relevance to two cardiovascular syndromes deemed to possess a clear inflammatory component of clinical significance; heart failure and systemic inflammatory response to cardiac surgery.

Although many studies have previously measured circulating levels of various pro- and anti-inflammatory factors in such patients these measurements were required in this current study in order to establish the extent of the systemic inflammatory response in the study populations included in this thesis.

3.2 STUDY N°1 – EFFECT OF DETERIORATING CLINICAL STATUS ON PLASMA CYTOKINES LEVELS IN HEART FAILURE PATIENTS.

3.2.1 BACKGROUND

Supported by a large body of both scientific and clinical evidence it has now been reasonably established that heart failure and the progression of the disease are associated with low grade systemic inflammation and the expression of pro-inflammatory molecules such as TNF α and IL-1 β . The belief in the cytokine hypothesis of heart failure has led to the use anti-inflammatory strategies in the treatment of this syndrome. Unfortunately, to date, such approaches have been largely unsuccessful and in some cases have led to an increase in mortality. This data brings into question the role of elevated circulating pro-inflammatory cytokines in the setting of heart failure and prompts further investigation into the complexity of the inflammatory response and the possible counteracting anti-inflammatory factors associated with this disease.

3.2.2 AIMS

To determine the profile of circulating pro- and anti-inflammatory cytokines, their endogenous inhibitors and the balance between them in patients presenting for cardiac surgery with varying degrees of heart failure focusing on changes during deteriorating functional status.

3.2.3 STUDY DESIGN

Three groups of patients with varying degrees of heart failure and ventricular dysfunction were included in this study:

Patients with coronary artery disease undergoing routine coronary artery bypass grafting surgery (CABG) all of whom were considered to have good ventricular function and few symptoms of heart failure (CABG patients).

Patients with advanced but stable heart failure undergoing cardiac transplantation (Tx). All of these patients had moderate to severe heart failure with poor ventricular function but were clinically stable on the transplant waiting list (Tx patients).

Patients with severe decompensated, acutely deteriorating heart failure requiring emergency implantation of left ventricular assist device (LVAD) for survival (LVAD patients).

Individual cytokines concentrations and the molar ratios of endogenous inhibitors to pro-inflammatory cytokines were determined in the plasma of these patients immediately prior to surgery.

3.2.4 METHODS

3.2.4.1 Patients

3.2.4.1.1 CABG patients

This study included 26 patients undergoing routine CABG.

- Three were female and 23 were male
- Mean age 62.9 ± 1.8 years
- Ten patients were in NYHA class I, 12 in NYHA class II and four were in NYHA class III
- Patients with ejection fraction of $< 30\%$ were excluded from this patient population

3.2.4.1.2 Tx patients

The study included 15 consecutive patients undergoing cardiac transplantation. These patients did not meet the criteria for LVAD implantation.

- Thirteen patients were male and 2 were female
- Mean age 45.3 ± 3.7 years
- Diagnosis included:
 - Dilated cardiomyopathy (8)
 - Ischaemic heart disease (6)
 - Post partum cardiomyopathy (1)

- Thirteen patients were in NYHA Class III and two in NYHA class IV
- These patients required minimal support from inotropes (n=3) or intra-aortic balloon pump (IABP, n=1)

3.2.4.1.3 LVAD patients

The study included 14 consecutive patients undergoing LVAD implantation because of deteriorating clinical status with evidence of secondary organ dysfunction in the context of low cardiac output and increased filling pressure despite appropriate medical treatment.

- All 14 patients were male
- Mean age 38.0 ± 4.3 years
- Diagnosis included:
 - Dilated cardiomyopathy (11)
 - Ischaemic heart disease (1)
 - Ischaemic heart disease with post infarct ventricular septal defect rupture (2)
- All patients were in NYHA Class IV
- Patients had deteriorated over a period of 2.3 ± 0.4 days
- All patients required inotropic support and 8 were also supported by IABP

	CABG	Tx	LVAD
Age (years, mean $\pm$ s.e.m)	62.9 $\pm$ 1.8	45.3 $\pm$ 3.7	38.0 $\pm$ 4.3
Male/Female (number of patients)	23 / 3	13 / 2	14 / 0
NYHA class I/II/III/IV (number of patient)	10/12/4/0	0/0/13/2	0/0/0/14
Diagnosis (number of patients)			
Dilated cardiomyopathy (DCM)	0	8	11
ischaemic Heart disease (IHD)	26	6	1
IHD with post infarct ventricular septal defect rupture	0	0	2
Post partum cardiomyopathy	0	1	0
Number of patients on Inotropes	0	3	14
Number of patients with IABP	0	1	8

Table 3.1. Study No 1 - Patient details

3.2.4.2 Collection and processing of samples

Central venous blood was collected into EDTA containing tubes at the time of the operation after insertion of the arterial line but before induction of anaesthesia. Samples were placed on ice and transported to the laboratory. Samples were centrifuged at 800 x

g for 10 minutes. Plasma supernatants were removed and stored at -80°C , in small aliquots to avoid freeze thawing, until cytokine analysis.

3.2.4.3 Cytokine measurements

Plasma cytokine concentrations were measured by one of two methods

3.2.4.3.1 ELISA

Unless stated otherwise commercially available cytokine ELISA kits (Quantikine® R&D Systems) were used for the individual measurement of $\text{TNF}\alpha$, IL-1 β , IL-4, IL-6, IL-10, sTNFR1, sTNFR2, sIL-1R2 and IL-1RA. Plasma cytokine concentrations were determined according to the manufacturer's instructions.

Where cytokines levels were below the sensitivity of the assay (Table 3.2) but an OD reading greater than the zero standard was achieved then a calculated concentration was obtained by extrapolating the linear standard curve. These concentrations were always considered as low.

Assay sensitivity levels (determined by the ELISA manufacturer; R&D Systems) are obtained by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

3.2.4.3.2 Multiplex Bead Array Assay using Flow Cytometry.

Where stated some cytokine measurements were made using a commercially available multiplex kit (BD™ CBA from Becton Dickinson) allowing simultaneous analysis of multiple cytokines from one small sample. Cytokine concentrations were determined according to the manufacturer's instructions using Becton Dickinson FACScan and analysis software.

3.2.4.4 Data analysis

Plasma cytokine concentrations are expressed as pg/ml or ng/ml. Molar ratios of endogenous inhibitors to cytokines were obtained using pMolar values.

Box and Whisker plots represent median and 10th, 25th, 75th and 90th percentiles. Circles below and above the whiskers represent 5th and 95th percentiles.

Non-parametric analysis of data was used to determine statistical significant of any differences. Kruskal-Wallis One Way Analysis of Variance on Ranks followed by

Dunn's post hoc analysis method for multiple comparisons was used for all pairwise comparisons or comparisons to control data of multiple data points. For comparison between two data sets Mann-Whitney Rank Sum Test was used. A p value less than 0.05 was considered to be statistically significant.

3.2.5 RESULTS – STUDY N^o1

Patients in the CABG group were significantly older than those in the LVAD and the Tx groups ($p < 0.001$).

3.2.5.1 Pro-inflammatory cytokines

3.2.5.1.1 Plasma TNF α

Plasma TNF α levels were low and in some cases below the assay sensitivity (< 4.4 pg/ml) in the CABG (median: 1.8 (0.0 – 7.2) pg/ml $n=26$) and the Tx (median: 3.6 (1.8 – 7.8) pg/ml, $n=15$) patients with no significant difference between these two groups. There were significantly elevated levels of TNF α in the plasma of the LVAD patients (median: 8.5 (4.1 – 32.9) pg/ml, $n=14$, $p < 0.001$) compared to both CABG and Tx patients (Figure 3.1a, Table 3.2).

3.2.5.1.2 Plasma IL-1 β

Plasma IL-1 β levels were low and in some cases below the assay sensitivity (< 1.0 pg/ml) with no significant difference detected between the three groups of patients (CABG: 0.0 - 8.1 pg/ml, Tx: 0.0 - 1.2 pg/ml, LVAD: 0.0 and 6.8 pg/ml, $p=0.870$, Figure 3.1b, Table 3.2).

3.2.5.2 Anti-inflammatory cytokines

3.2.5.2.1 Plasma IL-4

Plasma IL-4 levels were below the level of detection in all patients (< 10 pg/ml, data not shown).

3.2.5.2.2 Plasma IL-6

Plasma IL-6 levels in the LVAD patients ranged between 6.3 and 129.8 pg/ml (median: 91.0, $n=14$). This was significantly higher than IL-6 levels found in the plasma of the

Tx patients (median: 3.5 (0.7 – 15.8) pg/ml, n=13, p<0.05) and in the CABG patients (1.3 – 9.2 (median: 1.8) pg/ml, n=8, p<0.05) (Figure 3.2a, Table 3.2). IL-6 levels in the plasma samples of the CABG patients were measured using a BD CBA kit.

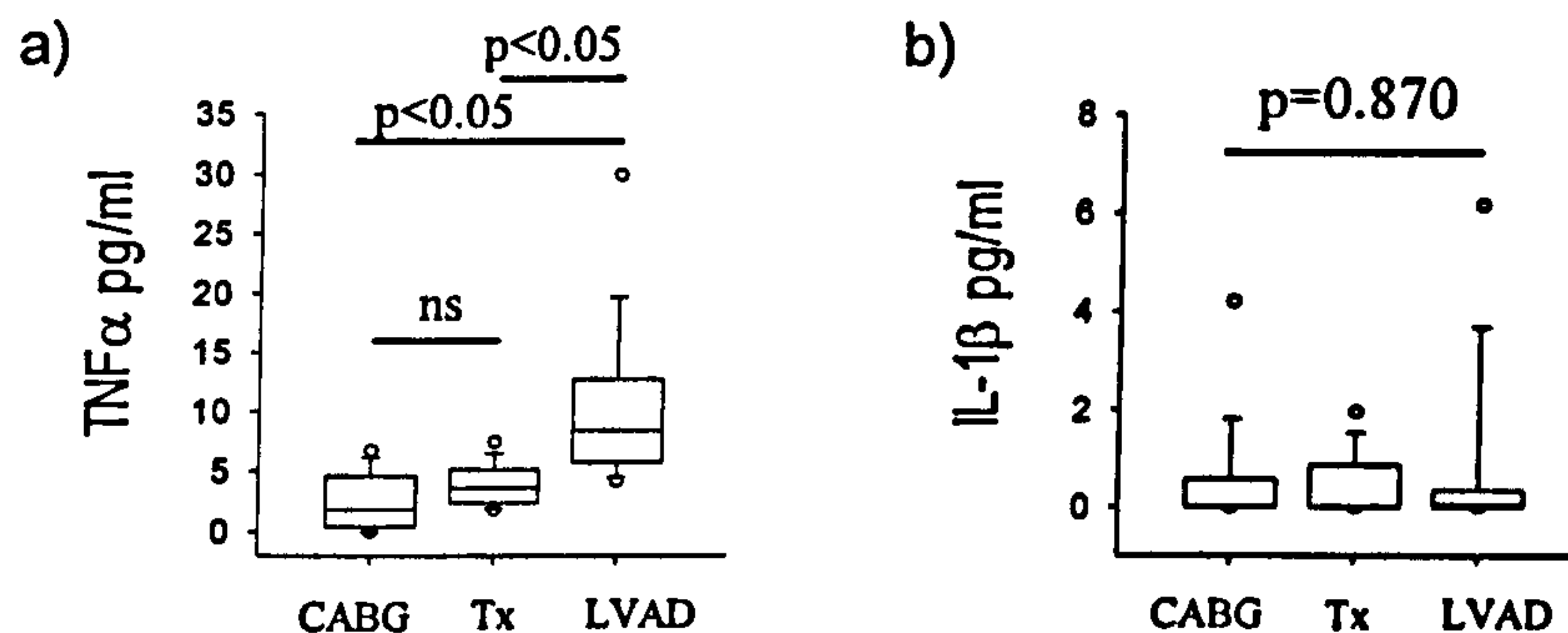


Figure 3.1 Pro-inflammatory cytokine concentrations in plasma of patients presenting for cardiac surgery. Patients undergoing routine CABG (n=26), compared to patients with stable advanced heart failure undergoing heart transplant (Tx, n=15) and deteriorating heart failure patients undergoing left ventricular assist device implantation (LVAD, n=14). a) TNFα. b) IL-1β.

3.2.5.2.3 Plasma IL-10

Circulating IL-10 levels were significantly elevated in the LVAD patients (0.0 – 85.9 (median: 12.1) pg/ml, n=12) compared to the Tx patients (0.0 – 30.4 (median: 1.7) pg/ml, n=12, p<0.05). There was no significant difference between IL-10 levels in the Tx and the CABG patients (0.0 – 7.2 (median: 5.4) pg/ml, n=22, p>0.05, (Figure 3.2b, Table 3.2). IL-10 levels in the plasma samples of the CABG patients were measured using a BD CBA kit.

3.2.5.3 Soluble receptors

3.2.5.3.1 Plasma sTNFR1

Circulating sTNFR1 levels ranged between 316.7 and 1663.4 (median: 904.0) pg/ml (n=26) in the CABG patients. In patients with advanced heart failure (Tx and LVAD) plasma sTNFR1 levels were significantly increased (p<0.05) above that observed in the CABG patients (LVAD: 627.7 - 9623.2 pg/ml (median: 3059.2, n=14) and Tx: 518.0 - 6794.0 pg/ml (median: 2833.0, n=15). No difference was observed between the Tx and the LVAD patients (Figure 3.3a, Table 3.2).

3.2.5.3.2 Plasma sTNFR2

Plasma sTNFR2 concentrations were significantly elevated in the Tx (median: 9087.0 (2203.0 – 17409.0) pg/ml, $n=15$, $p<0.05$) and LVAD (median: 5428.8 (1577.5 and 25190.4) pg/ml, $n=14$, $p<0.05$) patients compared to the CABG patients (median: 2100.0 (967.0 – 3153.2) pg/ml $n=26$). There was no difference in the circulating levels of sTNFR2 between the Tx and the LVAD patients ($p>0.05$, Figure 3.3b, Table 3.2).

3.2.5.3.3 Plasma total sTNFR

The concentration of total sTNFR (sTNFR1 + sTNFR2) in the circulation of the CABG patients was significantly less than those found in patients with end stage heart failure (Figure 3.3c, Table 3.2)

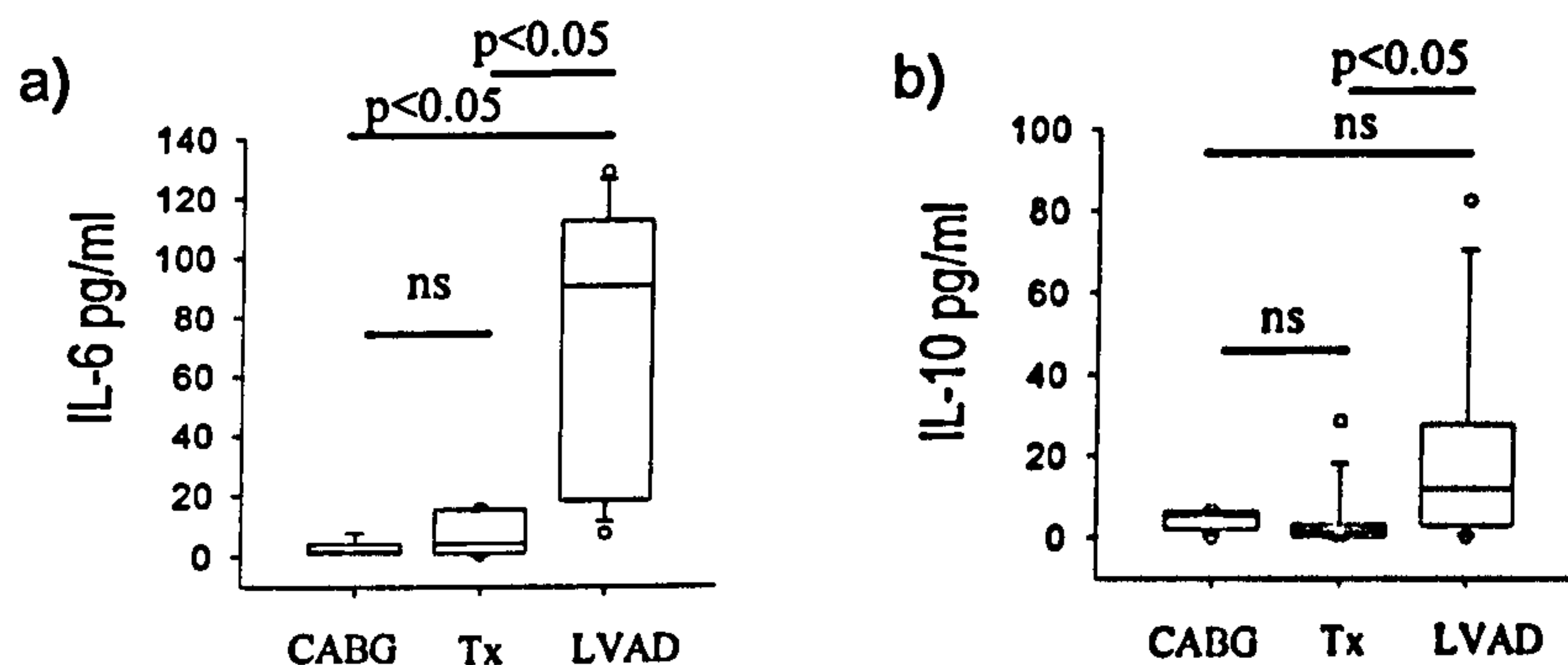


Figure 3.2. Anti-inflammatory cytokine concentrations in plasma of patients presenting for cardiac surgery. Comparison between patients undergoing routine CABG and patients with severe end stage heart failure requiring heart transplantation (Tx) or LVAD implantation. a) IL-6. b) IL-10.

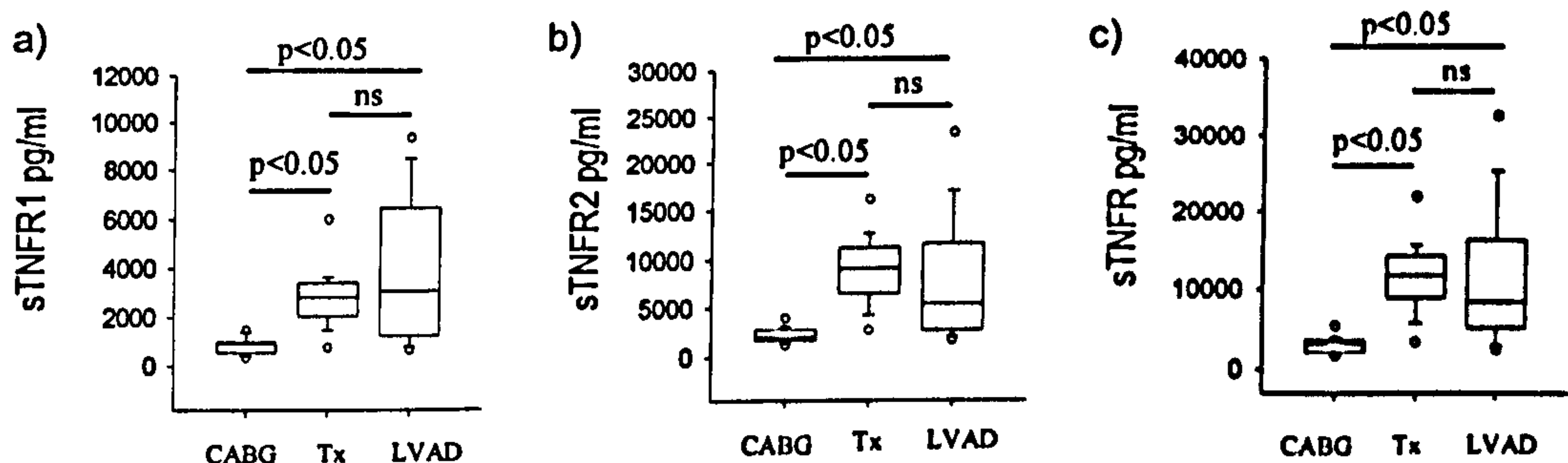


Figure 3.3. sTNFR concentrations in plasma of patients presenting for cardiac surgery. Comparison between patients with severe end stage heart failure requiring cardiac transplantation (Tx) or LVAD implantation and patients with CAD undergoing routine CABG. a) sTNFR1. b) sTNFR2. c) Total sTNF Receptors.

3.2.5.4 Receptor antagonists

3.2.5.4.1 Plasma IL-1RA

In both the LVAD and Tx heart failure patients plasma IL-1RA levels were variable ranging between 0.16 – 56.09 (median: 0.91) ng/ml (n=14) and 0.22 – 11.16 (median: 0.39) ng/ml (n=12), respectively with no significant difference between the two groups. In patients undergoing routine CABG IL-1RA plasma levels were less variable ranging from 0.64 – 2.4 (median: 0.89) ng/ml (n=26) and were significantly higher than those observed in Tx patients ($p<0.05$).

In the Tx and LVAD patients, increased plasma concentrations of IL-1RA significantly correlated with circulating concentrations of IL-10 ($r=0.746$, $p=0.004$ and $r=0.840$, $p<0.001$, respectively) and in the LVAD patients also with IL-6 ($r=0.538$, $p=0.045$).

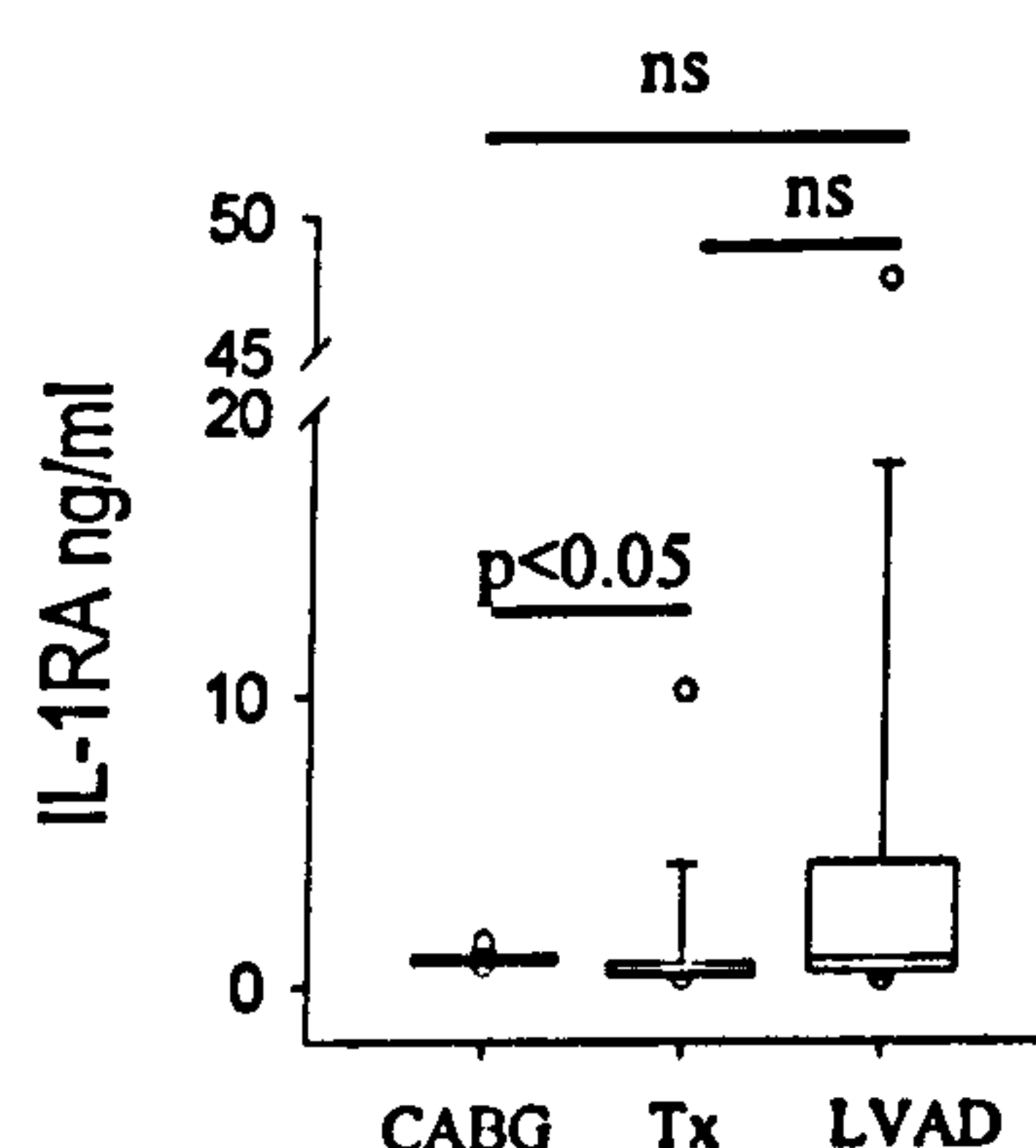


Figure 3.4. IL-1RA concentrations in plasma of CABG, Tx and LVAD patients immediately prior to surgery.

3.2.5.5 Balance between pro and anti-inflammatory factors

3.2.5.5.1 Molar ratio of soluble TNF α receptors to TNF α (sTNFR/TNF α)

Where TNF α levels were undetectable in a plasma sample a concentration of 0.03pM (~0.5 pg/ml) was assigned for the purpose of obtaining a molar ratio of sTNFR to TNF α . Where this was necessary it is likely an overestimation of the TNF α concentration.

3.2.5.5.1.1 sTNFR1/TNF α

The molar ratio of plasma sTNFR1 to TNF α (sTNFR1/TNF α) did not significantly differ between the CABG (median: 292.3 (26.9 – 1564.0), n=26), the Tx (median: 419.5

(90.7 – 1047.1), n=15) or the LVAD (median: 169.7 (21.5 – 527.6), n=14) patients (p=0.068, Figure 3.5a).

Cytokine (Assay sensitivity)	CABG Median (range) pg/ml	Tx Median (range) pg/ml	LVAD Median (range) pg/ml
TNF α (4.4pg/ml)	1.8 (0.0 – 7.2) 73% < 4.4	3.6 (1.8 – 7.8) 67% < 4.4	8.5 (4.1 – 32.9) 7% < 4.4
IL-1 β (1pg/ml)	0.0 (0.0 – 8.1) 81% < 1.0	0.0 (0.0 – 2.0) 73% < 1.0	0.0 (0 – 6.8) 79% < 1.0
IL-6 (0.7pg/ml)	1.8 (1.3 – 9.3)*	3.5 (0.7 – 15.6)	91.0 (6.3 – 129.8)
IL-10 (3.9pg/ml)	5.4 (0.0 – 7.5)*	1.7 (0.0 – 30.4) 75% < 3.9	12.1 (0.0 – 85.9) 29% < 3.9
sTNFR1 (0.77pg/ml)	904.0 (316.7 – 1663.4)	2833.0 (518.0 – 6794.0)	3059.2 (627.7–9623.2)
sTNFR2 (0.6pg/ml)	2100.0 (967.0 – 3153.2)	9087.0 (2203.0 – 17409.0)	5428.8 (1577.5 – 25190.4)
IL-1RA (22pg/ml)	855.7 (640.0 – 2400.0)	386.5 (215.7 – 11156.7)	912.3 (159.0 – 56090.0)

Table 3.2 Summary of the cytokine concentrations in the plasma of CABG, Tx and LVAD patients immediately prior to surgery. Median values with ranges in brackets. * samples analysed using BD CBA.

3.2.5.5.1.2 sTNFR2/TNF α

The molar ratio of plasma sTNFR2 to TNF α (sTNFR2/TNF α) was significantly lower in the LVAD patients (median: 306.6 (100.9 – 789.2), n=14) compared to the Tx (median: 1055.8 (289.3 – 2305.9), n=15, p<0.05) and the CABG (median: 482.1 (86.3 – 3216.3), n=26, p<0.05) patients (Figure 3.5b).

3.2.5.5.1.3 Total sTNFR/TNF α

The molar ratio of total plasma sTNFR to TNF α (sTNFR/TNF α) was significantly lower in the LVAD patients (median: 450.8 (161.0 – 1316.8), n=14) compared to that observed in Tx (median: 1471.8 (380.1 – 3353.0), n=15, p<0.05) and CABG (median: 742.6 (118.1 – 4780.3), n=26, p<0.05, Figure 3.5c) patients.

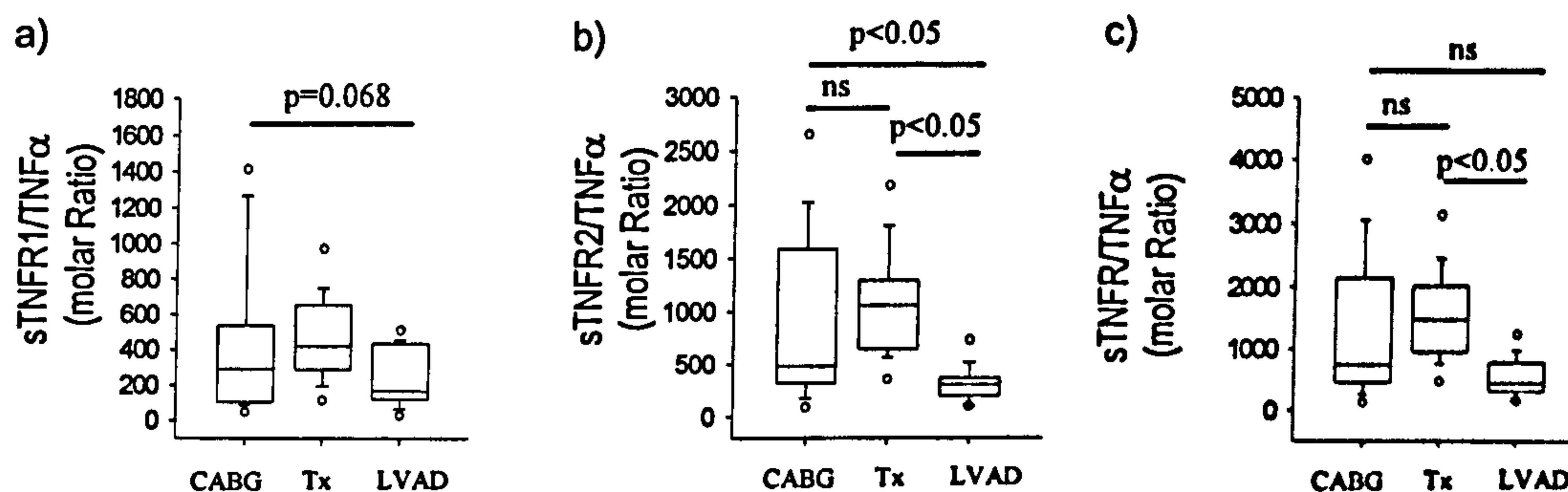


Figure 3.5. Molar ratios of sTNF receptors to TNF α in plasma of CABG, Tx and LVAD patients before surgery. a) sTNFR1 to TNF α . b) sTNFR 2 to TNF α . c) Total sTNFR to TNF α .

3.2.5.5.2 Ratio of IL-1RA to IL-1 β

Where plasma IL-1 β levels were below the level of detection the concentration was given a value of 0.01pM ($\sim$ 0.2pg/ml) for the purpose of determining the molar ratio of plasma IL-1RA to IL-1 β . Where this was necessary it is likely that this is an overestimation of the IL-1 β concentration.

The molar ratio of IL-1RA to IL-1 β was between 98.5 and 14117.7 (median: 4470.6) in the CABG patients. These values were not significantly different to those observed in the Tx (median: 1719.6 (143.7 – 4239.2) or in the LVAD patients (3234.3 (47.7 – 81705.8)). The molar ratio values of IL-1RA to IL-1 β obtained for the LVAD patients was highly variable compared to those for the other two groups of patients and were significantly higher than those observed in the Tx group (p<0.05, Figure 3.6).

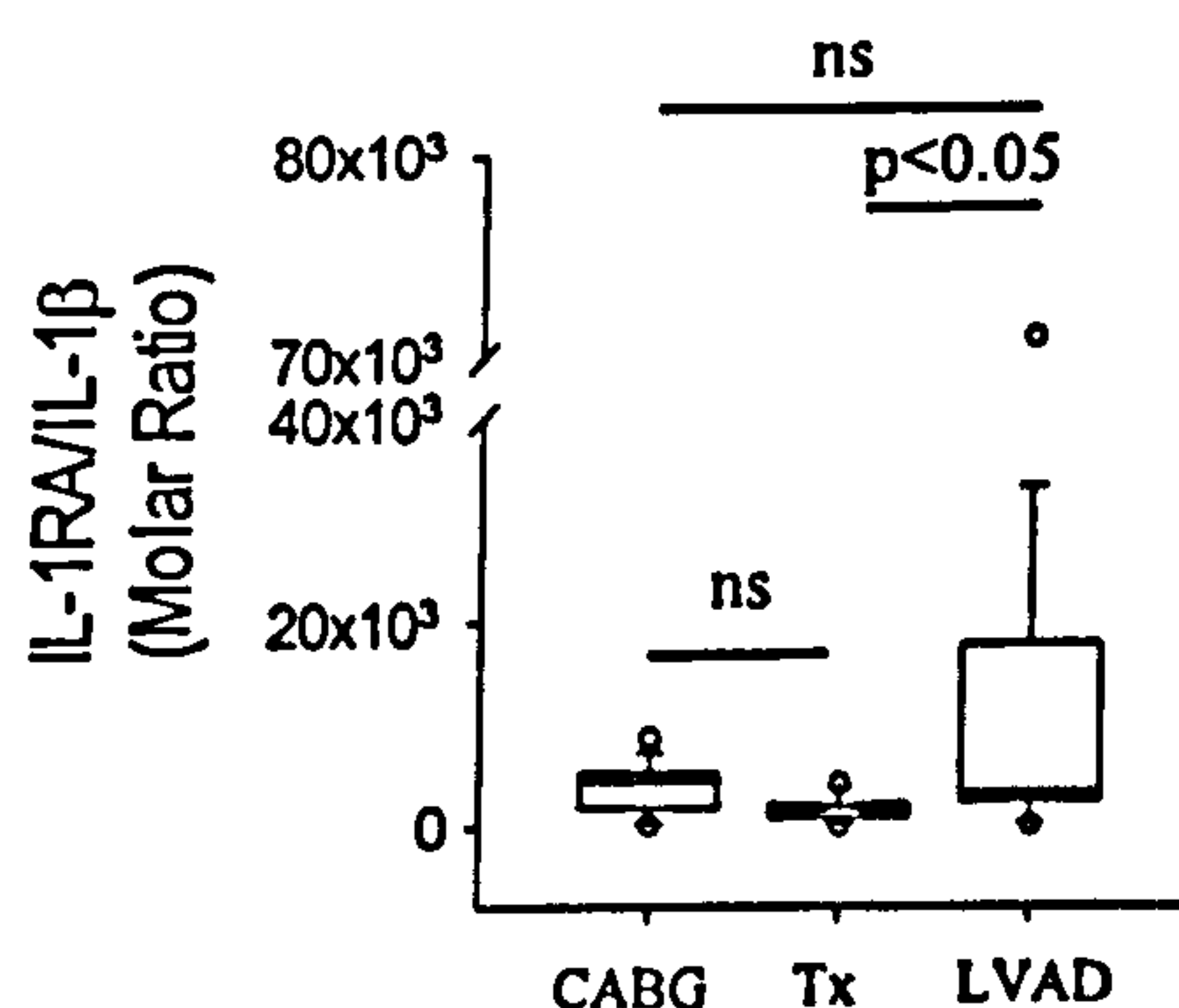


Figure 3.6. Molar ratio of IL-1RA to IL-1 β in plasma of CABG, Tx and LVAD patients immediately prior to surgery.

3.2.6 DISCUSSION

This data shows that there is a significant shift in the circulating cytokine profile of patients during the progression of heart failure.

3.2.6.1 Cytokine profile in CABG patients

In patients with coronary artery disease undergoing routine CABG pro-inflammatory cytokines IL-1 β and TNF α were undetectable in the circulation. The concentrations of the other factors measured (IL-6, IL-10, sTNFR and IL-1RA) were within the 'normal' range (Normal range determined by the manufacturers of the ELISA kits used in this study (R & D Systems) quantifying plasma cytokine levels in at least 40 normal, healthy subjects using the Quantikine® ELISA).

All the patients within the CABG study group had left ventricular ejection fraction of greater than 30% and clinically showed few signs of heart failure. The absence of abnormal levels of circulating cytokines suggests that there is no on-going pro-inflammatory state in the circulation of these patients.

3.2.6.2 Cytokine profile in transplant (Tx) patients

In patients with end stage heart failure undergoing cardiac transplantation plasma levels of the pro-inflammatory cytokines, IL-1 β and TNF α were low and in some cases undetectable. Similar to the situation in the CABG patients circulating levels of the anti-inflammatory cytokines IL-6 and IL-10 were within the 'normal' range. Interestingly plasma levels of soluble TNF α receptors, both type 1 and type 2 were significantly elevated in these patients compared to the CABG patients although the sTNFR to TNF α ratios remained similar. Circulating IL-1RA levels however were significantly reduced in the transplant patients compared to those observed in the CABG patients.

All of the patients in the transplant group had advanced, but stable end stage heart failure (NYHA classification III and IV). The circulating cytokine profile of these patients suggests that there are no obvious signs of an on-going systemic pro-inflammatory cytokine response despite poor LV function and advanced signs of heart failure. Indeed the predominant alteration observed in the circulation of these patients compared to the CABG patients was increased sTNFR1 and sTNFR2.

3.2.6.3 Cytokine profile in LVAD patients

Consistent with the very earliest observations of TNF α in heart failure (Levine et al., 1990b) there were significantly greater concentrations of TNF α in the circulation of deteriorating heart failure patients than in the circulation of stable end stage heart failure patients or CABG patients undergoing CABG. In addition there were also increased circulating levels of IL-6 and IL-10 in the LVAD patients compared to the other groups of stable patients.

Despite the increased levels of TNF α in the LVAD patients there was no additional increase in sTNFR compared to the Tx patients thus resulting in a reduced ratio of sTNFR to TNF α indicating a shift towards increased TNF α activity in the deteriorating heart failure patients.

There was no difference in plasma IL-1RA levels in the LVAD patients compared to the CABG or the Tx patients. However, the molar ratio of IL-1RA/IL-1 β was significantly greater in the LVAD patients suggesting a shift towards increased anti-IL-1 β activity.

All of the patients in the LVAD group of this study were in NYHA class IV and had advanced decompensated heart failure. All patients required LVAD implantation because of deteriorating clinical status with evidence of secondary organ dysfunction in the context of low cardiac output despite appropriate and maximal medical treatment (including inotropes and intra-aortic balloon pump). The circulating cytokine profile of these patients suggests that there is increased pro-inflammatory situation with respect to TNF α . However, there were increased concentrations of IL-10 and IL-6 and an increased ratio of IL-1RA/IL-1 β suggesting that there is a possible counteracting anti-inflammatory activity in the circulation of these patients.

3.2.6.4 General discussion

3.2.6.4.1 TNF α and soluble TNF α receptors

Increased TNF α has consistently been demonstrated in the circulation of patients with heart failure and numerous experimental studies suggest that this pro-inflammatory cytokine plays a pivotal role in the structural and functional alterations to the heart that are observed in this syndrome (section 1.2.3.1.1 and 1.2.3.1.2). The altered pattern of sTNFR and TNF α in the patients in this current study is intriguing. There appeared to be no increase in circulating TNF α levels in the transplant patients compared to the

CABG patients. In light of the conventional view that circulating TNF α levels increase with progression of heart failure and increasing NYHA functional class (Torre-Amione et al., 1996b) the lack of TNF α increase in this current study is interesting although not unique (Rumalla et al., 2002). Indeed some published studies have failed to detect differences in plasma TNF α levels between healthy volunteers and patients with heart failure (Loppnow et al., 2002). There are several possible reasons why no differences were detected between the CABG and the Tx patients in this present study. The TNF α levels measured in the plasma of the patients were at the lower range of the assay kit used and in some cases below the level of accurate detection (sensitivity 4.4pg/ml). Given the levels of TNF α previously reported (about 1-8pg/ml) and the differences observed between NYHA functional class (about 2pg/ml), a larger study population and a more sensitive assay kit would be required to accurately detect such differences. In addition, the patients in this study were grouped according to the surgical procedure that they were about to undergo. All the patients in the LVAD group were in NYHA IV, patients in the transplant group were in either III or IV and those in the CABG group were in either I, II or III. Because NYHA functional class was not the primary factor that determined the patients' groupings this study was not sufficiently powered to identify the small changes in circulating cytokine levels between patients in different NYHA functional class. However, there was a significant increase in plasma TNF α in the LVAD patients indicating that progression of heart failure to a severely decompensated functional status is associated with an apparent systemic inflammatory response.

A previous study demonstrated that the myocardium may be the source of increased TNF α in patients with severe decompensated heart failure with both mRNA and protein levels increased compared to stable end stage heart failure patients (Birks et al., 2001). Interestingly, the concentration of TNF α in the systemic circulation of those patients were higher than the local concentration in the myocardium and the differences between the two patient groups were greater when the serum concentrations were considered suggesting that the systemic inflammatory response may be greater than the local one. In addition that data also suggests that sources other than the myocardium may contribute to the increase in circulating TNF α observed in the setting of heart failure. Studies by Anker and colleagues have demonstrated that endotoxin concentrations are increased in the circulation of patients with severe heart failure and have proposed a hypothesis whereby increased mesenteric venous congestion results in increased bowel

permeability and subsequent translocation of bacterial endotoxin into the systemic circulation. Through the activation of Toll-like receptors on cells within the vasculature, circulating endotoxin may be responsible for the increased concentration of circulating inflammatory factors observed in patients with severe heart failure (Anker et al., 1997; Niebauer et al., 1999).

In transplant patients there was an increase in sTNFR above that observed in the CABG patients. However sTNFR levels did not further increase in the decompensated LVAD patients despite the increase in TNF α levels. This resulted in a reduced molar ratio of sTNFR/TNF α in the LVAD compared to the Tx patients supporting the proposal that there is a shift from anti- to pro-inflammatory, with respect to TNF α during the progression from stable to decompensated end stage heart failure. However, despite the reduced ratio of sTNFR /TNF α in these LVAD patients there was still a significant molar excess (sTNFR1/TNF α 21-527 fold and sTNFR2/TNF α 100-789 fold) of soluble receptor over TNF α suggesting that the TNF α bioactivity may be sufficiently buffered (Engelberts et al., 1991; Gerain et al., 1997; Van Zee et al., 1992).

Soluble TNF α receptors represent the extracellular domain of the type 1 and type 2 TNF α receptors (sTNFR1 and sTNFR2 respectively) that are cleaved from the cell. These soluble receptors retain their affinity for TNF α but lack the ability to induce cellular signalling events thus acting as inhibitory decoy receptors. Increased shedding of the sTNFR is generally considered to be a compensatory mechanism in response to increased TNF α that serves to desensitise the tissue to TNF α by reducing the number of cellular receptors and by reducing the bioavailability of TNF α .

In the LVAD patients increased TNF α levels seemed to correlate with increased sTNFR2 but not sTNFR1 suggesting that at least in the case of one of the receptors increased TNF α may be responsible for its cleavage from the cell. However there was no evidence of increased TNF α in the transplant patients in which circulating sTNFR were also increased. One potential possibility for this observation is that the levels of plasma TNF α under these situations was underestimated because TNF α bound to sTNFR was not recognised by the assay method used. This concept has been previously tested by Kapadia et al who demonstrated that levels of immunoreactive TNF α measured using the ELISA kits used in this present study (Quantikine from R&D Systems), in contrast to some other kits, were not masked by either type of soluble

receptor (Kapadia et al., 1994). Thus, mechanisms other than increased TNF α are may be responsible for the observed increase in TNF α receptor shedding.

The shedding of sTNFR receptors can be induced by a number of mechanisms other than TNF α binding to the type 1 cellular receptor. IL-10 and IL-6 can both increase TNFR shedding leading to increase circulating levels and decreased cellular receptors (Joyce et al., 1994; Tilg et al., 1994). Indeed the mechanism by which IL-10 and IL-6 induce an anti-inflammatory effect is in part due to their ability to increase shedding of TNFR and thus reduce the bioactivity of TNF α . In the LVAD patients the plasma concentration of both of these cytokines significantly correlated with increased plasma levels of sTNFR1 suggesting that in these patients IL-10 and/or IL-6 may contribute to the increase in circulating sTNFR1. However this does not explain the increase sTNFR observed in the Tx group of patients since no detectable increased in circulating IL-10 or IL-6 were observed in these patients. It is entirely possible that low levels of cytokines in these patients are capable of inducing a biological effect *in vivo*, such as TNF α receptor shedding at concentrations that are not detectable by the assay system used in this present study.

Given that virtually all nucleated cells express TNF α receptors the source of the soluble forms in the setting of heart failure and the mechanism of release are at present unknown. TNF α receptor protein expression has been shown to be reduced in the failing myocardium and on circulating PMN and monocyte of patients with moderate to severe heart failure (Rumalla et al., 2002) suggesting that these are possible sources of the increased sTNFR observed in the circulation of the heart failure patients in the current study.

The significance of increased sTNFR plasma levels in patients with severe heart failure is unclear. They may provide an increased circulating buffer against increased levels of TNF α , serving to reduce tissue expression and to act as soluble decoy receptors. They may simply reflect an ongoing inflammatory state in the tissue and circulation or, given that increased circulating sTNFR1 levels appear to be the single best predictor of mortality in patients with heart failure (Rauchhaus et al., 2000), they may have an adverse effect in the setting of heart failure. Indeed, in addition to the inhibitory action of sTNFR1 and 2 on TNF α bioactivity, sTNFR2 can bind to trimeric TNF α and increase its stability by preventing the monomerisation and subsequent inactivation. In this manner sTNFR can act as a slow release reservoir of TNF α prolonging it's half life

(in the circulation $\text{TNF}\alpha$ $t_{1/2} \sim 6$ minutes) and biological function (Aderka et al., 1992). In this manner sTNFR2 may actually be considered to be pro-inflammatory.

3.2.6.4.2 IL-1 β and IL-1RA

IL-1 β was undetectable in most of the plasma samples used in this study suggesting that there is little systemic contribution from this cytokine in the progression of heart failure. Previous studies have demonstrated increased myocardial IL-1 β mRNA in patients with severe decompensated heart failure compared to stable end stage heart failure patients. Whether this increase is significant with regard to the production of biologically active protein is unclear but this may represent local production of IL-1 β that is not reflected in the systemic circulation (Birks et al., 2001).

IL-1RA is a naturally occurring specific inhibitor of IL-1 β that has been shown to inhibit all described IL-1 β activities due to competitive binding to the IL-1 type 1 receptor. Binding of IL-1RA to the IL-1 receptor does not trigger the formation of a tri-molecular complex of ligand/IL-1R1/IL-1R AcP and thus blocks signal transduction (Schreuder et al., 1997). IL-1 β , in contrast to IL-1RA interacted with two additional sites on the receptor thought to be responsible for induction of biologic responses in target cells (Ju et al., 1991).

The IL-1RA plasma levels in the patients with heart failure (both Tx and LVAD) were considerably variable with some patients expressing plasma IL-1RA in the 2-50 ng/ml range. Normal plasma levels of IL-1RA are generally in the range of 50-1000 pg/ml suggesting that in some patients there is significantly increased production but this does not appear to be a general feature of increased severity and progression of heart failure. The strong correlation between IL-1RA and both IL-10 and IL-6 levels suggest that the changes in IL-1RA levels may be a secondary to increased levels of these cytokines in some patients rather than as a compensatory response to counteract increased IL-1 β levels which were not apparent in these study patients.

IL-1RA production from a number of different cell types can be modulated by a variety of pro- and anti-inflammatory factors (IL-1 β , $\text{TNF}\alpha$, LPS, IL-4, IL-6) that act both alone and in a synergistic manner (Ascione et al., 2003; Ascione and Angelini, 2003; Cassatella et al., 1994; Marie et al., 1996; Ohmori et al., 1996; Smith, Jr. et al., 1994; Tilg et al., 1994). Thus, the precise nature of the inducing stimuli or the source of IL-1RA in some of these patients is unknown.

3.2.6.4.3 IL-6

In agreement with other studies IL-6 levels were elevated in the plasma of the decompensated heart failure patients compared to patients with less severe heart failure (transplant and CABG patients). Evidence suggests that the role or consequences of increased IL-6 in the plasma of heart failure patients is a detrimental one. In clinical studies IL-6 in the circulation and in the myocardium increases in direct relation to decreasing functional status and are highly predictive of increased mortality. Furthermore, increased circulating IL-6 levels compared with other inflammatory mediators are the strongest and most consistent risk factor for cardiovascular events (Koukkunen et al., 2001; Spinale, 2002; Vasan et al., 2003b). In further support of this notion, overexpressing both IL-6 and IL-6R leads significant LV hypertrophy with increased LV wall thickness and cardiomyocyte size (Hirota et al., 1995). This has also been demonstrated in isolated cardiomyocytes in which continuous and extensive activation of gp130 and STAT3 cause a dramatic increase in cell size (Hirota et al., 1995) and protein synthesis (Kunisada et al., 1998). Furthermore, Gab1-SHP2 interaction following gp130 activation plays a critical role in the activation of ERK5 in cardiomyocytes inducing the formation of eccentric hypertrophy characterized not only by increased cell length and surface area but also by regulation of embryonic gene expression (Nakaoka et al., 2003). This data implicates IL-6 in promoting the changes to the structure of the heart during the progression of heart failure. In addition, IL-6 can also induce angiotensinogen synthesis in the liver through Jak/STAT3 dependant mechanisms (Sherman and Brasier, 2001) and increase angiotensin (Ang) type 1 receptor expression in vascular tissue leading to an increase in Ang II-mediated effects (vasoconstriction, oxidative stress and the development of endothelial dysfunction, Wassmann et al., 2004).

IL-6 synthesis and release can be stimulated from cardiac myocytes, fibroblasts and smooth muscle cells by Ang II and adrenergic stimulation *in vitro* (Han et al., 1999; Jeron et al., 2003; Murray et al., 2000; Sano et al., 2000). Indeed the hypertrophic effects of angiotensin II has been shown to be mediated by IL-6 family of cytokines through gp130 stimulation (Sano et al., 2000). Consistent with this finding, high-dose ACE inhibitor therapy (enalapril) in patients with severe heart failure has been associated with a significant decrease in IL-6 plasma concentrations and activity and reduced LV wall thickness. However a persistent immune activation (elevated TNF α , sTNF receptors, IL-1 β , IL-1RA, soluble adhesion molecules) remained in these patients

advocating a central role for IL-6 and not TNF α and IL-1 β in the structural changes to the ventricles that occurs in heart failure (Gullestad et al., 1999).

There is also a growing body of evidence that suggests that IL-6 signalling via the gp130 receptor and STAT3 activation provides a critical myocyte survival pathway *in vivo*. Mice with a cardiomyocyte-restricted deletion of gp130 have apparently normal hearts but fail to develop compensatory hypertrophy to pressure overload. These animals rapidly progress to cardiac dilation and failure caused by massive cardiac myocyte apoptosis compared to control animals that demonstrate compensatory hypertrophy (Hirota et al., 1999a). Furthermore cardiac-restricted deletion of STAT3 leads to increased susceptibility to doxorubicin-induced heart failure with increased apoptosis. These animals also spontaneously develop cardiac dysfunction with age (Jacoby et al., 2003). This data not only suggests a pro-survival role of IL-6/gp130/STAT3 pathway in preventing myocyte hypertrophy but also indicates the critical role of myocyte apoptosis in the transition from compensatory hypertrophy to heart failure.

3.2.6.4.4 IL-10

IL-10 plasma levels have been variably observed in heart failure patients and its relationship to disease progression is unclear. Stumpf et al demonstrated that IL-10 expression was significantly reduced in patients with severe heart failure contributing to the pro-inflammatory status of these patients and suggested that patients with a high TNF α /IL-10 ratio could benefit from recombinant IL-10 therapy (Stumpf et al., 2003). In contrast Yamaoka et al reported increased plasma levels of IL-10 in patients with severe heart failure, a response believed to be aimed at counteracting the increased TNF α in patients with advanced heart failure (Yamaoka et al., 1999). Other studies have demonstrated that circulating IL-10 levels were not different between control patients and patients with heart failure irrespective of disease severity (Bolger et al., 2002a; Gullestad et al., 2001b). The data presented in this thesis demonstrates, like the study of Yamaoka, that IL-10 levels were significantly elevated in the deteriorating LVAD patients compared to either the Tx or the CABG patients again implying that there is a significant anti-inflammatory component in the circulation of these severely decompensated patients. In the setting of heart failure the increased plasma levels of IL-10 have the potential to exert a significant anti-inflammatory effect by inhibiting monocyte production of cytokines such as IL-1 β and TNF α , which is critical to the role

of IL-10 as an anti-inflammatory cytokine. Indeed Bolger et al demonstrated that IL-10 can inhibit LPS-induced TNF α production from PBMCs isolated from patients with HF (Bolger et al., 2002b). IL-10 also inhibits the production of some CC and CXC chemokines (Moore et al., 2001b). Combined with the ability to inhibit the induction of monocyte CD18 and CD62-L expression (Mtairag et al., 2001) and increase IL-1RA production and TNFR cleavage, IL-10 can significantly limit an inflammatory reaction. Also of importance in the setting of heart failure and LV remodelling is the ability of IL-10 to modulate the production of MMP2 and MMP9 and enhance TIMP production (Mtairag et al., 2001), the balance of which appears to be tipped in favour of increased protease activity and extracellular matrix degradation in the patients with heart failure (Spinale et al., 2000, 2002b; Wilson et al., 2002).

Further support for the potential beneficial effect of IL-10 in the setting of heart failure comes from trials in which the intravenous administration of immunoglobulin to patients with chronic HF increased plasma levels of IL-10 that was accompanied by improvements in left ventricular ejection fraction (Gullestad et al., 2001a). However IL-10 expression, in the patients in this current study, only appears to be increased in the severely decompensated patients in which dysregulated inflammatory/immune activation is most pronounced. Under these circumstances this response is likely to be an attempted compensatory one that has, unfortunately occurred too late in the progression of the disease to be of significant benefit.

3.2.6.4.5 Influence of factors unrelated to heart failure status

Due to the observational nature of this present study and the limited number of patients undergoing LVAD implantation or transplantation due to heart failure it was not possible to perform a match study. It is therefore important to note that the three patient populations are not necessarily balanced for other underlying differences unrelated to LV function and heart failure.

3.2.6.4.5.1 Age

Ageing is associated with a low-grade inflammatory activity with increased circulating levels of IL-1 β , TNF α , IL-6, IL-1RA and sTNFR (Bruunsgaard et al., 2001). In addition secretion of pro-inflammatory cytokines from stimulated monocytes has been shown to be increased in elderly people (Fagiolo et al., 1993; O'Mahony et al., 1998). The CABG patients in this current study were significantly older than both the Tx and LVAD

patients, however the circulating pro-inflammatory cytokine levels were higher in the latter, younger patients. In addition plasma cytokine levels in the CABG patients appeared to fall within the normal range implying that age is an unlikely contributing factor to the observations reported in this thesis.

3.2.6.4.5.2 Medical treatment

β -blockers are a widely used therapy in the treatment of heart failure and have been shown to modulate plasma cytokine levels in heart failure patients (Ohtsuka et al., 2001, 2002). However none of the patients in this present study were receiving β -blocker therapy and thus this class of drugs is not responsible for the changes in plasma cytokine levels observed. The use of inotropic therapy also has the potential to influence the inflammatory profile of these patients. Several studies have shown that these drugs, in particular phosphodiesterase III inhibitors (PDEI) can modulate plasma levels of inflammatory cytokines in heart failure patients (Liu and Zhao, 1999b; Sliwa et al., 2002b). All the LVAD patients in this study were receiving inotropic therapy, of which five were receiving PDEI. Thus, these drugs could potentially explain the altered circulating cytokine profile of these LVAD patients compared to the Tx and CABG patients. However, the small number of patients in this study and the varied inotropic drug regimens of these patients prevent any meaningful conclusions in this respect and this issue will require further investigation.

With the exception of two patients, modulation of the renin–angiotensin system (either ACE inhibitors Enalapril or Captopril, or the Angiotensin II receptor blocker Losartan) formed part of the therapeutic regimen for the transplant patients in this study. Both clinical and experimental studies have demonstrated that these agents can suppress cytokine production and inflammation (Agha and Mansour, 2000; Fukuzawa et al., 1997; Peeters et al., 1998; Schindler et al., 1995). These studies suggest that pharmacological agents aimed at reducing the effects of the RAS could be responsible for the low cytokine levels in the Tx patients in this present study and may therefore be related to their clinically beneficial effects.

3.2.6.4.5.3 Disease etiology: ischaemic versus dilated cardiomyopathy

Increased circulating cytokine levels have been shown to be consistently higher in patients with ischaemic cardiomyopathy than in patients with dilated cardiomyopathy (Deswal et al., 2001). However, disease etiology is unlikely to be influencing the

cytokine results in this present study since all but one patient in the LVAD group, in which IL-6, IL-10 and TNF α were highest, had DCM. In addition, statistical analysis revealed that there was no difference in any cytokine measurements between heart failure patients with ischaemic or dilated etiologies (data not shown).

Despite the evidence presented above it is unlikely that other factors independent of severity and progression of heart failure can totally explain the altered circulating cytokine profile in these patients.

3.2.7 CONCLUSION

In this present study, although circulating TNF α levels were increased in patients with severe decompensated heart failure compared to stable heart failure patients undergoing cardiac transplantation or patients undergoing routine CABG, there was also the presence of a considerable anti-inflammatory component that, in some circumstances, was greater than that observed in patients with less advanced disease. Such anti-inflammatory factors have the potential to prevent or at least reduce the cellular effects of the increased TNF α . This observation brings into question the true role of inflammatory cytokines in the setting of heart failure and questions whether the increased levels observed in the circulation are sufficient to contribute to the systemic manifestations of heart failure and disease progression. If the inflammatory effects of increased circulating pro-inflammatory cytokines are effectively neutralised by counter-acting factors and their presence in the circulation are simply an indirect measure of disease severity then treatment strategies aimed at inhibiting their activity would be futile.

3.3 STUDY N^o2 - EFFECT OF CARDIAC SURGERY ON PLASMA CYTOKINES

3.3.1 BACKGROUND

Cardiac surgery is often the only option for the treatment of many heart diseases. For example, transplantation or insertion of mechanical assist devices for patients with severe heart failure and coronary artery bypass graft surgery for the treatment of coronary artery disease. However such surgical procedures are associated with elevated levels of circulating pro-inflammatory cytokines and the development of postoperative systemic inflammatory response syndrome.

Numerous studies have reported on the inflammatory response to cardiac surgery and have measured circulating levels of pro-inflammatory cytokines such as TNF α , IL-6 and IL-8. However, as with research into the inflammatory mechanisms associated with heart failure the role of counteracting anti-inflammatory factors and the ratios between pro- and anti-inflammatory factors have received less comprehensive attention.

3.3.2 AIMS

To determine the influence of cardiac surgery with CPB on circulating cytokine levels with particular focus on the balance between pro-inflammatory cytokines and their endogenous inhibitors.

To examine the effect of cardiac surgery on cytokine production in whole blood induced by an *in vitro* inflammatory insult (endotoxin).

3.3.3 STUDY DESIGN

Individual cytokine concentrations and the molar ratios of endogenous inhibitors to pro-inflammatory cytokines were determined in the plasma of patients undergoing CABG with CPB before and 6 hours after surgery.

To investigate the influence of cardiac surgery on a controlled, *in vitro* inflammatory insult, whole blood, taken immediately before and 6 hours after surgery was treated with bacterial endotoxin and individual cytokine concentrations and molar ratios of pro-inflammatory cytokines to endogenous inhibitors were determined.

3.3.4 METHODS

3.3.4.1 Patients

9 patients undergoing CABG with CPB

- 7 were male and 2 were female
- Mean age 64.1 ± 1.9 years

3.3.4.2 Anaesthetic and operative techniques

Anaesthetic pre-medication included morphine 10 mg, metoclopramide 10 mg, and temazepam 10 mg administered the morning of the operation. The general anaesthetic technique comprised a low-intermediate dose of opioid (usually fentanyl, 8-15 μ g/kg) and a propofol infusion (3 mg/kg per hour). Additional monitoring by transesophageal echocardiography was undertaken when necessary.

3.3.4.3 Cardiopulmonary bypass

Anticoagulation was achieved with 300 U/kg of heparin. The activated clotting time was maintained above 480 seconds. Heparin effects were reversed with protamine at the end of the procedure. The operation was performed through a median sternotomy, and CPB was instituted with a single right atrial cannula and an ascending aorta perfusion cannula. Standard bypass management included membrane oxygenators and non-pulsatile flow of 2.4 L/min per m² with a mean arterial blood pressure of greater than 50 mmHg. Myocardial protection was achieved with intermittent cold blood cardioplegia (4:1 blood-crystalloid ratio).

3.3.4.4 Collection and processing of samples

Central venous blood was collected from the patients, into heparin containing tubes, in the anaesthetic room after insertion of the arterial line and before induction of anaesthesia (PreCPB). Post CPB samples were collected six hours after the termination of CPB. All samples were placed on ice and transported to the laboratory. The bloods were divided and treated as follows:

- Native plasma – whole blood was centrifuged immediately to obtain plasma which was stored in small aliquots at -80°C.
- LPS Plasma – Whole blood was treated with LPS (1 ng/ml) for 6 hours at 37°C before centrifugation to separate plasma. As a control, blood was incubated at 37°C for 6 hours in the absence of LPS. Plasma was stored at -80°C until cytokine analysis.

3.3.4.5 Cytokine measurements

Commercially available cytokine ELISA kits (Quantikine®) for the individual measurement of TNF α , IL-1 β , sTNFR1, sTNFR2, and IL-1RA in plasma were purchased from R&D Systems. Plasma cytokine concentrations were determined according to the manufacturer's instructions.

3.3.4.6 Data analysis

Analysis of data was carried out as described in section 3.2.4.4

3.3.5 RESULTS - STUDY N°2

3.3.5.1 Pro-inflammatory cytokines

3.3.5.1.1 Plasma TNF α

In this study native pre CPB plasma TNF α levels were above the sensitivity of the assay (4.4pg/ml) in only 2 of the 9 patients (4.5 and 22.4pg/ml). TNF α levels in native plasma increased in one of these patients (from 4.5 to 7.2) and decreased in the other (from 22.4 to 14.8 pg/ml) 6 hours after CPB. Post CPB plasma TNF α concentrations were increased in two patients with previously undetectable levels (15.5 and 20.3 pg/ml). Grouped data showed no significant difference between pre and post operative plasma TNF α concentrations ($p=0.723$, $n=9$, Figure 3.7a and b).

Six hours control incubation of whole pre CPB blood resulted in the generation of detectable levels of TNF α in all but one sample (0.0-132.8 (median: 39.7) pg/ml). Plasma levels of TNF α increased further in all samples following 6 hours *in vitro* stimulation of whole pre CPB blood with 1ng/ml LPS (1492.6 – 9782.9 (median: 5266.8) pg/ml, $p<0.05$, Figure 3.7c).

In contrast to pre CPB samples, 6 hours control incubation of post CPB blood caused TNF α levels to increase in only five samples, four of which had detectable levels in native post CPB plasma. *In vitro* stimulation of post CPB blood with LPS (1ng/ml) resulted in the generation of detectable TNF α levels in all samples (57.1 – 3644.2 (median: 1170.0) pg/ml). LPS stimulated TNF α production was significantly less in post CPB blood compared to blood collected before CPB ($p=0.001$, Figure 3.7c).

3.3.5.1.2 Plasma IL-1 β

IL-1 β levels in pre CPB plasma were above the sensitivity of the assay (1pg/ml) in only a minority of patients ($n=2$, 3.0 and 1.7pg/ml), one of which also had detectable TNF α levels. IL-1 β levels in these two patients appeared to decrease 6 hours after CPB (2.0 pg/ml and not detectable, respectively). Post CPB plasma from four other patients exhibited increased IL-1 β levels from undetectable pre CPB levels to 1.3, 4.1, 24.5 and 3.8 pg/ml. Grouped data showed no significant difference between pre and post operative plasma IL-1 β concentrations ($p=0.215$, $n=9$, Figure 3.8a and b).

Of the patients exhibiting increased plasma levels of IL-1 β following CPB none showed any change in TNF α levels. There appeared to be no correlation between the concentration of IL-1 β and TNF α in the native plasma of these patients before or after surgery.

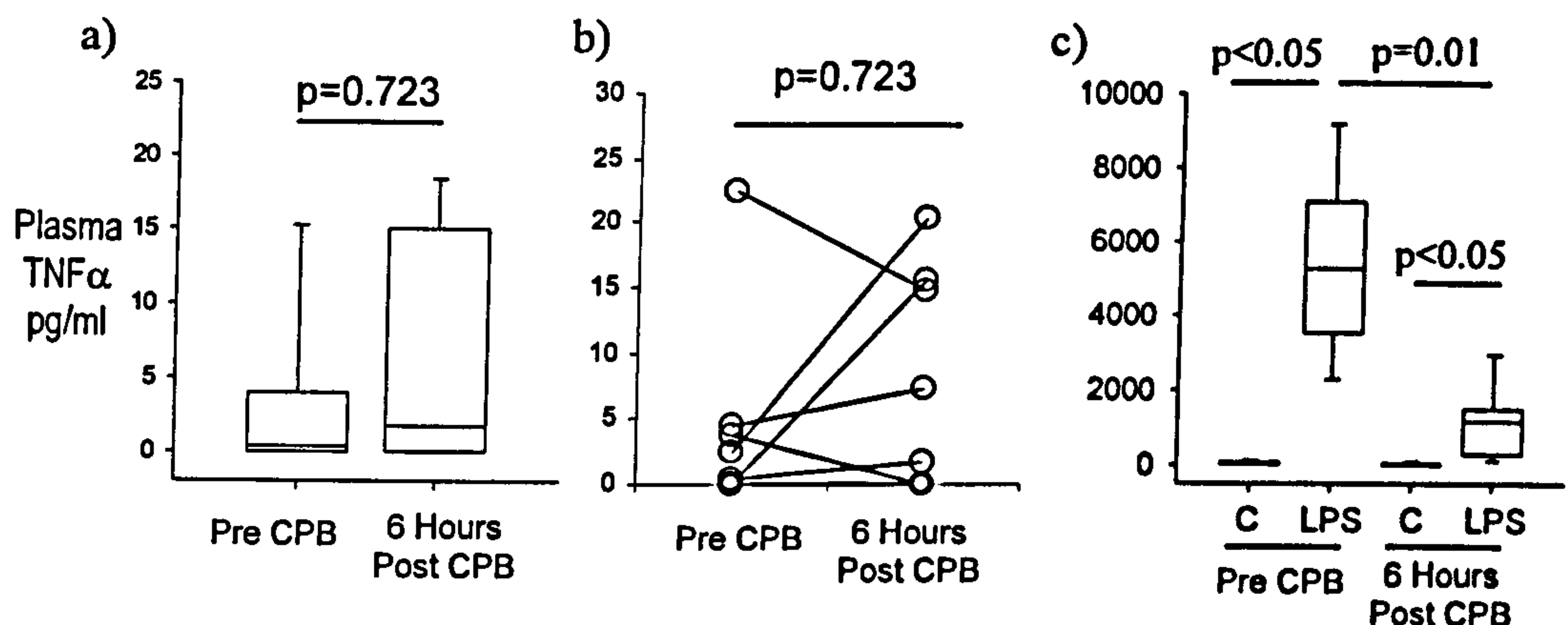


Figure 3.7. TNF α concentrations in the plasma of patients undergoing CABG. a) boxplots and b) individual data points from each patient of TNF α levels in native plasma before and 6 hours after surgery. c) Effect of 6 hours *in vitro* stimulation of whole blood with LPS before and after surgery (c=plasma from control incubated blood. LPS= plasma from 1ng/ml LPS stimulated blood).

Six hours control incubation of pre CPB blood resulted in an increase in IL-1 β levels in all but two patients (median: 2.9 (0.0 – 16.6) pg/ml). IL-1 β levels significantly increased further following *in vitro* stimulation of whole blood with LPS (median: 5425.0 (370.0 – 11783.3) pg/ml, $p<0.05$, Figure 3.8c).

Control incubation of post CPB blood for 6 hours resulted in the generation of detectable levels of IL-1 β in all but one sample (median: 2.37 (0.76 - 27.9) pg/ml) with significantly greater increases observed following *in vitro* stimulation with LPS (median: 856.7 (113.3 – 4425.0) pg/ml, $p<0.05$, Figure 3.8c). In all but one case, *in vitro* stimulation with LPS induced a greater increase in IL-1 β generation in pre CPB samples compared to post CPB samples ($p=0.027$).

There was a significant correlation between LPS stimulated increase in TNF α and IL-1 β ($r=0.674$, $p=0.002$).

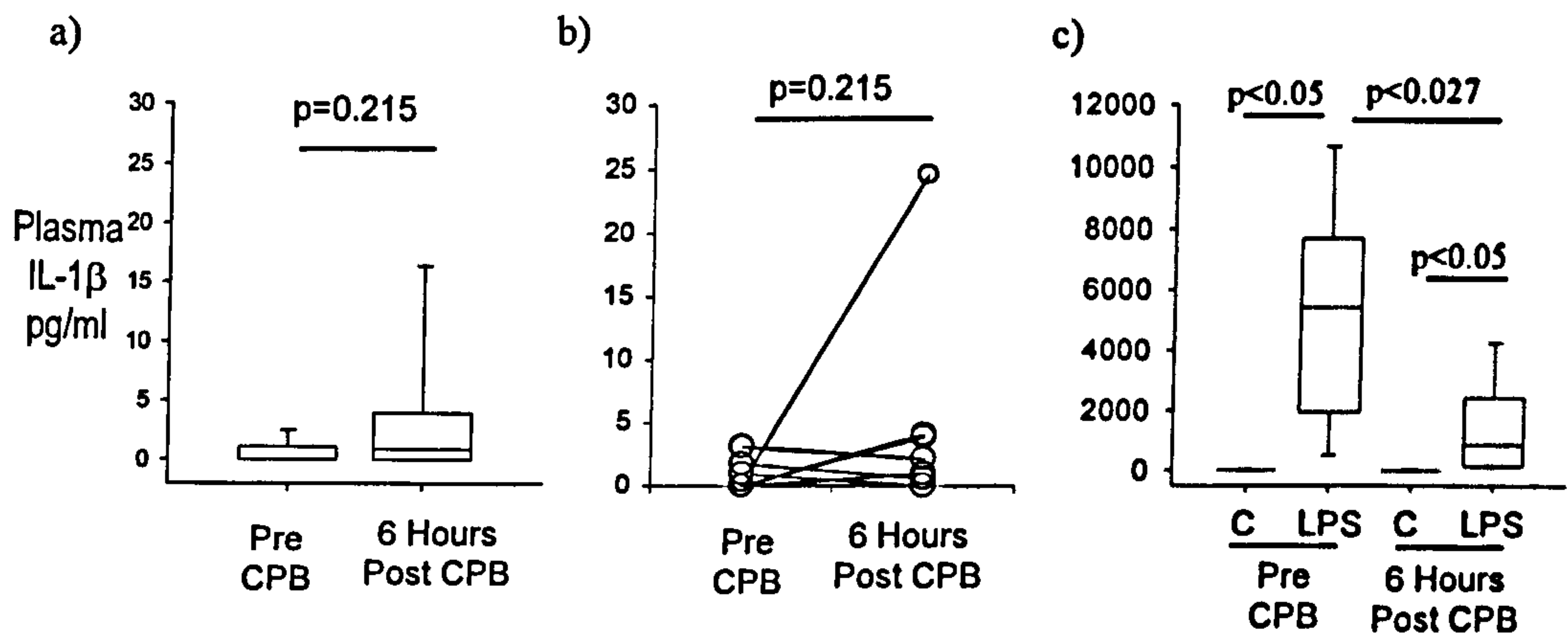


Figure 3.8. IL-1 β concentrations (pg/ml) in the plasma of patients undergoing CABG (n=9) a) boxplots and b) individual data points from each patient of IL-1 β levels in native plasma before and 6 hours after surgery. c) Effect of 6 hours *in vitro* stimulation of whole blood with LPS before and after surgery (c=plasma from control incubated blood. LPS= plasma from 1ng/ml LPS stimulated blood).

3.3.5.2 Soluble cytokine receptors

3.3.5.2.1 Plasma sTNFR1

Before CPB plasma levels of soluble TNFR1 varied between 1400.0 and 7326.7 (median: 1903.7) pg/ml. In all patients sTNFR1 increased in native plasma 6 hours after CPB (median: 4680.0 (2326.7 – 10093.3) pg/ml, $p=0.004$, Figure 3.9a and b).

Incubation of whole blood for 6 hours in either the presence or absence of LPS did not significantly alter the plasma levels of sTNFR1 either before CPB (median: 2520.0 (1860.0 – 3053.3) and median: 2296.67 (1820.0 – 8106.67) pg/ml respectively, $p>0.05$) or after CPB (median: 5400.0 (2866.8 – 10780.0) and median: 5086.7 (3240.0 – 13253.3) and pg/ml, respectively, $p>0.05$, Figure 3.9c).

Post CPB plasma levels of sTNFR1 were significantly higher than pre CPB levels regardless of *in vitro* manipulation.

3.3.5.2.2 Plasma sTNFR2

Before CPB native plasma levels of sTNFR2 ranged between 1203.5 and 5000.0 (median: 2836.2) pg/ml. Although these levels tended to increase in most patients after CPB there was no statistical difference between pre and post CPB native plasma levels of sTNFR2 (Figure 3.10 a and b).

Further treatment of blood from these patients with LPS failed to alter plasma levels of sTNFR2 and pre and post CPB samples remained similar regardless of treatment.

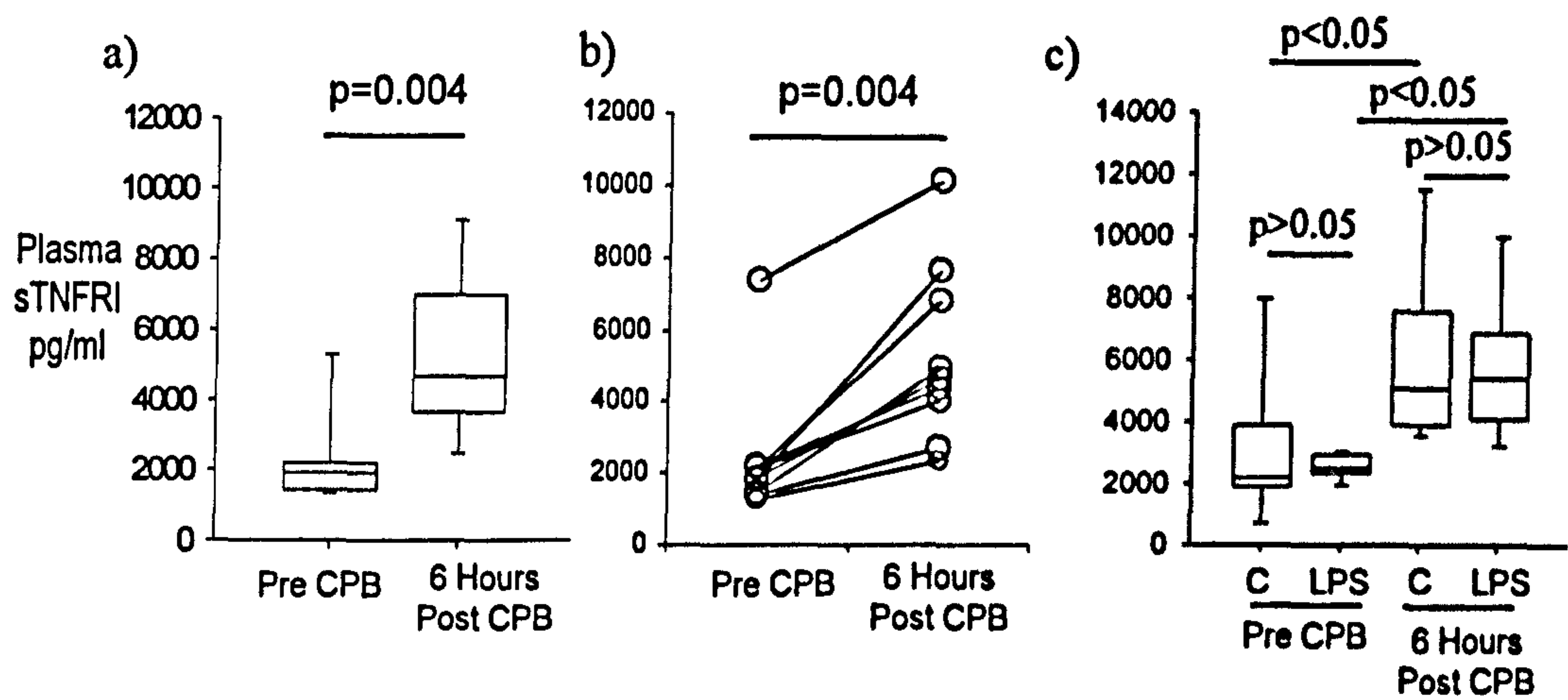


Figure 3.9. Plasma levels of sTNFR1 before and after CABG surgery with CPB. a) boxplot and b) individual data points from each patient of sTNFR1 levels in native plasma before and 6 hours after surgery. c) Effect of 6 hours *in vitro* stimulation of whole blood with LPS before and after surgery. (c=plasma from control incubated blood. LPS= plasma from 1ng/ml LPS stimulated blood).

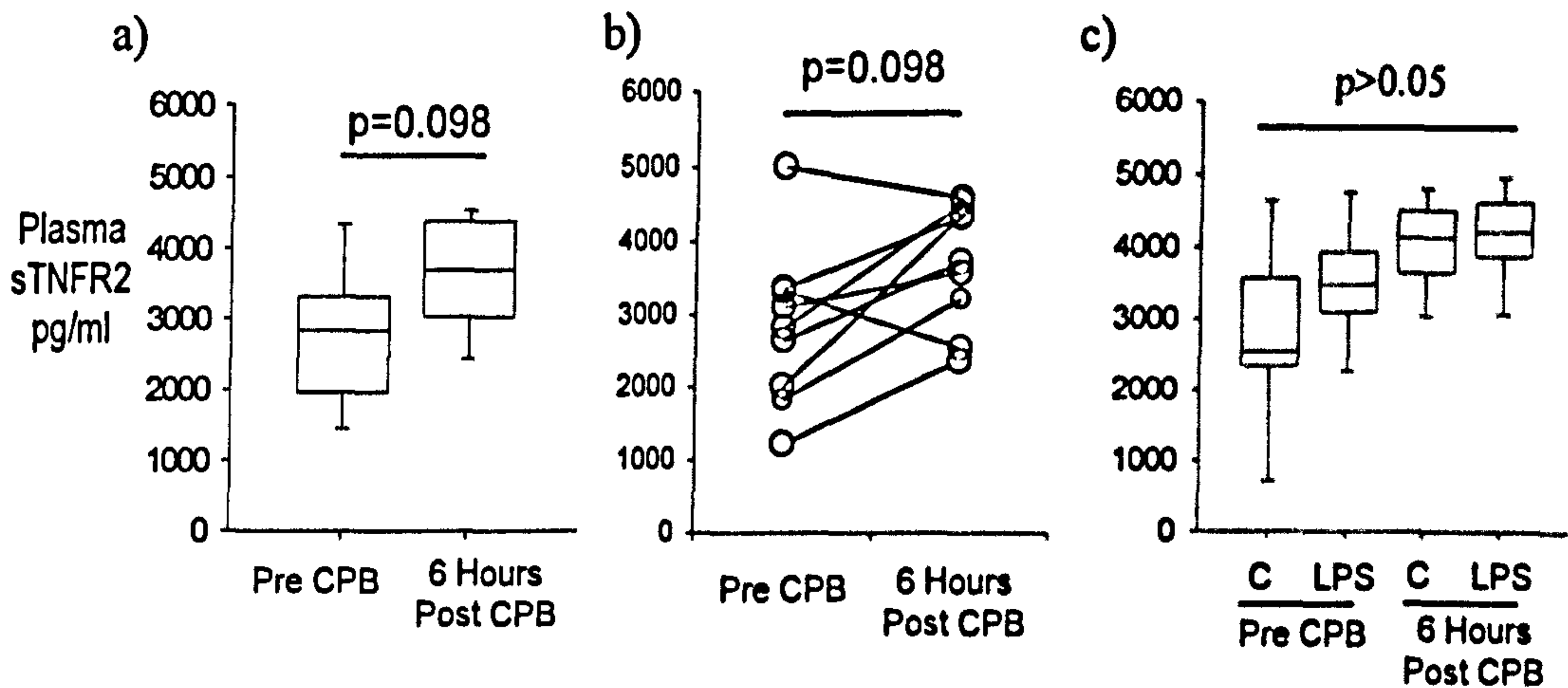


Figure 3.10 Plasma levels of sTNFR2 before and after CABG with CPB. a) boxplot and b) individual data points from each patient of sTNFR2 levels in native plasma before and 6 hours after surgery. c) Effect of 6 hours *in vitro* stimulation of whole blood with LPS before and after surgery. (c=plasma from control incubated blood. LPS= plasma from 1ng/ml LPS stimulated blood).

3.3.5.3 Receptor antagonists

3.3.5.3.1 Plasma IL-1RA

IL-1RA levels in pre CPB native plasma ranged between 912.9 and 6212.9 (median: 1027.1) pg/ml. These levels significantly increased in all patients after CPB with levels ranging from 3.7 to 47.5 (median: 42.3) ng/ml ($p= 0.001$, Figure 3.11a and b).

Although six hours control incubation of whole pre CPB blood resulted in an increase in IL-1RA above that seen in native plasma in all but one sample (median: 4441.4 (612.0 – 39455.7) pg/ml) Dunn’s post hoc statistical analysis failed to detect a statistically significant difference on grouped data in this respect. IL-1RA levels further increased in all samples following stimulation with LPS (median: 46.1 (41.6 – 48.4) ng/ml, $p< 0.05$, Figure 3.11c).

In post CPB samples no statistically significant increase in IL-1RA, above native levels, was seen, regardless of *in vitro* treatment.

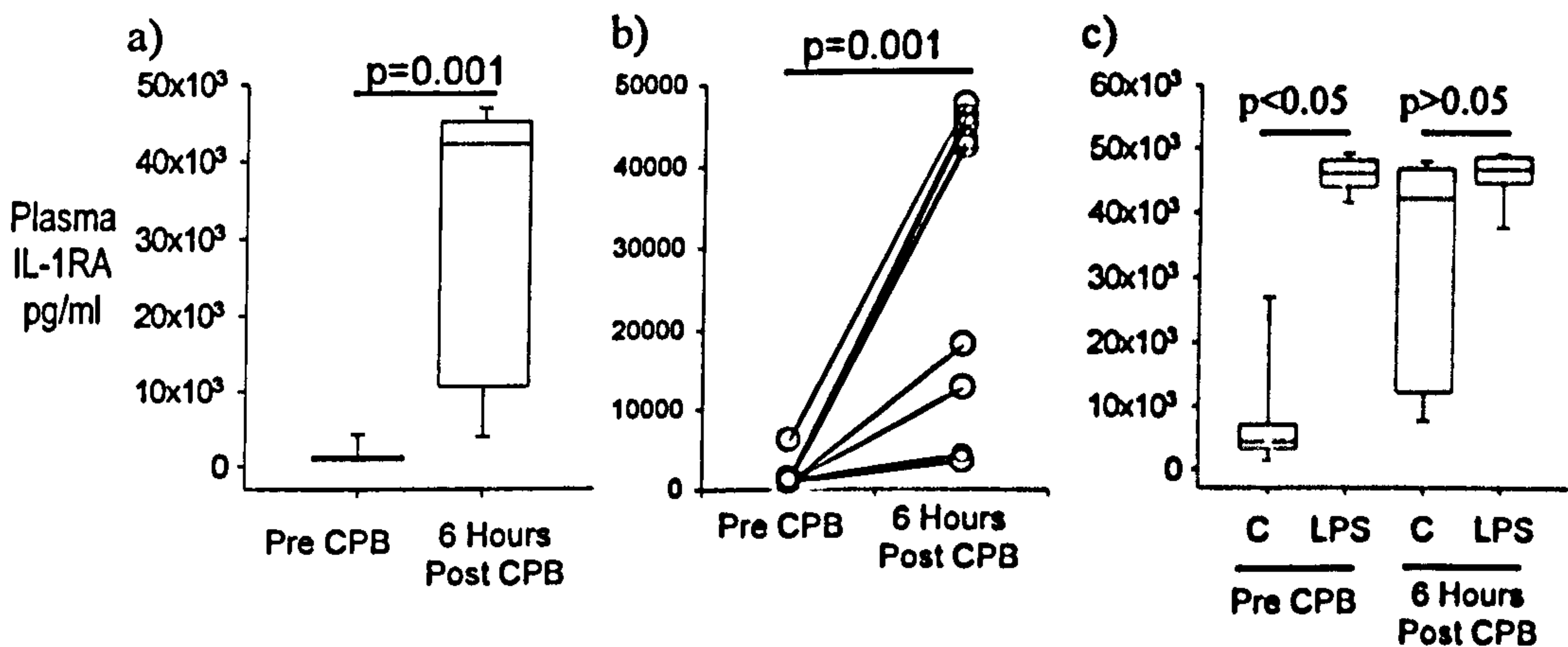


Figure 3.11 Plasma levels of IL-1RA before and after CABG surgery with CPB. a) boxplot and b) individual data points from each patient of IL-1RA levels in native plasma before and 6 hours after surgery. c) Effect of 6 hours *in vitro* stimulation of whole blood with LPS before and after surgery. (c=plasma from control incubated blood. LPS= plasma from 1ng/ml LPS stimulated blood).

3.3.5.4 Cytokine balance

Cytokine ratios were determined by an identical method to that used for Study N°1 (page 121 and page 123)

3.3.5.4.1 Molar Ratio of sTNFR1/TNF α

In native pre CPB plasma there was a 1414.8 (range 185.2 – 2422.2) fold molar excess of sTNFR1 to TNF α . On an individual basis there was considerable variation in the response to CPB with respect to the balance between sTNFR1 and TNF α , however as a group there was no significant alteration following CPB (median: 2508.8 (136.3 – 4881.5) pg/ml, $p=0.216$, Figure 3.12a and b).

In pre CPB samples the molar ratio of sTNFR1 to TNF α was reduced in all samples following 6 hours control incubation of whole blood (median: 36.8 (8.9 – 677.1)) and was reduced further by *in vitro* stimulation with LPS (median: 0.3 (0.15 – 0.81), $p<0.05$).

Similarly, in post CPB samples the ratio of sTNFR1 to TNF α was lower in all samples following 6 hours control incubation compared to native plasma and was reduced further following *in vitro* LPS stimulation (median: 2.0 (1.4 – 87.1) $p<0.05$, Figure 3.12c).

There was a trend towards higher ratio of sTNFR1 to TNF α in post CPB samples following *in vitro* manipulation however this did not reach statistically significant levels.

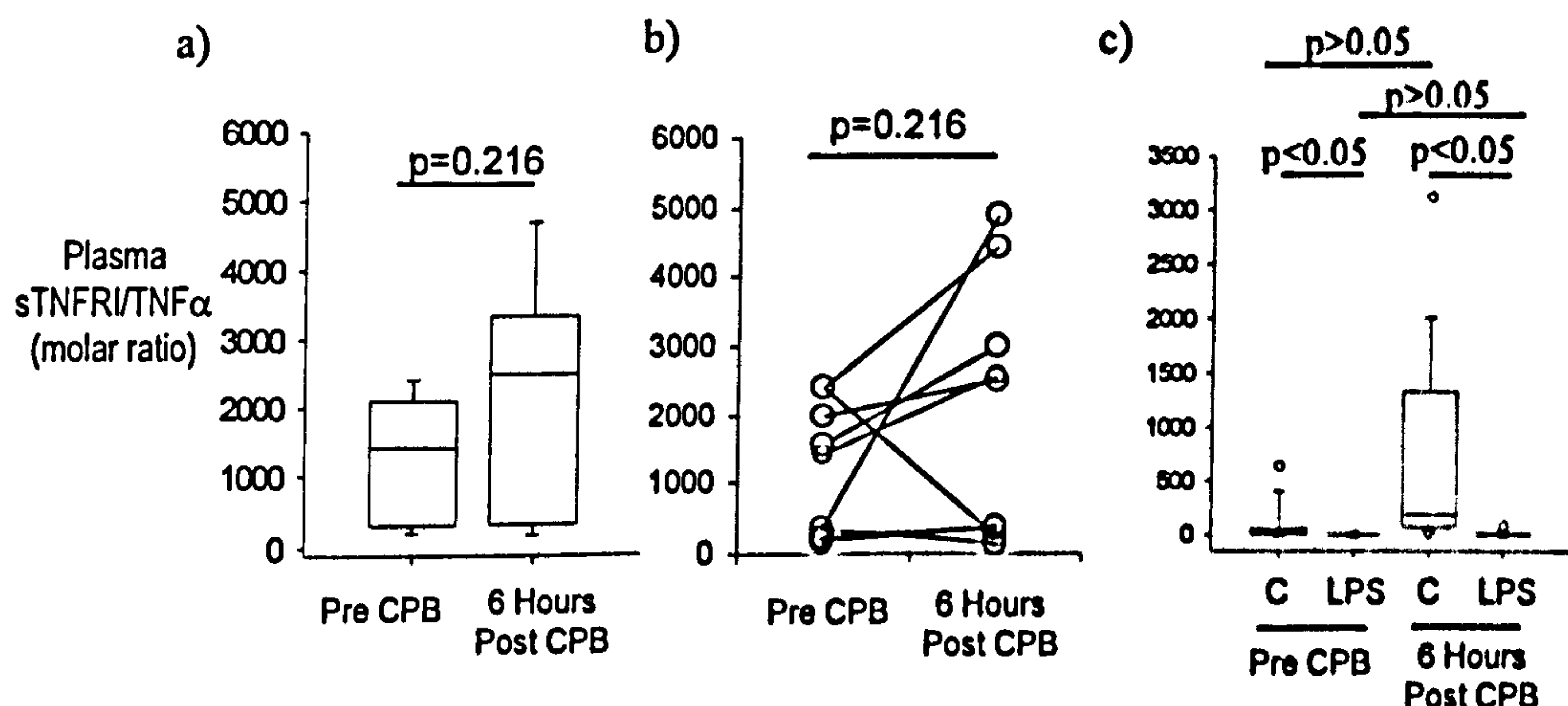


Figure 3.12. Molar ratio of sTNFR1/TNF α in the plasma of patients before and after CABG surgery with CPB. a) boxplot and b) individual data points from each patient of sTNFR1/TNF α levels in native plasma before and 6 hours after surgery. c) Effect of 6 hours *in vitro* stimulation of whole blood with LPS before and after surgery. (c=plasma from control incubated blood. LPS= plasma from 1ng/ml LPS stimulated blood).

3.3.5.4.2 Molar ratio of sTNFR2/TNF α

Similar to sTNFR1/TNF α there was a considerable molar excess of sTNFR2 to TNF α (94.8 – 2363.5 (median: 1002.9) in pre CPB native plasma. The effect of cardiac surgery with CPB on sTNFR2/TNF α was variable among patients and no overall effect on grouped data was observed (91.0 – 3090.5 (median: 626.3), $p=0.930$, Figure 3.13a and b).

In pre CPB samples the molar ratio of sTNFR2 to TNF α was reduced in all samples following 6 hours control incubation of whole blood (median: 34.5 (0.13 -572.1) and was reduced further by *in vitro* stimulation with LPS (0.15 – 0.59 (median: 0.3), $p<0.05$).

Similarly, in post CPB samples the ratio of sTNFR2 to TNF α was lower following 6 hours control incubation compared to native plasma (median: 183.6 (15.2 – 1228.5)) and was reduced further following *in vitro* LPS stimulation (0.59 – 20.8 (median: 1.5) $p<0.05$, Figure 3.13c).

Similar to sTNFR1/TNF α there was a trend towards higher ratio of sTNFR2/TNF α in post CPB samples however this did not reach statistically significant levels.

3.3.5.4.3 Molar ratio of total sTNFR/TNF α

The molar ratio of total sTNFR to TNF α followed the same trend as the results for the individual receptors with no difference observed, on the grouped data, between the pre and post-operative values.

The ratio of total sTNFR/TNF α was reduced compared to that in native plasma by control incubation of whole blood and was reduced further by stimulation with LPS. There was a trend towards a higher ratio of total sTNFR/TNF α in post CPB samples however this did not reach statistically significant levels (Figure 3.14).

3.3.5.4.4 Molar ratio of IL-1RA/IL-1 β

There was a considerable molar excess of IL-1RA to IL-1 β in native pre CPB plasma (median: 7022.0 (441.0 – 47791.2)), which increased in 7 of the 9 patients following cardiac surgery with CPB (28560.4 (180.4 – 365593.4), $p=0.052$, Figure 3.15a and b).

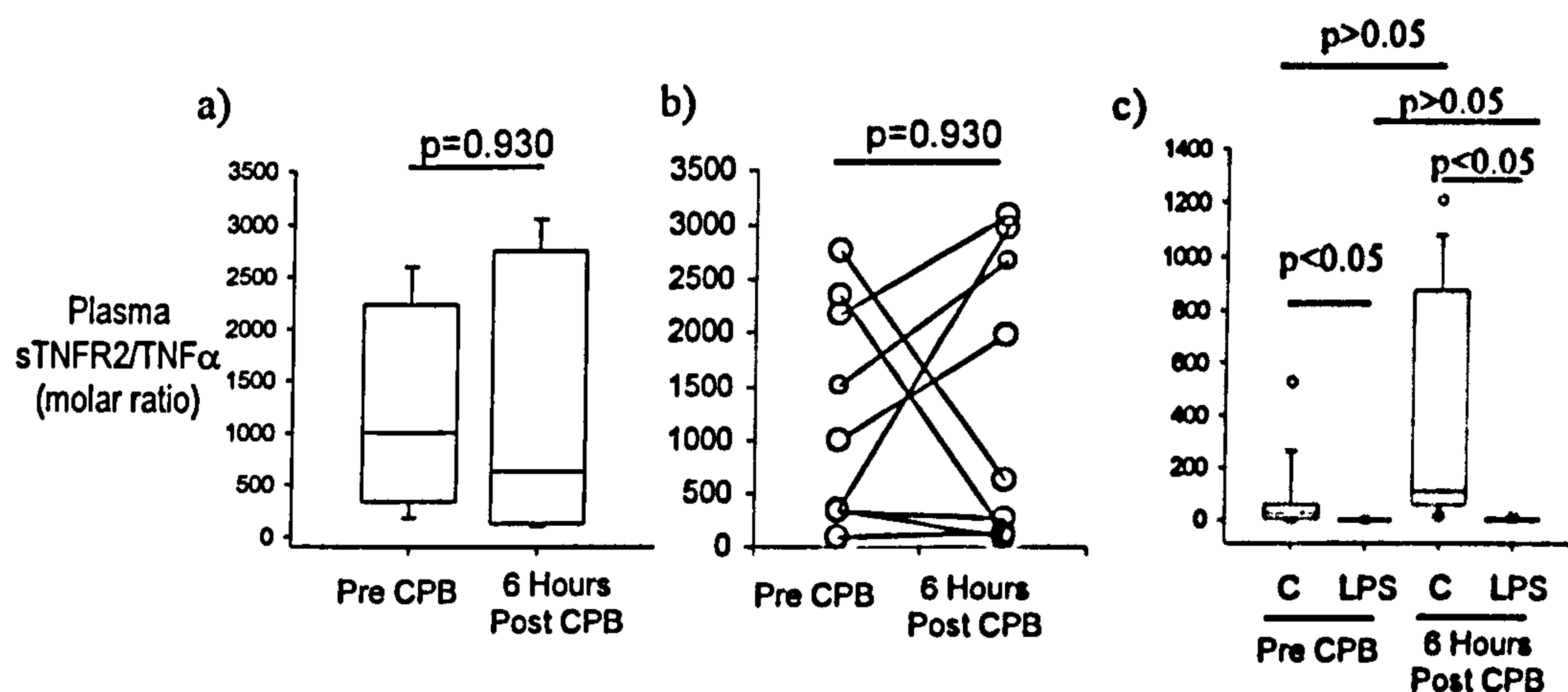


Figure 3.13. Molar ratio of sTNFR2/TNF α in the plasma of patients before and after CABG surgery with CPB. a) boxplot and b) individual data points from each patient of sTNFR2/TNF α levels in native plasma before and 6 hours after surgery. b) Effect of 6 hours *in vitro* stimulation of whole blood with LPS before and after surgery. (c=plasma from control incubated blood. LPS= plasma from 1ng/ml LPS stimulated blood).

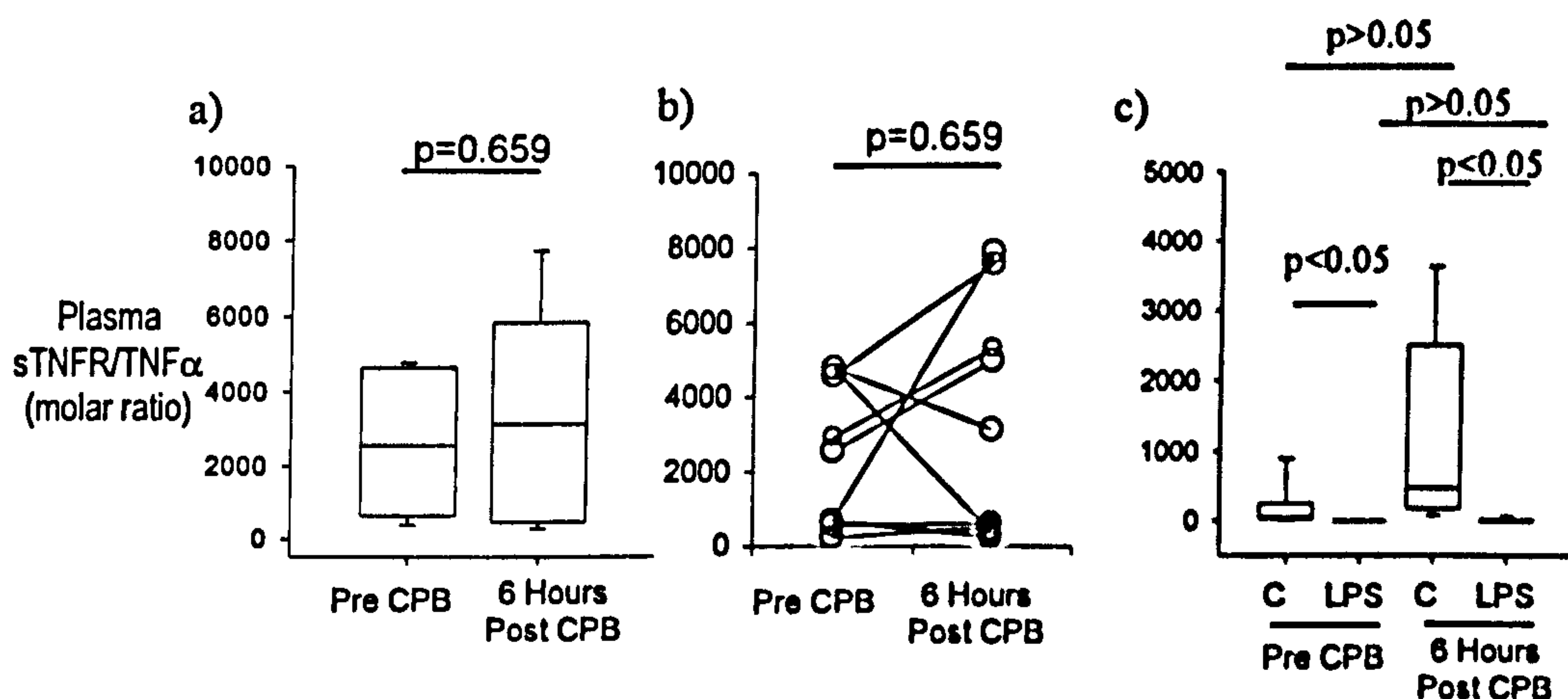


Figure 3.14. Molar ratio of total sTNFR/TNF α in the plasma of patients before and after CABG surgery with CPB. a) boxplot and b) individual data points from each patient of total sTNFR/TNF α levels in native plasma before and 6 hours after surgery. b) Effect of 6 hours *in vitro* stimulation of whole blood with LPS before and after surgery. (c=plasma from control incubated blood. LPS= plasma from 1ng/ml LPS stimulated blood).

Treatment of both pre and post CPB blood with LPS caused a significant reduction in the ratio of IL-1RA/IL-1 β (median: 8.8 (4.0 – 136.6) and median 57.7 (10.7 – 362.0) respectively, $p<0.05$ from native and LPS 0 ng/ml, Figure 3.15c). The ratio of IL-1RA/IL-1 β following LPS stimulation of whole blood was higher in all post CPB samples than in the corresponding pre CPB samples although this did not reach a statistically significant levels.

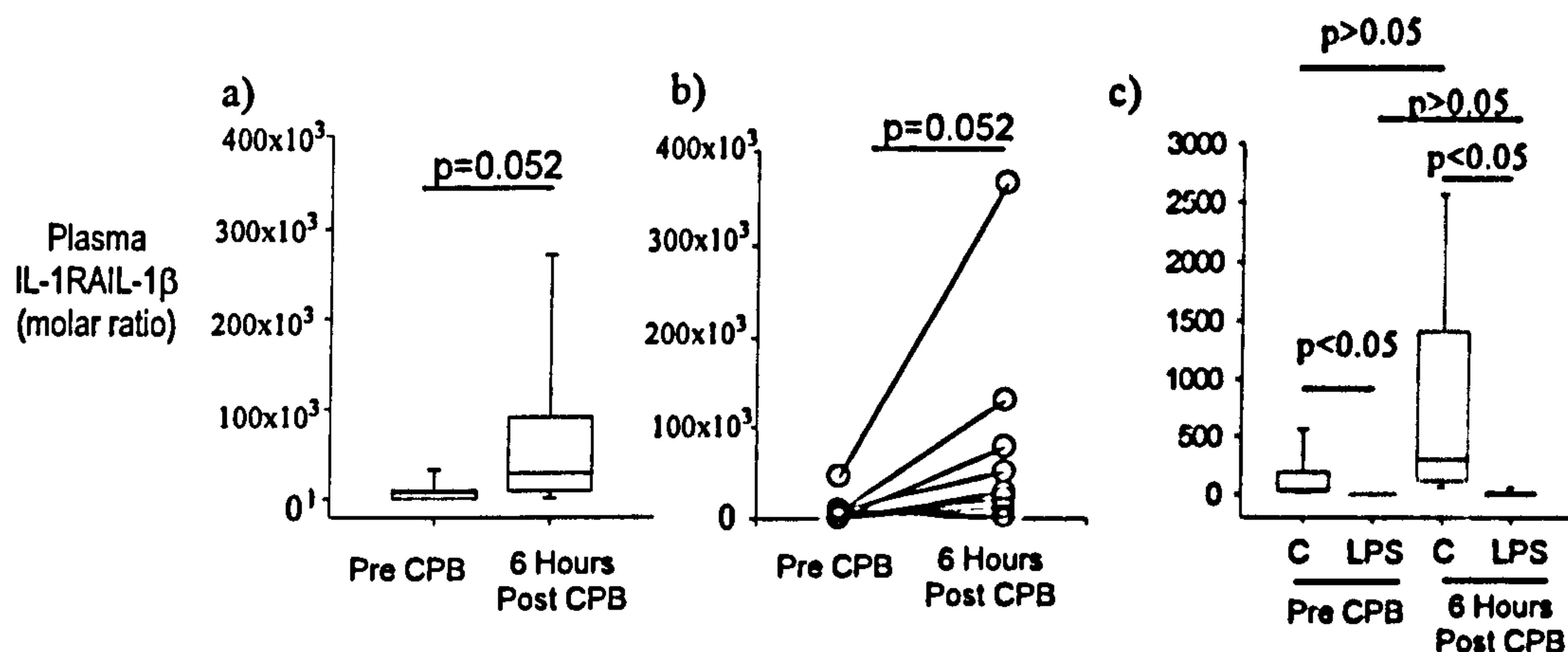


Figure 3.15. Molar ratio of IL-1RA/IL-1 β in the plasma of patients before and after CABG surgery with CPB. a) Effect of cardiac surgery on IL-1RA/IL-1 β molar ratio in native plasma. b) Effect of *in vitro* stimulation of whole blood with LPS on the molar ratio of IL-1RA/IL-1 β . (c=plasma from control incubated blood. LPS= plasma from 1ng/ml LPS stimulated blood)

3.3.6 DISCUSSION

These data demonstrate that there is a significant shift in the profile of circulating cytokines, towards an anti-inflammatory response, in patients following cardiac surgery.

3.3.6.1 Native plasma

In this small group of patients studied no statistically significant changes in the plasma levels of the pro-inflammatory cytokines IL-1 β or TNF α were observed on grouped data following cardiac surgery. In contrast, counter-acting endogenous inhibitors of these cytokines, sTNFR and IL-1RA were significantly increased following cardiac surgery.

Although not significant when the data is grouped, small increases in plasma levels of IL-1 β and TNF α were observed in the postoperative period in three and four patients, respectively. This data suggests that cardiac surgery with CPB does not universally trigger a systemic inflammatory response; however an exaggerated inflammatory response may develop in some patients, which could potentially contribute to postoperative complications.

Many studies have described the induction of a systemic inflammatory response following cardiac surgery with CPB and there are numerous reports that consistently demonstrate increased circulating levels of IL-6 and IL-8 following CPB. Less

consistent are the studies that investigate systemic expression of TNF α and IL-1 β during and after CPB.

One of the major contributing factors to the initiation of the systemic inflammatory response associated with CPB is the activation of blood cells as a result of contact with the non-endothelial surface of the bypass circuit and the oxygenator or increased circulating endotoxin levels. A number of studies have investigated the possibility that cytokines are produced in the circulation by mononuclear cells however the results, as with those that have directly measured plasma cytokine concentrations are variable. Zimmermann et al failed to demonstrate increased mRNA for either TNF α or IL-1 β in PBMC from patients undergoing cardiac surgery with CPB (Zimmermann et al., 2003). In contrast, Okubo observed a significant increase in PBMC mRNA expression for IL-1 β , TNF α , IL-8 and IL-10 in patients after cardiopulmonary bypass (Okubo et al., 2003).

The lack of detectable pro-inflammatory cytokines in the circulation of the majority of patients in this study following cardiac surgery does not definitively mean that no inflammatory response is initiated during cardiac surgery. For instance, local production of inflammatory cytokines may occur, such as in the myocardium that is not reflected in the systemic circulation. Indeed several studies suggest that the major source of TNF α during cardiac surgery with CPB is the ischaemic myocardium (Meldrum et al., 2003; Wan et al., 1996a). With this in mind it is highly likely that adverse effects of increased pro-inflammatory cytokines can occur at the site of production presumably without necessarily being observed in the circulation.

In addition, the timing of the sample collection may be such that these cytokines have been cleared from the systemic circulation by the kidneys or have been sequestered in tissue. However, the studies that demonstrate increased circulating TNF α and IL-1 β following cardiac surgery with CPB generally suggest that the time point of blood sampling in this current study is appropriate to detect any increases (Franke et al., 2002a; Roth-Isigkeit et al., 1998, 1999b, 2001b; Schulze et al., 2000a).

Clinically, the post operative course of all nine patients in this study was uneventful and proceeded without complications suggesting that even in the patients where pro-inflammatory cytokines were detected these levels were either clinically insignificant or compensatory mechanisms controlled their activity.

In this respect circulating levels of specific counteracting anti-inflammatory factors (sTNFR and IL-1RA) were quantified in these patients. Following CPB there was a considerable anti-inflammatory response with circulating levels of sTNFR and IL-1RA increasing in all nine patients. As discussed above (section 3.2.6.4) the increased circulating levels of these molecules can occur in response to a number of chemical and physical factors including pro- and anti-inflammatory cytokines and adhesion of leucocytes to matrix and endothelial cells suggesting that a number of factors could be responsible for their increased expression in the circulation following CPB. The plasma levels of soluble cytokine receptors have been suggested to serve as a sensitive marker of the activation of pro-inflammatory cytokines and may therefore be reflective of local pro-inflammatory cytokine release that is not apparent in the systemic circulation.

3.3.6.1.1 Influence of factors other than cardiac surgery

There is large volume of data in the literature concerning the effects of CPB on circulating levels of cytokines and much of it is conflicting. There are a number of variable factors that could be responsible for the differences observed between and within investigating centres that could potentially influence the outcome of such studies.

3.3.6.1.1.1 Anaesthetic agent

Both *in vitro* (Rossano et al., 1992; Larsen et al., 1998; Takaono et al., 2002) and *in vivo* (Crozier et al., 1994; Gilliland et al., 1997; Brix-Christensen et al., 1998; Taylor et al., 1997) studies have suggested that various anaesthetic agent can influence both basal and stimulated cytokine responses. However, the data generated from these studies are often conflicting and no general consensus concerning pro- or anti-inflammatory effects of specific agents has been forthcoming. In this regard it is worth noting that the choice of anaesthetic regimens could potentially influence the cytokine response to cardiac surgery and may account for the variation of results among study centres. All the patients in this current study received the same type of anaesthetic agent and thus is unlikely to account for any variability observed within this patient population.

3.3.6.1.1.2 Patient age

It has been suggested that patient ages can have a significant influence of the extent of the inflammatory response stimulated by surgical trauma (Kudoh et al., 2001). However, a study carried out by Roth-Isigkeit et al found that the cytokine release of elderly patients (>65 years) was not significantly different from younger patients (<50

years) in the perioperative course of CABG surgery (Roth-Isigkeit et al., 1998). In this current study the patients ranged in age from 54.8 – 71.5 years and there was no relationship between the ages of the patients and the cytokine responses to cardiac surgery with CPB.

3.3.6.2 *In vitro* stimulated whole blood

To contrast the effects of CPB to a controlled inflammatory situation, whole blood was stimulated with LPS for 6 hours prior to separating the plasma.

Treatment of pre operative blood with LPS for 6 hours caused a significant production of TNF α and IL-1 β from the blood cells. IL-1RA levels were also increased by LPS treatment however sTNFR levels were not. In post operative blood samples LPS treatment also caused an increase in the production of IL-1 β and TNF α from the blood cells however this was considerably less than that observed in pre operative blood. sTNFR and IL-1RA concentrations in post operative samples were not altered by LPS treatment.

Reduced responsiveness of blood cells to endotoxin challenge (endotoxin tolerance) has previously been shown following trauma (Keel et al., 1996), repeated challenge with endotoxin (Granowitz et al., 1993) and in patients with sepsis (Munoz et al., 1991). In addition, Dehoux et al demonstrated that LPS-induced TNF α , IL-6, IL-8 and IL-10 from whole blood was reduced in blood samples taken during the course of CPB and that this effect could be mimicked in blood cells from healthy volunteers circulating in a simulated bypass circuit (Dehoux et al., 2000). That data suggests that contact of blood with non-physiologic surfaces is sufficient to induce LPS hyporeactivity. Furthermore, the effect was in part due to increased IL-10 levels, the inactivation of which partially restored the pre CPB response to LPS although this effect was restricted to the *in vivo* study since no increase in IL-10 was observed in the simulated bypass circuit. Because of the use of the isolated bypass circuit and blood from healthy volunteers the authors could rule out any potential influence of anaesthetic factors or endocrine mediators such as glucocorticoids in the observed response. These and other factors such as prior exposure to endotoxin during CPB and increased plasma levels of anti-inflammatory factors such as IL-10, IL-6 and glucocorticoids induced by the surgical procedure may explain or be responsible for the observed alteration in LPS responsiveness in post CPB blood in this current study.

Soluble TNFR levels were not affected by *in vitro* incubation of whole blood in either the absence or presence of LPS. This was an intriguing result since LPS treatment is considered to be an important stimulus for proteolytic cleavage of these receptors from the cell membrane of leucocytes. In addition, the increase in TNF α production in these samples would also be expected to induce receptor shedding as a compensatory response. However, the effects of leucocyte activation by various agents on the shedding of soluble TNF α receptors appear confusing and often conflicting.

A rapid increase in plasma levels of sTNFR2 and, to a lesser extent sTNFR1 has been shown following intravenous endotoxin administration to healthy volunteers reaching a maximum at 3 hours. In addition, incubation of whole blood with endotoxin (1ng/ml) increased shedding of both receptors from monocytes and granulocytes with a concurrent increase in plasma levels and reduced cellular TNF α binding (Van Der et al., 1995). TNF α -induced shedding of both receptors from neutrophils and monocytes has also been shown to occur within 15 to 30 minutes of treatment with maximum effects achieved following 60 minute (Dri et al., 2000; Gasparini et al., 2003; Lantz et al., 1994; Porteu and Hieblot, 1994). This data suggests that LPS treatment of whole blood in this present study should yield similar responses. Other studies however have suggested that LPS-induced shedding of sTNFR from isolated human monocytes requires a much longer incubation period (24-48 hours) (Dickensheets et al., 1997b; Leeuwenberg et al., 1994a, 1994b).

Furthermore, several studies have suggested that selective shedding of TNF α receptors from different cell types occurs with monocytes and neutrophils selectively releasing sTNFR2 in response to LPS or TNF α stimulation and endothelial cells selectively releasing sTNFR1 (Joyce and Steer, 1995; Lien et al., 1995; Porteu and Hieblot, 1994).

Given the above information one would expect to see an increased in at least sTNFR2 if not both receptors following *in vitro* treatment of whole blood with LPS whether from the neutrophils or the monocytes and whether as a direct result of LPS stimulation or indirectly as a result of increased TNF α . The reason why this was not observed is unclear. Internalisation of the TNFR1 receptors is believed to be the principle mechanism through which this receptor is down regulated in response to increased TNF α stimulation (Porteu and Hieblot, 1994) and this may, in part explain the current observation. In addition, this data suggests that the mechanisms responsible for the increase levels of sTNFR1 in the native plasma in response to cardiac surgery do not

involve the shedding of leucocyte receptors in response to soluble factors that can be induced by LPS stimulation, such as TNF α and IL-1 β .

The phenomenon of LPS hypo-responsiveness following cardiac surgery with cardiopulmonary bypass has been suggested to be due to a deactivation of monocytes (Grundmann et al., 2000) since removal of all plasma factors and subsequent cultures of PBMC only partially restored the ability of these cells to secrete TNF α in response to LPS. This may partially explain the current finding since LPS-induced pro-inflammatory cytokine production from neutrophils has been shown to be less than 1.5% of that produced by PBMC, on a per cell basis. In addition neutrophil contribution to anti-inflammatory cytokine concentrations (sTNFRs and IL-1RA) have been shown to be around 10-15% of that produced by PBMC (Xing and Remick, 2003). Taken together, these studies suggest that CPB may cause deactivation of monocytes, but not neutrophils. As a consequence LPS treatment of whole blood will result in a significant reduction of pro-inflammatory cytokines release with a lesser reduction in anti-inflammatory factors because of the greater contribution of the neutrophils.

It was interesting to observe that 6 hours *in vitro* control incubation of whole blood from these current CPB patients resulted in the accumulation of TNF α and IL-1 β in the sample, increasing the plasma concentrations to detectable levels, which, in some cases was quite considerable. There are two possible explanations for this observation. The *in vitro* manipulation of the blood (pipetting and prolonged incubation under static conditions in plastic vessel) activated the blood cells stimulating the production of TNF α and IL-1 β . Alternatively the cells in the blood may already be producing low levels of cytokines. Under static *in vitro* conditions the cytokines are allowed to accumulate in the absence of active removal or tissue sequestration. The accumulation of TNF α in the samples *in vitro* was generally greater in the pre CPB samples compared to the post CPB samples suggesting that whatever the reason for the accumulation of TNF α over the 6 hours it is attenuated by surgery/CPB.

In addition, in patients that demonstrated increased TNF α or IL-1 β levels in the native plasma following surgery there were not always greater cytokine levels in the postoperative samples following *in vitro* control incubation. This data suggests that the blood leucocytes are not the source of the increased pro-inflammatory cytokines observed in some of the post-operative native plasma samples.

In a similar manner IL-1RA also accumulated in the pre CPB blood over the 6 hour time period however in contrast to IL-1 β and TNF α there was a greater concentrations in the post CPB sample. This data suggests that cardiac surgery with CPB induces leucocytes to produce IL-1RA and further substantiates the conclusion that in these patients there is a significant anti-inflammatory response.

In contrast soluble TNF α receptor concentrations were not altered from those in the native plasma following any *in vitro* manipulation. The result suggests that the blood leucocytes are perhaps not the source of the increased soluble receptors observed during cardiac surgery with CPB since one might expect to see continued shedding from these cells *in vitro*. The increase in sTNFR1 observed in native plasma following cardiac surgery may be a result of receptor shedding in tissue and serve as a surrogate marker of a local inflammatory response that is not manifested in the systemic circulation.

3.3.7 CONCLUSION

In patients undergoing coronary artery bypass graft surgery utilising CPB there was no detectable systemic inflammatory response with respect to increased pro-inflammatory cytokines. Contrary, the principle response to cardiac surgery utilising cardiopulmonary bypass was an anti-inflammatory one.

3.4 STUDY N°3 – ROLE OF CPB IN THE CYTOKINES RESPONSE TO CARDIAC SURGERY

To achieve desirable condition under which a surgeon can perform open-heart surgery the heart must be still and flaccid in order that it can be easily manipulated and the operating field must be free of blood. To this end the heart must be arrested and its function, and that of the lungs, must be maintained artificially by the cardiopulmonary bypass machine. The changes in inflammatory factors associated with cardiac surgery are frequently attributed to the use of CPB. Such a procedure forces the blood into contact with the non-endothelial surface of the extracorporeal circuit of the CPB machine and renders the heart and the lungs ischaemic during the course of the surgery, causing injury/damage when reperfused. Thus, what is advantageous to the surgeon comes at a price to the patient.

In order to avoid these problems associated with the use of CPB alternative methods of coronary artery surgery have been developed, termed 'off pump' coronary artery bypass

graft surgery (OPCAB), that avoid the use of the extra-corporeal circuit and the associated global myocardial ischaemia.

In 1994, Borst et al developed the Octopus Tissue Stabilizer® which utilises suction to immobilise the epicardium (Borst et al., 1996). The Octopus Tissue Stabilizer consists of two suction paddles that are placed in parallel on either side of the coronary artery. Once a suction pressure of 400 mmHg is reached, the target site is effectively immobilised. This method restrains the anastomosis site during otherwise normal beating of the heart. The Octopus Tissue Stabilizer has continued to evolve with further design improvements that include a lower profile device with flexible heads and disposable, transparent paddles that are incorporated into a one-piece system which mounts directly on to the sternal retractor.

A recent study compared the early clinical outcome following OPCAB technique (n=2223) with conventional CABG using CPB (n=5163) and demonstrated a significant clinical benefit of OPCAB. Morbidity (pulmonary, renal and myocardial dysfunction) and mortality were significantly lower in the OPCAB patients compared with the CPB patients (Al Ruzzeh et al., 2003). However, whether this apparent benefit of avoidance of CPB is as a result of an altered cytokine response is unclear.

3.4.1 AIMS

To further explore the time course of the cytokine response (pro- and anti-inflammatory) to cardiac surgery and to determine whether the use of cardiopulmonary bypass (CPB) significantly contributes to the observed changes.

To determine the effects of CPB on neutrophil and monocyte activation (CD11b expression and NFκB activation) during coronary artery bypass surgery.

3.4.2 STUDY DESIGN

Plasma cytokine concentrations, the balance between pro-inflammatory cytokines and their endogenous inhibitors and leucocyte activation were measured at various time points in patient undergoing coronary surgery comparing the response of patients undergoing CABG with CPB to that of patients undergoing OPCAB utilising the Octopus®3 (Medtronic, Minneapolis, MN, USA).

3.4.3 METHODS

3.4.3.1 Patients

This study included the 26 patients with CAD with good left ventricular function undergoing CABG surgery described in section 3.2.4.1.1. The patients were randomised prospectively into two groups.

Those undergoing conventional CABG utilising cardiopulmonary bypass (CPB).

Those undergoing off pump coronary artery bypass graft surgery utilising Octopus®3 (OPCAB).

	CPB (n=13)	OPCAB (n=13)
Age (years, mean ± sem	62.3 ± 3.6	63.5 ± 2.8
Male/female	12/1	11/2

Table 3.3. Study No 3 - Patient details

3.4.3.2 Anaesthetic and operative techniques

Anaesthetic pre-medication included morphine 10 mg, metoclopramide 10 mg, and temazepam 10 mg administered the morning of the operation. The general anaesthetic technique comprised a low-intermediate dose of opioid (usually fentanyl, 8-15 µg/kg) and a propofol infusion (3 mg/kg per hour). Anaesthesia was maintained with nitrous oxide and isoflurane or sevoflurane.

3.4.3.2.1 Cardiopulmonary bypass.

Anticoagulation was achieved with 300 U/kg of heparin. The activated clotting time was maintained above 480 seconds. Heparin effects were reversed with protamine at the end of the procedure. The operation was performed through a median sternotomy, and CPB was instituted with a single right atrial cannula and an ascending aorta perfusion cannula. Standard bypass management included membrane oxygenators and non-pulsatile flow of 2.4 L/min per m² with a mean arterial blood pressure of greater than 50 mm Hg. Myocardial protection was achieved with intermittent cold blood cardioplegia (4:1 blood-crystalloid ratio).

3.4.3.2.2 OPCAB

Anticoagulation was achieved with 150 U/kg of heparin. The activated clotting time was maintained above 250 seconds. Heparin effects were reversed with protamine at the end of the procedure. The operation was performed through a median sternotomy with the Octopus®3 stabilisation device. Mean arterial blood pressure was maintained between 50 and 70 mm Hg during the procedure by means of repositioning the heart and the selective use of vasoconstrictors. Other measures to improve cardiac output, such as elevating the feet and increasing the heart rate, were used as appropriate. A standby perfusionist with a primed bypass circuit was available for all cases.

3.4.3.3 Collection and processing of samples

Central venous blood was collected into EDTA or heparin containing tubes at following time points.

t-1: Pre operative – after insertion of arterial line and before induction of anaesthesia

t-2: 15 minutes after initiation of CPB or application of Octopus®3

t-3: 60 minutes after initiation of CPB or application of Octopus®3

t-4: 2 hours after initiation of CPB or application of Octopus®3

t-5: 5 hours after initiation of CPB or application of Octopus®3

t-6: 24 hours after initiation of CPB or application of Octopus®3

Samples were placed on ice and transported immediately to the laboratory

3.4.3.3.1 Analysis of leucocyte activation

Leucocyte activation was analysed by two methods. The upregulation of surface CD11b expression (a component of the leucocyte adhesion molecule Mac-1) and the activation of the transcription factor NFκB.

3.4.3.3.1.1 CD11b

Leucocyte CD11b expression was measured using immunofluorescent antibody staining of whole blood followed by flow cytometry.

For each time point 90 µl of whole blood was placed into each of two 12 x 75 mm polypropylene tubes. 10 µl of mouse anti-human CD11b (clone ICRF 44, IgG₁, Serotec) was added to tube one from each time point to give a final concentration of 10µg/ml. To

tube two for each time point 10µl of mouse IgG₁ isotype control (clone MOPC 21, Sigma-Aldrich) was added to a final concentration of 10µg/ml. The samples were vortexed and incubated on ice for 15 minutes. Samples were washed by dilution in 3ml PBS and centrifugation at 365 x g for 5 minutes to pellet the cells and allow removal of the supernatant by aspiration. Following two washes, FITC-conjugated rabbit anti-mouse secondary antibody (Sigma- Aldrich) was added to all tubes to a final dilution of 1 in 64. Samples were vortexed and incubated on ice for 15 minutes. After washing the cells twice in PBS the red cells were lysed using COULTER® whole cell lysing reagent. 1ml of working strength COULTER CLONE® immunolyse was added to the cell pellet in each tube, vortexed and incubated for 1 minute to lyse the erythrocytes. 250 µl of COULTER CLONE® fixative was added to each tube and vortexed to fix the remaining leucocytes. The Samples were washed for a final time and the leucocytes were resuspended in PBS. CD11b staining was analysed immediately using Coulter EPICS®-XL flow cytometer. Leucocyte populations were identified by characteristic forward and side scatter profiles and gates isolating the distinct populations were set accordingly (Figure 3.16). Mean fluorescent intensity of CD11b versus MOPC 21 isotype control were presented as the Relative Fluorescence Intensity (RFI) (ratio of experimental mean fluorescence intensity over MOPC 21 irrelevant isotype control mean fluorescence intensity) where an RFI of 1.00 = no expression).

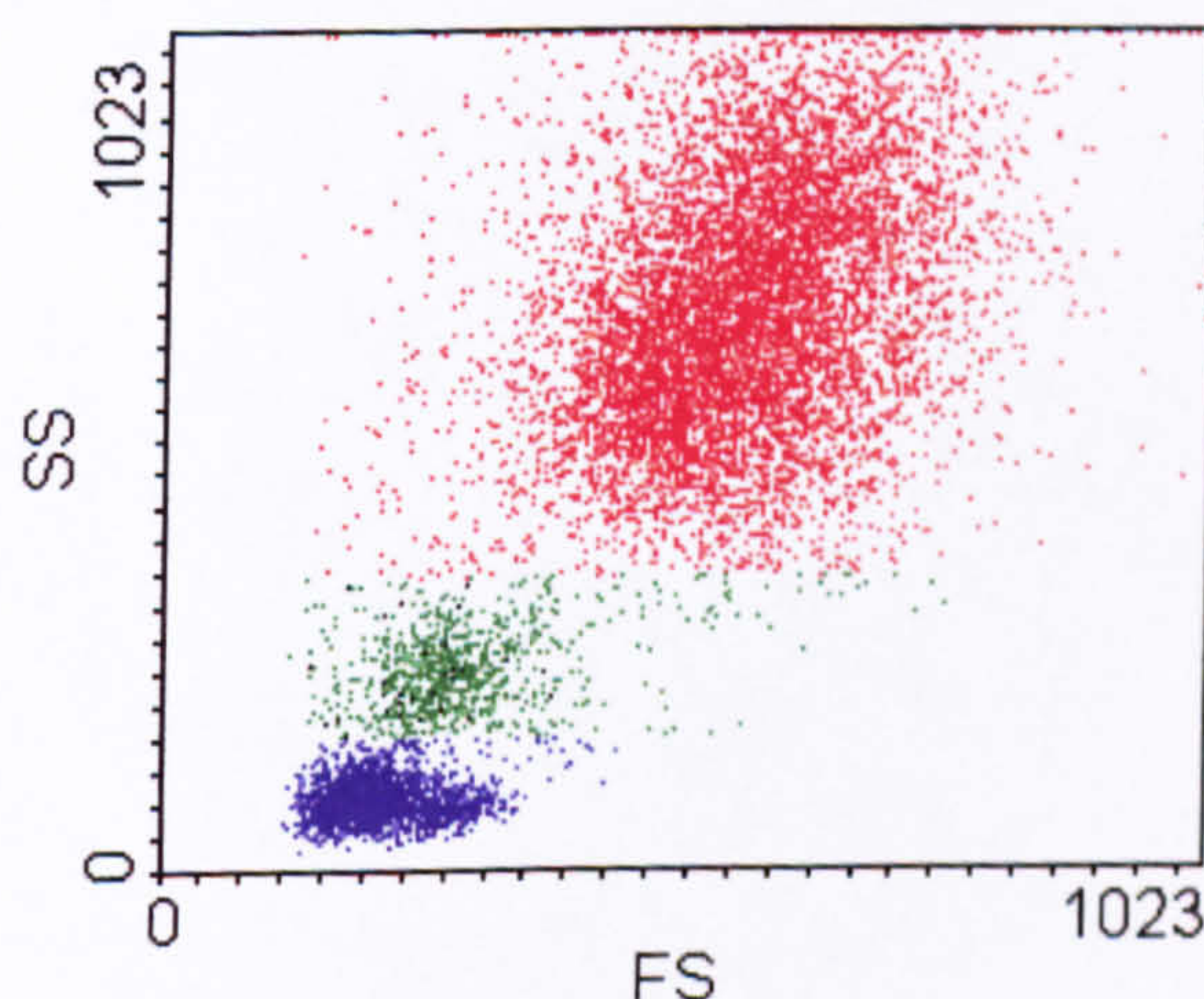


Figure 3.16. Dot plot demonstrating the distinct forward and side scatter characteristics of leucocytes prepared for flow cytometry analysis as described in section 3.4.3.3.1.1. Red = neutrophils, Green = monocytes, blue = lymphocytes

3.4.3.3.1.2 *NFκB*

Monocytes *NFκB* activation was monitored by immunoperoxidase staining of isolated mononuclear cells.

Mononuclear cells were isolated from whole blood using lymphoprep™. 5 ml of diluted whole blood (2.5ml heparinised blood plus 2.5ml sterile saline solution) was carefully layered onto 2.5ml of lymphoprep™ 1.077 ±0.001g/ml in a 15ml centrifuge tube. The samples were centrifuged for 20 minutes at 800 x g at room temperature. Centrifugation resulted in the sedimentation of aggregated erythrocytes to the bottom of the tube and the formation of a distinct band of mononuclear cells at the sample/medium interface. Mononuclear cells were carefully collected with a Pasteur pipette and diluted with sterile saline to reduce the density of the solution. The cells were pelleted by centrifugation at 200 x g for 10 minutes and resuspended in 1ml RPMI medium.

The cells were counted using a haemocytometer and the cell density adjusted to 12.5×10^4 cells per ml. Slides were prepared using a Shandon cytopsin and cytofunnels®. 200µl of cell suspension was used in each funnel chamber to put 2.5×10^4 cells per spot onto a poly-L-lysine microscope slide (VWR International). Slides were left to dry at room temperature before fixing the cells in ice cold acetone for 5 minutes. All acetone was evaporated from the slides before they were wrapped, air tight, in Clingfilm and stored at -80°C. *NFκB* activation in mononuclear cells was assessed by the presence of nuclear *NFκB* p65 determined by immunoperoxidase staining (Methods section 2.5) using mouse anti-human *NFκB* p65 (F-6) monoclonal antibody (Santa Cruz) at 6.7µg/ml overnight at 4°C. Mouse anti-CD68 monoclonal (clone EBM11, DakoCytomation) antibody at 0.46 µg/ml was used as a positive control staining for monocytes. Monocytes were distinguished from lymphocytes visually by their size 16-20 µm and their reniform or horseshoe-shaped nucleus. Lymphocytes are generally smaller than monocytes, 8-10 µm in diameter and their nucleus is round and large in comparison to the cell. *NFκB* activation in monocytes was analysed in a quantitative manner expressing the number of *NFκB* p65 positive nuclei as a percent of cells counted.

3.4.3.3.2 Plasma cytokine analysis

Plasma cytokine concentrations were measured by the methods described in section 3.2.4.3

3.4.3.4 Data analysis

Analysis of data was carried out as described in section 3.2.4.4

3.4.4 RESULTS – STUDY N°3

3.4.4.1 Leucocyte activation

3.4.4.1.1 Neutrophil CD11b

Neutrophil CD11b expression levels at the start of the operation were similar for both groups of patients with a median RFI of 24.2 (8.0 - 41.8, n=13) in the CPB patients and 17.8 (5.8 – 129.8, n=13) in the OPCAB group (p=0.538). Sixty minutes after the initiation of CPB (t3), neutrophil CD11b expression was significantly increased (median 42.0 (21.5 – 64.8) RFI, n=13, p<0.05) compared to baseline levels and remained elevated at 2 hours after the initiation of CPB (t4, 31.4 (22.2 - 54.0, n=13, p<0.05, Figure 3.17a). In contrast, neutrophils from OPCAB patients showed no evidence of increased CD11b expression compared to baseline levels (t2: 19.1 (8.0 – 67.8), t3: 21.5 (9.0 – 106.5), t4: 24.4 (8.4 – 63.4) RFI, n=13, p=0.816, Figure 3.17b).

3.4.4.1.2 Monocyte CD11b

Pre-operative baseline expression of CD11b on monocytes was not statistically different between the two groups of patients with a median RFI of 33.5 (12.3 – 62.3, n=13) in the CPB patients and 35.9 (8.2 – 68.8, n=12) in the OPCAB patients (p=0.474). In both groups monocyte CD11b expression remained unaltered throughout the time course studied (CPB: p=0.198, OPCAB: p=0.813, Figure 3.18).

3.4.4.1.3 Monocyte NFκB

In pre-operative blood (t1) from CPB patients (n=8) 20.2 (4.5 – 35.5)% of monocytes had detectable levels of NFκB p65 protein in their nuclei. This level significantly rose to 35.5 (18.0 – 73.4)% (p=0.039) 2 hours after the initiation of CPB (t4). In contrast monocytes nuclear NFκB p65 did not increase significantly in patients undergoing OPCAB (n=6) between t1 and t4 (median: 25.8 (11.8 – 45.9) and 32.8 (19.1 – 40.9)% respectively, (p=0.993, Figure 3.19).

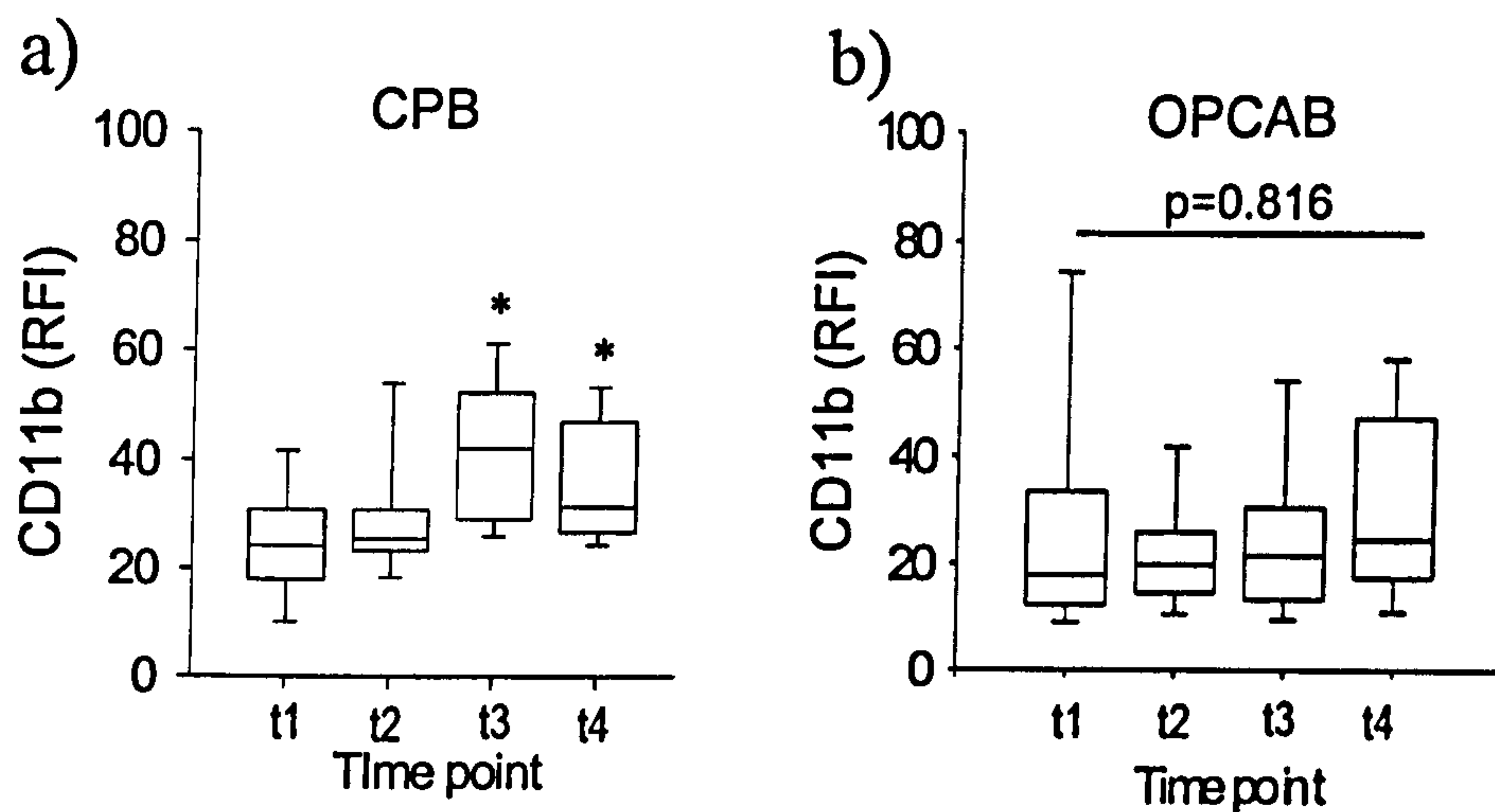


Figure 3.17. CD11b expression on neutrophils of patients undergoing CABG surgery. Effect of CABG surgery with (a, n=13) and without (b, n=13) CPB on the relative fluorescence intensity generated by positive CD11b staining of neutrophils. * denotes p value <0.05 from the t1 pre-operative CD11b expression.

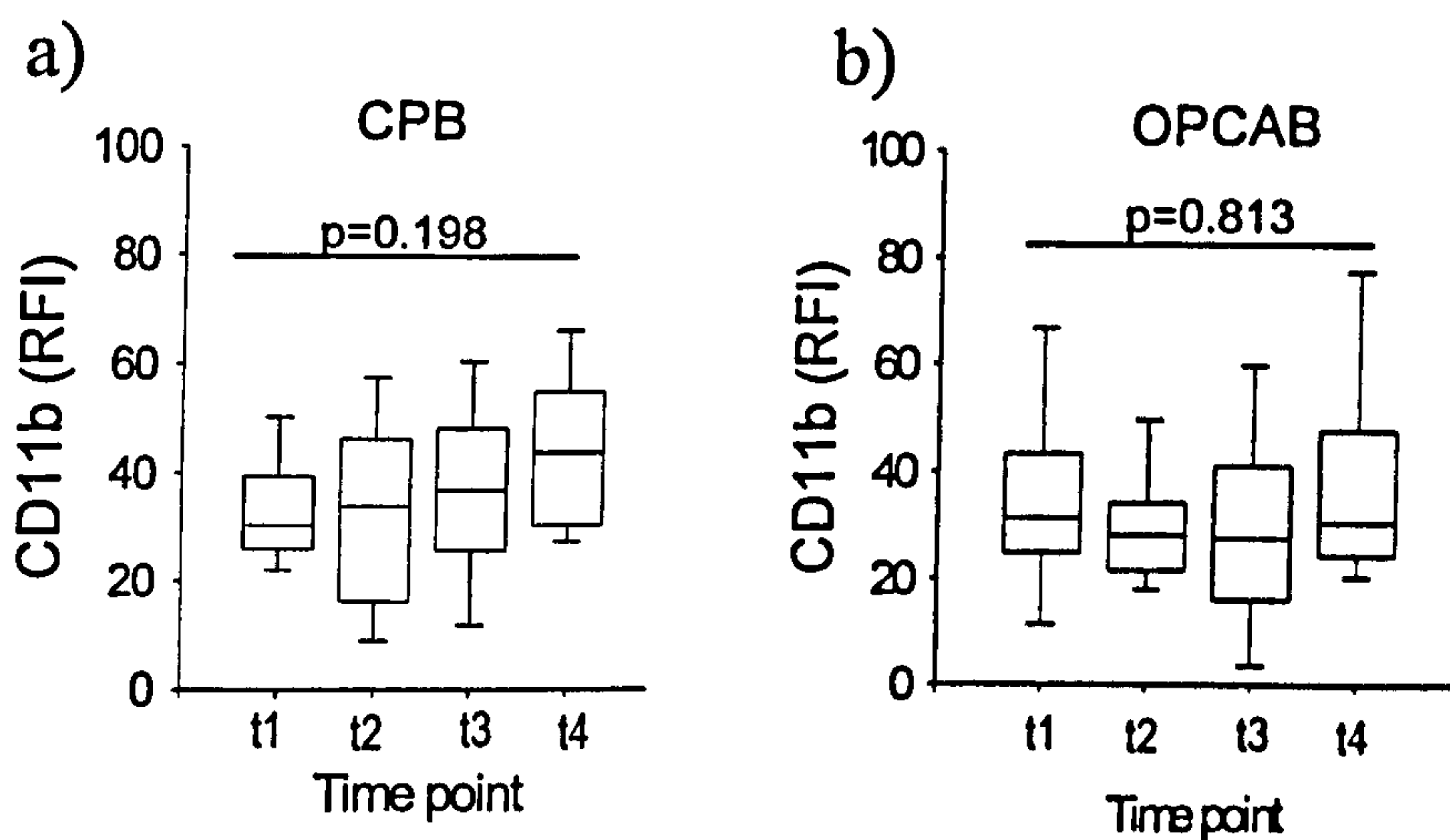


Figure 3.18. CD11b expression on monocytes of patients undergoing CABG surgery. Effect of CABG surgery with (a, n=13) and without (b, n=12) CPB on the relative fluorescence intensity generated by positive CD11b staining of monocytes.

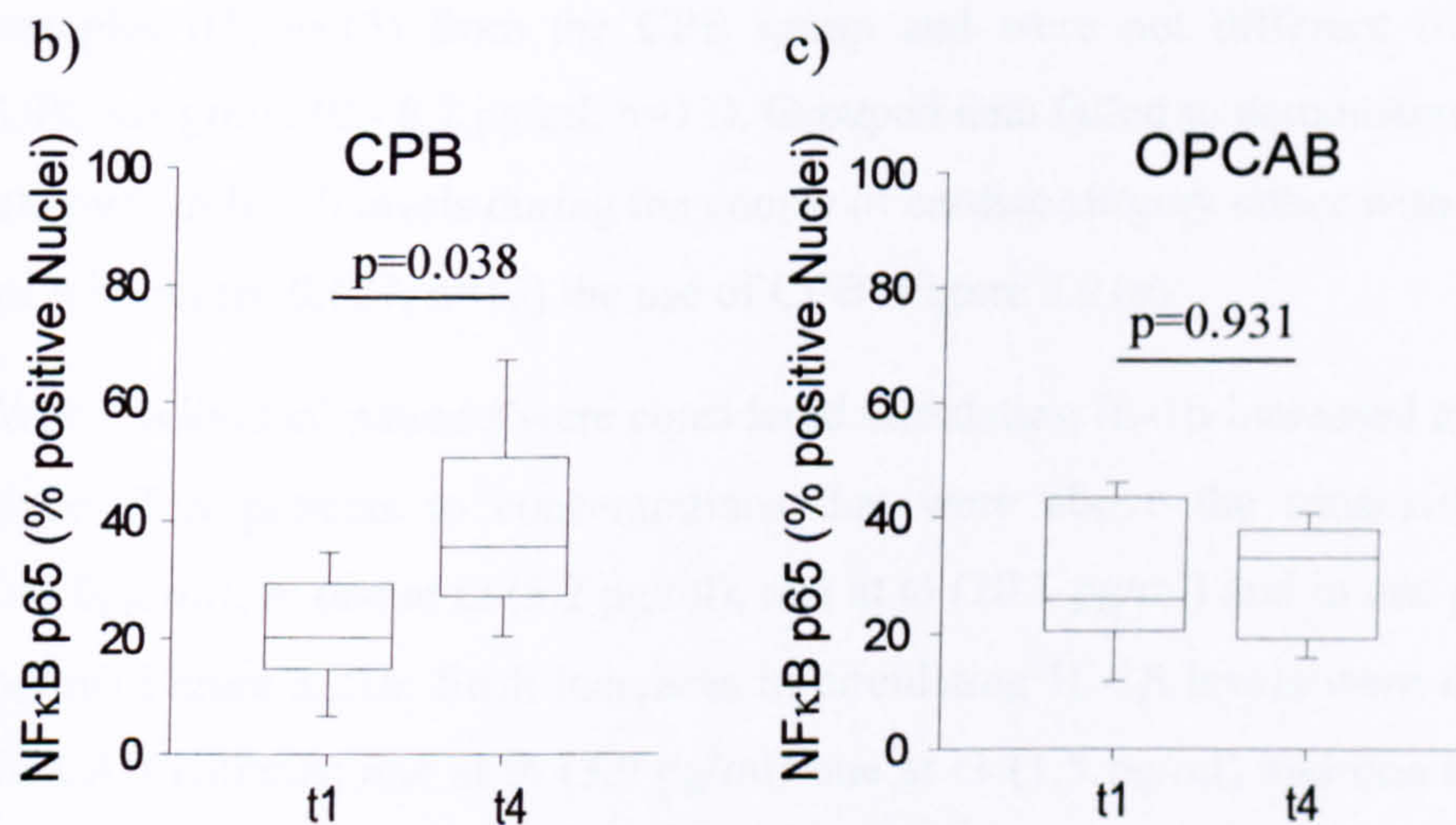
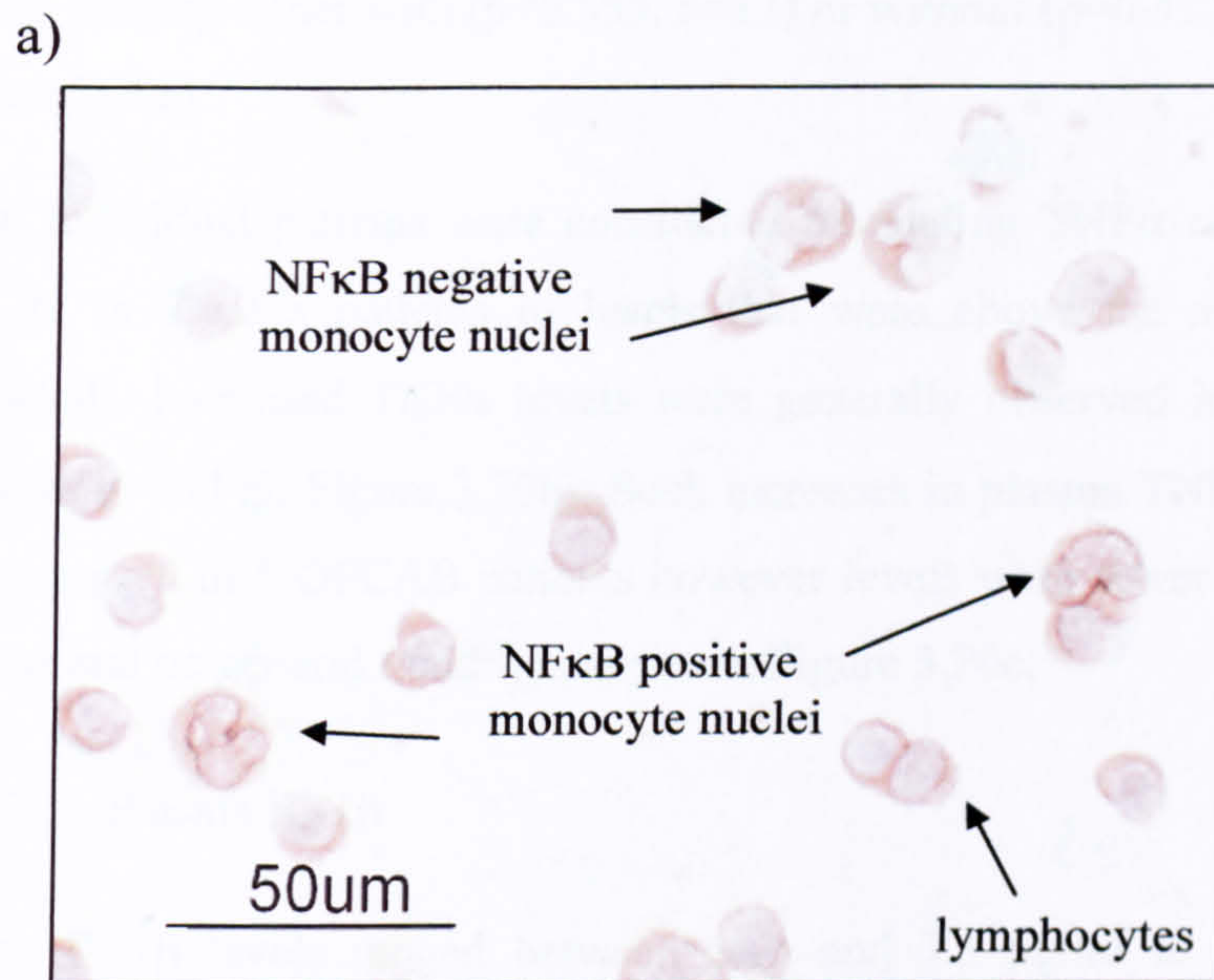


Figure 3.19. Effect of cardiac surgery on NFκB activation in monocytes. Representative immunoperoxidase staining of isolated PBMC demonstrating positive and negative NFκB p65 monocyte nuclei. Summary graph showing the percent of monocytes with detectable nuclear NFκB p65 at t1 and t4 in CPB (b, n=8) and OPCAB (c, n=6) patients.

3.4.4.2 Pro-inflammatory cytokines

3.4.4.2.1 Plasma TNFα

Plasma TNFα levels ranged between undetectable and 7.2 (median: 2.8) pg/ml in pre-operative baseline samples (t1, n=13) from the CPB group and were not different from that of the OPCAB group (median 0.8 (0 - 6.7 pg/ml, n=13, $p=0.427$). Grouped data failed to demonstrate any significant changes in TNFα levels during the course of

cardiac surgery either with ($p=0.555$, $n=13$) or without ($p=0.432$, $n=13$) the use of CPB (Figure 3.20a).

When individual patients were considered circulating TNF α concentrations increased from t1 in 7 CPB patients to levels that were above the sensitivity of the assay (4.4pg/ml). Increased TNF α levels were generally observed in the post CPB period (samples t4 and t5, Figure 3.20b). Such increases in plasma TNF α concentrations were also observed in 5 OPCAB patients however levels were lower than those of the CPB patients and no general trend was apparent Figure 3.20c.

3.4.4.2.2 Plasma IL-1 β

Plasma IL-1 β levels ranged between zero and 3.3 pg/ml in pre-operative baseline samples (t1, $n=13$) from the CPB group and were not different from those of the OPCAB group (0 - 8.1 pg/ml, $n=13$). Grouped data failed to demonstrate any significant changes in IL-1 β levels during the course of cardiac surgery either with ($p=0.521$, $n=13$) or without ($p=0.627$, $n=13$) the use of CPB (Figure 3.21a).

When individual patients were considered circulating IL-1 β increased above t1 levels in three CPB patients to concentrations that were above the sensitivity of the assay (>1.0 pg/ml); in one at t2 (5.2 pg/ml), one at t3 (20.1 pg/ml) and in one patient at t4 (4.4 pg/ml) Figure 3.21b. Such increases in circulating IL-1 β levels were detected in three OPCAB patients; one at t4 (3.9 pg/ml) one at t3 (1.5 pg/ml) and one at t2 (3.6 pg/ml) (Figure 3.21c).

3.4.4.3 Anti-inflammatory cytokines

Anti-inflammatory cytokines IL-4, IL-6 and IL-10 were quantified using BD CBA kit. Due to the restricted quantity of assay components cytokine measurements could only be made at four time points (t1, t4, t5 and t6).

3.4.4.3.1 Plasma IL-4

IL-4 plasma levels were low in all pre-operative baseline samples (0.0 - 5.6pg/ml in CPB patients ($n=7$) and 3.1 – 8.9pg/ml in OPCAB patients ($n=7$) and were not different between the two groups ($p=0.318$). Plasma IL-4 levels remained statistically unchanged in both groups of patients at t4, t5 and t6 ($p=0.507$ (CPB) and $p=0.905$ (OPCAB, Figure

3.22c)) however, there was a trend towards increased IL-4 two hours after the initiation of CPB (t4). No such trend was observed in the OPCAB patients.

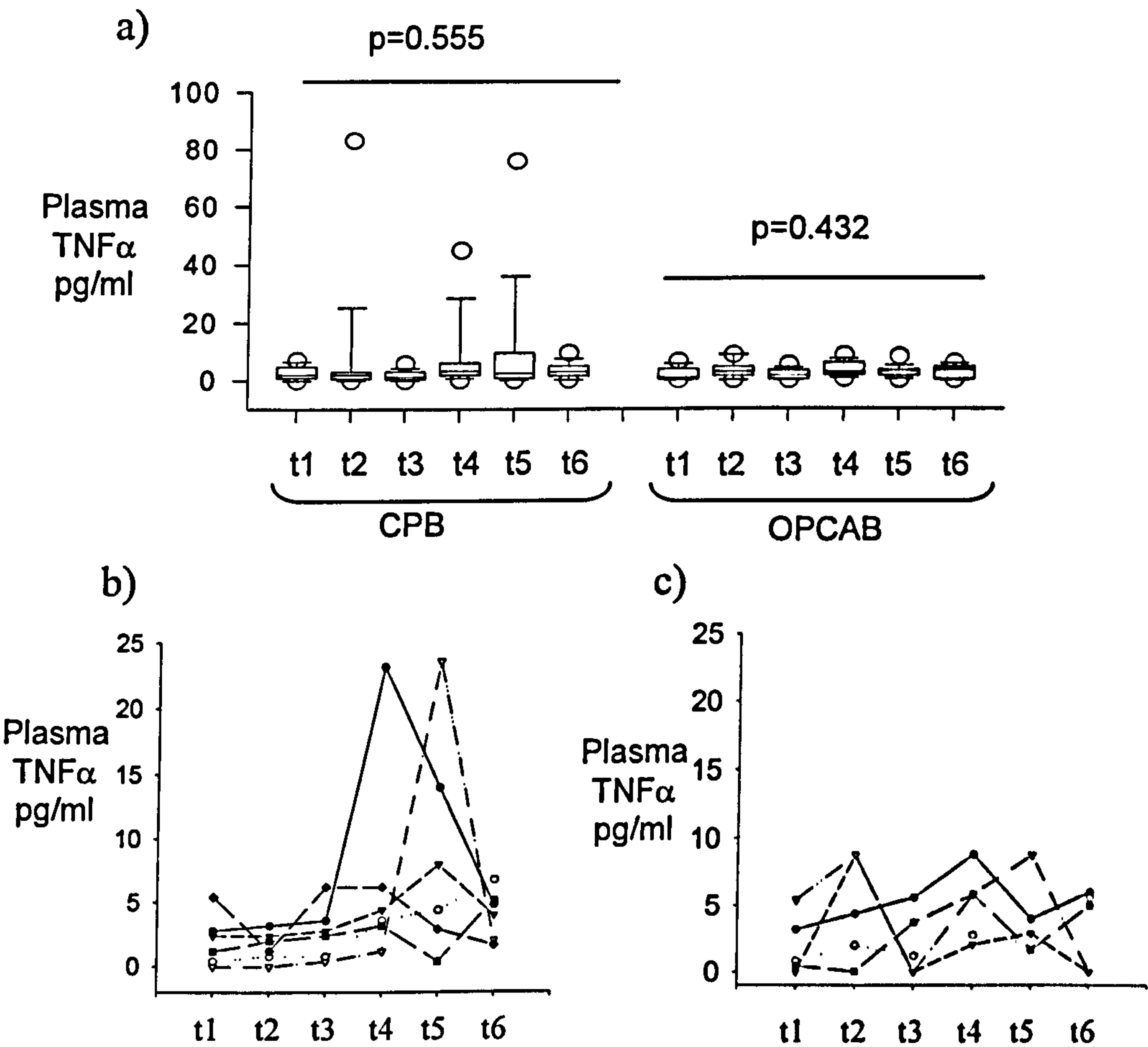


Figure 3.20. Effect of cardiac surgery with (CPB, n=13) and without (OPCAB, n=13) CPB on native plasma levels of TNFα a) Box and Whisker plots showing grouped data from the CPB and OPCAB patients. Individual data point from b) CPB patients and c) OPCAB patients in which TNFα levels increased. Data from one such CPB patient in which TNFα levels increased to 96.7, 48.8, 85.4 and 10.0pg/ml at t2, t4, t5 and t6 respectively is omitted from this graph for purposes of clarity.

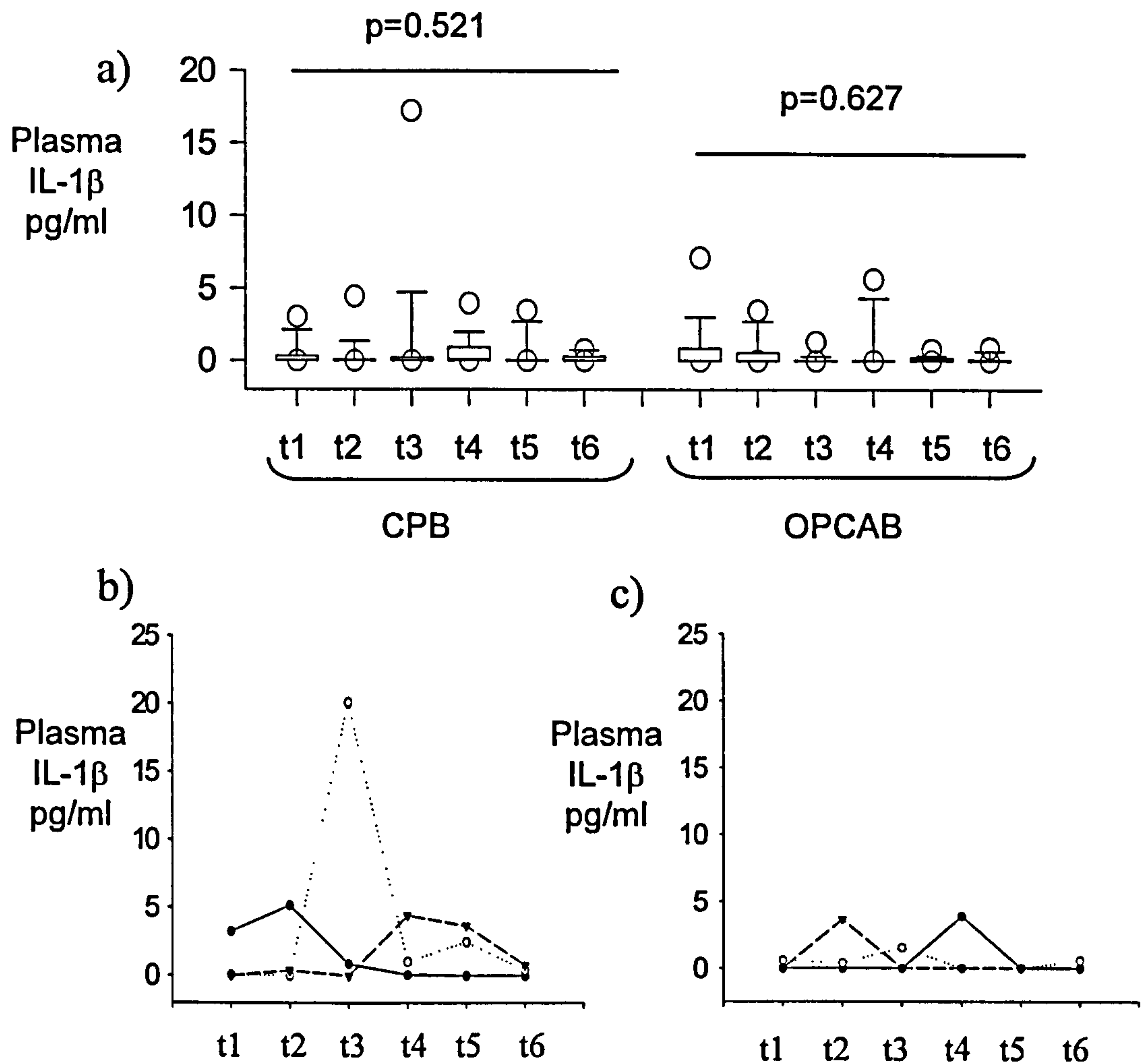


Figure 3.21. Effect of cardiac surgery with (CPB, n=13) and without (OPCAB, n=13) CPB on native plasma levels of IL-1 β a) Box and Whisker plots showing grouped data from the CPB and OPCAB patients. Individual data point from b) CPB patients and c) OPCAB patients in which IL-1 β levels increased.

3.4.4.3.2 Plasma IL-6

There was no difference in IL-6 levels in t1 samples between CPB and OPCAB patient (median: 3.4 (1.3 – 9.2) pg/ml (n=4) and median: 1.7 (1.4 – 3.6) pg/ml (n=4), respectively, $p=0.686$). Plasma IL-6 concentrations were significantly increased at five and 24 hours after the initiation of CPB (median: 1603.4 (83.0 - >5000.0) pg/ml, $p<0.05$, and median: 154.1 (105.1 – 1152.2) pg/ml, $p<0.05$, respectively) and after application of Octopus 3® (median: 316.8 (237.0 – 670.9) pg/ml, $p<0.05$, and 459.3 (284.0 – 598.5) pg/ml, $p<0.05$, respectively, Figure 3.22a). There was no statistical

difference in plasma IL-6 levels between the two groups of patients throughout the time course studied.

3.4.4.3.3 Plasma IL-10

Plasma levels of IL-10 in pre-operative baseline samples of CPB and OPCAB patients were not significantly different (median: 5.4 (1.2 – 6.4, n=11) and 5.4 (0.0-7.2, n=11) pg/ml, respectively, $p=0.870$). In CPB patients plasma concentrations of IL-10 were significantly greater than pre-operative baseline levels at two and five hours after the initiation of CPB (130.4 (11.2 – 1736.6) pg/ml, $p<0.05$ and (44.1 (4.8 – 875.5) pg/ml, $p<0.05$, respectively). Twenty four hours after the initiation of CPB plasma concentrations of IL-10 had returned to baseline levels (16.7 (3.8 – 28.6) pg/ml, $p>0.05$). Similar patterns of IL-10 plasma concentrations were observed in patients undergoing off pump surgery. At two and five hours after the application of the Octopus 3® IL-10 concentrations were significantly greater than those observed at baseline (57.7 (5.8 – 174.6) pg/ml, $p<0.05$ and 26.1 (4.3 – 393.1) pg/ml, $p<0.05$, respectively). In contrast to CPB patients IL-10 levels remained elevated at 24 hours (21.2 (4.6 – 40.7) pg/ml, $p<0.05$, n=10, Figure 3.22b). Statistically, there was no difference between the CPB and the OPCAB patients in terms of plasma IL-10 concentrations at any time point studied.

3.4.4.4 Soluble receptors

3.4.4.4.1 Plasma sTNFR1

Plasma levels of sTNFR1 were between 358.3 and 1395.8 (median: 908.0, n=13) pg/ml in CPB patients and between 316.7 and 1663.4 (median: 900.0, n=13) pg/ml in OPCAB patients ($p=0.876$). In both groups of patients circulating sTNFR1 levels remained unchanged in t2 and t3 samples. In CPB and OPCAB patients sTNFR1 plasma levels were significantly elevated at t4 (median: 1604.5 (562.5 – 2362.5) pg/ml, $p<0.05$ and 1333.3 (575.0 – 3033.3) pg/ml, $p<0.05$, respectively), and remained elevated in t5 and t6 samples (1529.5 (862.5 – 3484.8) and 1615.2 (1008.3 – 2541.7) pg/ml, respectively in the CPB patients, $p<0.05$ and 1787.5 (762.5 – 4090.2) and 2140.2 (600.0 – 2541.7) pg/ml respectively in OPCAB patients, $p<0.05$). There was no significant difference between CPB and OPCAB groups at any time point with respect to plasma sTNFR1 levels (Figure 3.23a).

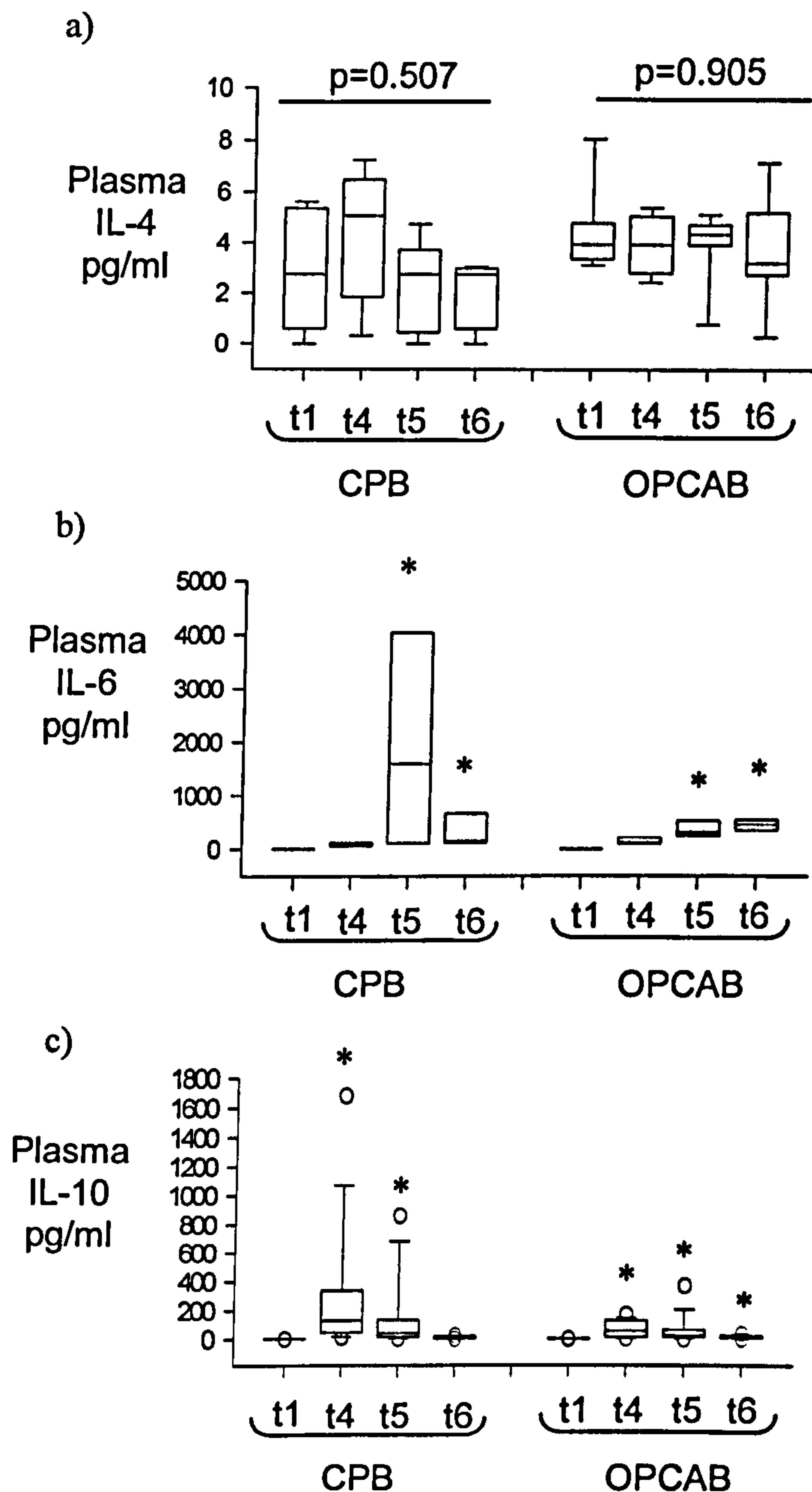


Figure 3.22. Effect of cardiac surgery with and without CPB on native plasma levels of the anti-inflammatory cytokines. a) IL-4, (b) IL-6, (c) IL-10. * Denotes p value < 0.05 from t1 pre-operative control.

3.4.4.4.2 Plasma sTNFR2

Pre-operative plasma sTNFR2 levels were 2020.2 (1461.7 – 4030.9) pg/ml and 2202.1 (967.0 – 3960.6) pg/ml in CPB and OPCAB patients respectively, $p=0.837$. In CPB patients although circulating sTNFR2 tended towards increased levels after CPB, similar to the pattern of sTNFR1 expression, statistically they did not deviate from pre-operative control levels at any time point studied ($p>0.05$, $n=13$). In OPCAB patients

circulating sTNFR2 levels began to increase 2 hours after the application of Octopus 3® and were significantly greater than pre-operative baseline levels by 24 hours after the application of Octopus 3® (t6, median: 3418.1 (1953.2 – 5160.6) pg/ml, n=13, p<0.05, Figure 3.23b).

3.4.4.4.3 Plasma sIL-1R2

High pre-operative concentrations of circulating sIL-1R2 were observed in all patients with no difference existing between the two groups (median: 23.2 (11.4 – 43.3) ng/ml, n=13 in CPB patients versus 22.2 (16.0 – 33.7) ng/ml, n=13 in OPCAB patients, p=1.00). These high plasma levels of sIL-1R2 decreased at the start of CPB reaching significantly lower levels one hour later (t3, median: 13.9 (2.25 – 24.33) ng/ml, p<0.05, n=13). Plasma sIL-1R2 levels remained lower than pre-operative levels over the following 24 hour time period, although these values were not statistically significant (t4 = 14.4 (6.8 – 23.4) ng/ml, t5 = 17.3 (4.7 – 34.5) ng/ml and t6 = 17.2 (9.9 – 29.1) ng/ml). In OPCAB patients although there appeared to be a decrease in plasma levels of sIL-1R2 following the application of Octopus 3® at no time point were these values statistically different from t1 (t2=17.0 (2.38 – 39.72) ng/ml, t3= 15.8 (2.3 – 31.2) ng/ml, t4 = 14.5 (9.56 – 30.6) ng/ml, t5 = 19.5 (8.7 – 30.6) ng/ml and t6 = 25.0 (12.3 – 33.9) ng/ml, p=0.080, n=13, Figure 3.23c).

3.4.4.5 Receptor antagonists

3.4.4.5.1 Plasma IL-1RA

Plasma levels of IL-1RA were variable in both CPB and OPCAB patients with no difference between the two groups at t1 (median: 0.86 (0.74 – 1.26) ng/ml in CPB patients (n=13) versus 0.86 (0.64 – 2.40) ng/ml in OPCAB patients (n=13), p=0.701). IL-1RA plasma levels remained unaltered in both groups of patients until 2 hours after the initiation of CPB or application of Octopus3® when circulating levels significantly increased above pre-operative levels (1.52 (0.9 – 17.10) ng/ml, p<0.05, n=13 and 2.54 (1.2 – 18.03) ng/ml, p<0.05, n=13, respectively). IL-1RA plasma levels continued to increase to 11.7 (2.0 – 64.3) ng/ml in CPB patients and 10.2 (1.8 – 61.5) ng/ml in OPCAB patients at t5. By 24 hours plasma levels of IL-1RA had decreased although they were still significantly higher than pre-operative levels in both groups of patients (1.3 (1.0 – 11.2) ng/ml, n=13 p<0.05 in CPB patients and 2.1 (1.3 – 55.3) ng/ml, p<0.05, n=13 in OPCAB patients, Figure 3.24).

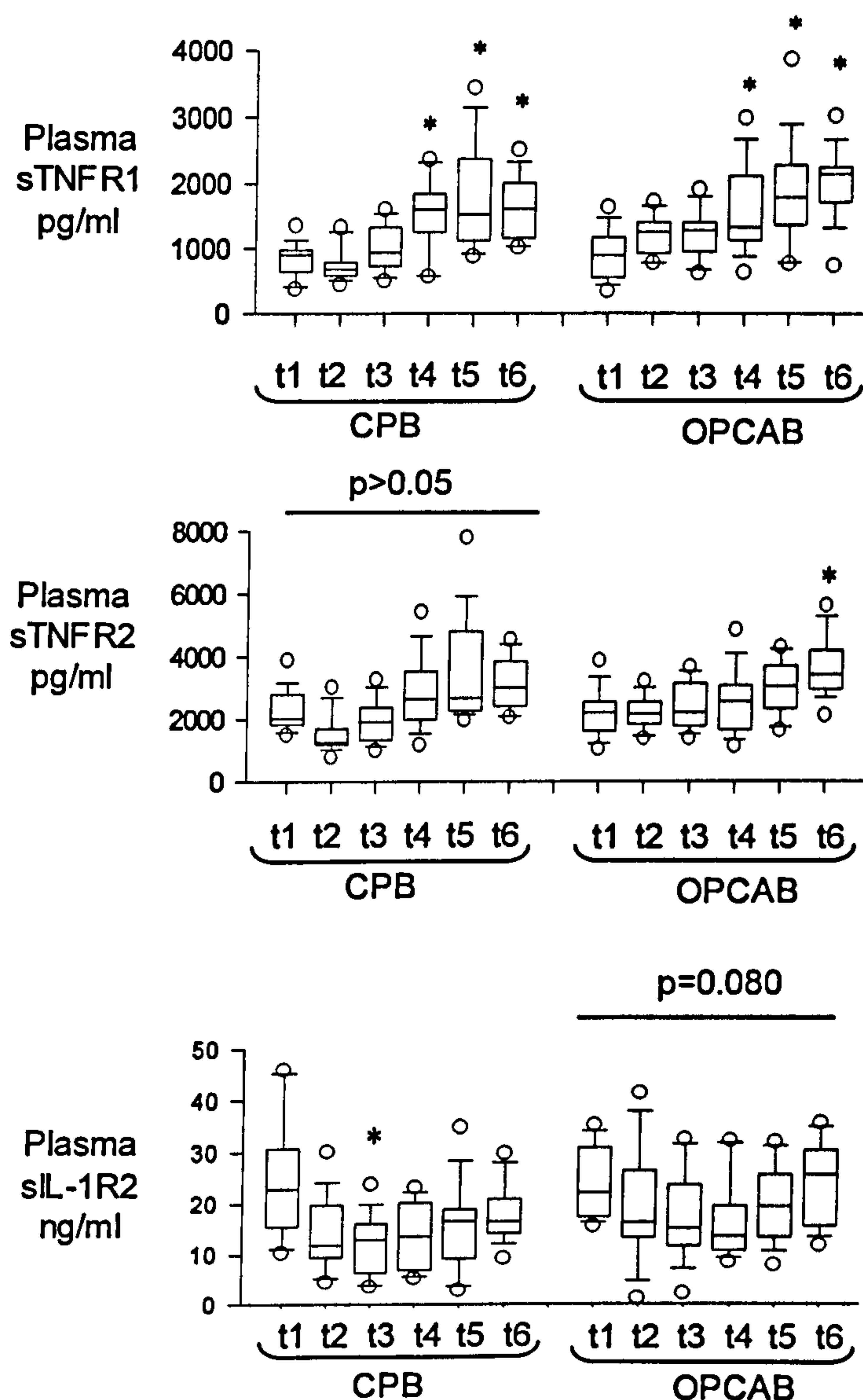


Figure 3.23. Effect of cardiac surgery with (CPB: n=13) and without (OPCAB: n=13) CPB on native plasma levels of soluble cytokine receptors. a) sTNFR1. b) sTNFR2 and c) sIL-1R2. * Denotes p value < 0.05 from t1 pre-operative control

3.4.4.6 Cytokine balance

Molar ratios were calculated as described for study N°1 (page 121 and page 123)

3.4.4.6.1 Molar ratio sTNFR1 to TNF α

There was 197.2 (55.0 – 1301.6) times molar excess of sTNFR1 to TNF α in the pre-operative plasma of patients undergoing cardiac surgery utilising CPB. The molar ratio of sTNFR1 to TNF α in the plasma of these patients did not significantly change over the time course studied ($p=0.663$, n=13). Similarly and not significantly different from

CPB patients, the molar ratio of sTNFR1 to TNF α in pre-operative plasma from OPCAB patient was 392.8 (26.9 – 1564.00, $p=0.238$) and remained unchanged throughout the time course studied ($p=0.736$, $n=13$, Figure 3.25a).

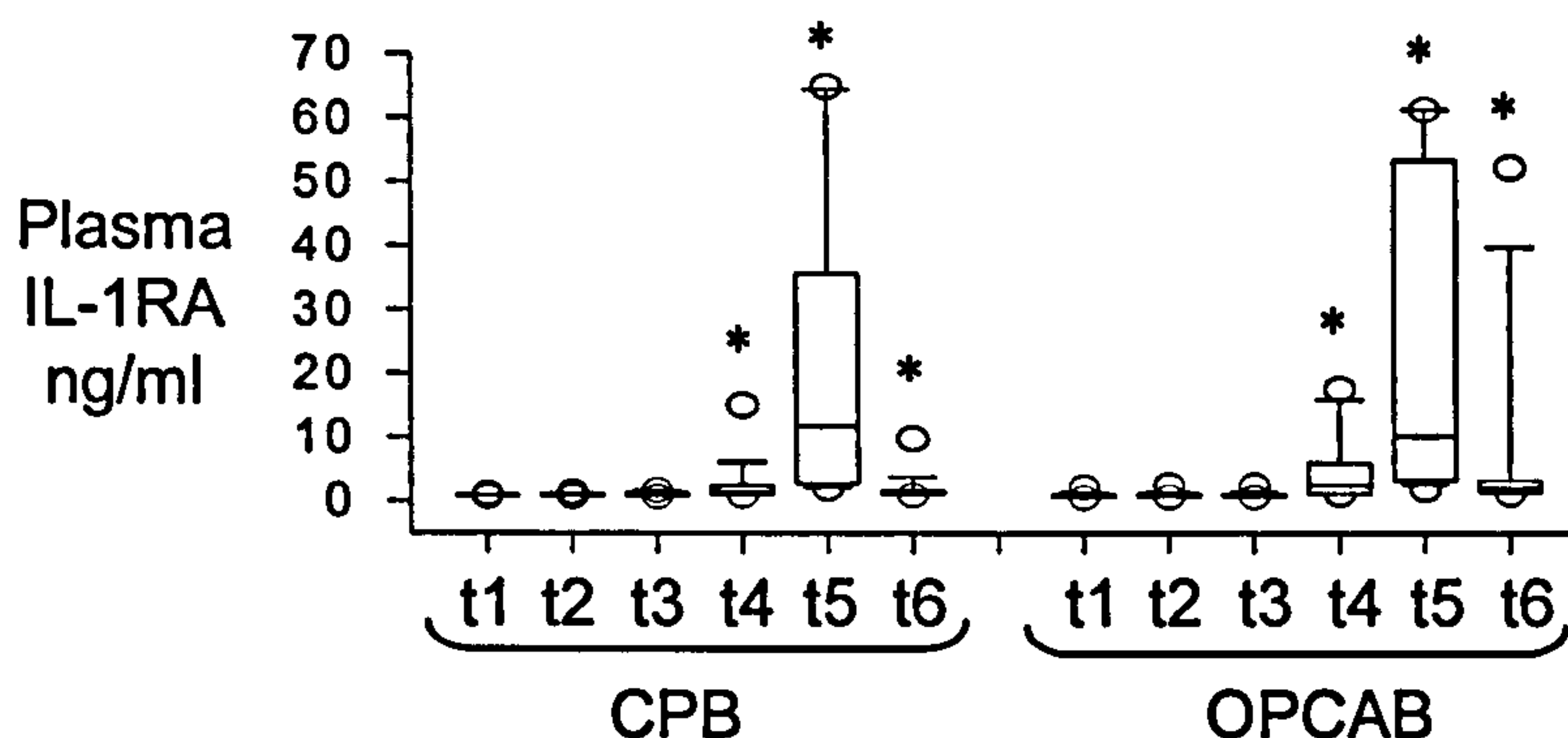


Figure 3.24. Effect of cardiac surgery with ($n=13$) and without ($n=13$) CPB on native plasma levels of IL-1RA. * Denotes p value < 0.05 from t1 pre-operative control

3.4.4.6.2 Molar Ratio sTNFR2 to TNF α

In CPB patients' plasma there was a pre-operative molar excess of sTNFR2 to TNF α of 376.31 (86.3 – 2502.5), which remained unchanged during the course of surgery and throughout the post-operative time course studied ($p=0.894$, $n=13$). Similarly, in OPCAB patients pre-operative plasma molar excess of sTNFR2 to TNF α was 673.1 (91.2 – 3216.3) and did not significantly change throughout the time course studies ($p=0.252$, $n=13$, Figure 3.25b).

3.4.4.6.3 Molar ratio total sTNFR to TNF α

The molar ratio of total sTNFR (sTNFR1 plus sTNFR2) to TNF α in plasma did not significantly deviate from pre-operative baseline levels in either CPB or OPCAB patients ($p=0.861$ and $p=0.476$, respectively, Figure 3.25c).

3.4.4.6.4 Molar Ratio sIL-1R2 to IL-1 β

The median pre-operative molar ratio of plasma sIL-1R2 to IL-1 β in the CPB patient group was 36524.8 (2064.4 – 90661.9) and in OPCAB group was 38297.9 (1334.9 – 68676.1, $p=0.798$). In both CPB and OPCAB patients these levels remained unchanged

during the time period studied ($p=0.755$, $n=13$ and $p=0.445$, $n=13$ respectively, Figure 3.26a).

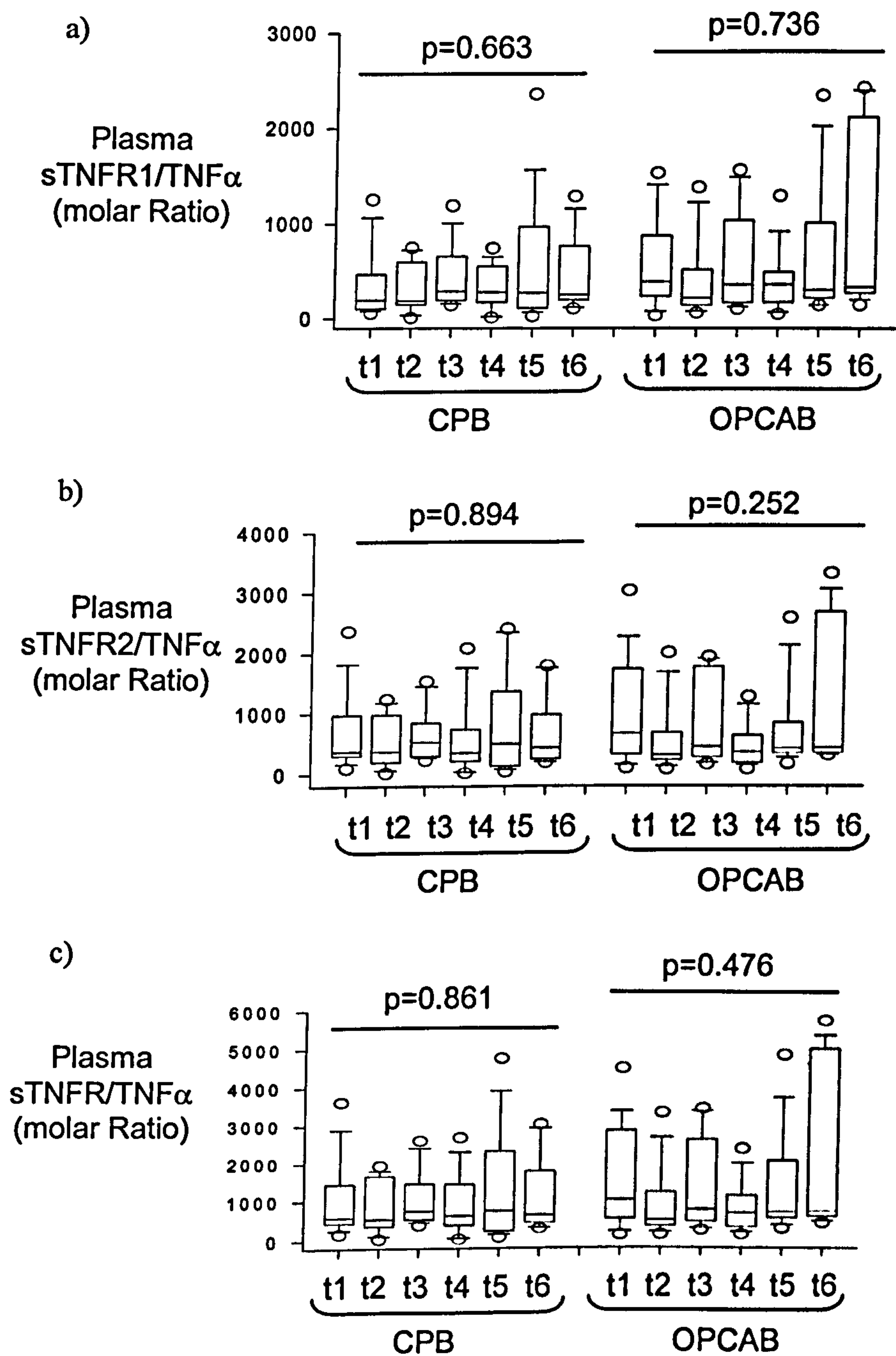


Figure 3.25. Effect of cardiac surgery with and without CPB on the molar ratios of soluble TNF receptors to TNF α in native plasma a) sTNFR1/TNF α . b) sTNFR2/TNF α and c) total sTNFR/TNF α

3.4.4.6.5 Molar ratio of IL-1RA to IL-1 β

In CPB patients at pre-operative baseline there was a 4470.59 (258.68 – 6470.52) times molar excess of IL-1RA to IL-1 β . This was not significantly different to pre-operative samples from OPCAB patients (median: 4470.59 (98.50 – 14117.65), $p=0.939$). In the CPB patient plasma the molar ratio of IL-1RA/IL-1 β was significantly greater than pre-operative levels 5 hours after the initiation of CPB (median: 34961.32 (396.66 – 56141.20), $p<0.05$, $n=13$). By 24 hours the ratio of IL-1RA to IL-1 β had returned to pre-operative levels (7411.76 (1422.67 – 14843.40), $p>0.05$, $n=13$). In OPCAB patients there was a significant increase in molar excess of IL-1RA to IL-1 β in plasma at t4 (13294.12 (215.78 – 106042.02), $p<0.05$). These levels increased further at t5 (60000.00 (3861.69 – 361294.12), $p<0.05$) and remained significantly greater than pre-operative levels 24 hours after the application of the Octopus3® (10411.76 (1418.19 – 325537.82), $p<0.05$, Figure 3.26b).

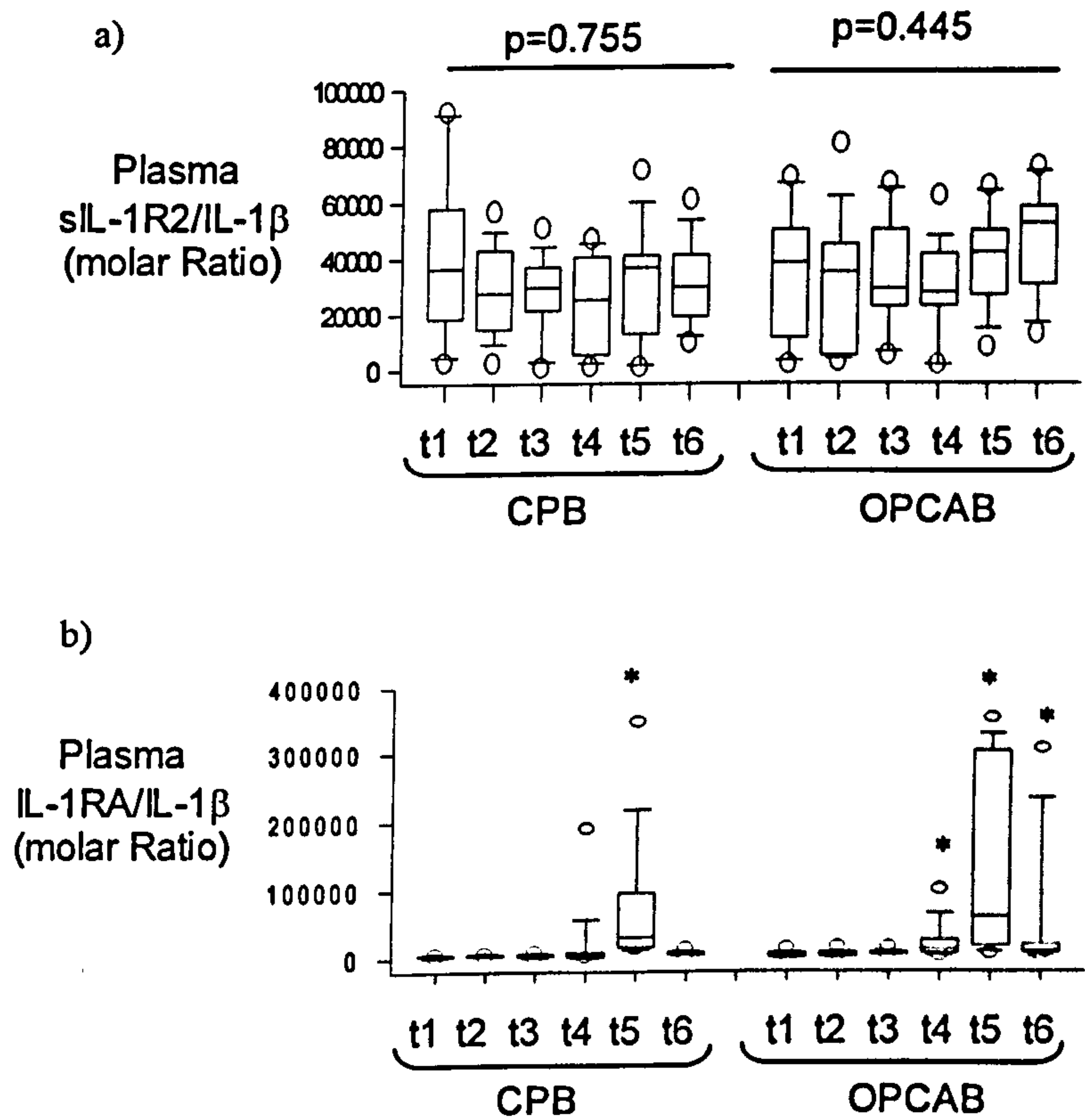


Figure 3.26. Effect of cardiac surgery with (CPB, $n=13$) and without (OPCAB, $n=13$) CPB on the molar ratios of endogenous inhibitors of IL-1 β sIL-1R2 and IL-1RA to IL-1 β in native plasma a) sIL-1R2/IL-1 β b) IL-1RA/IL-1 β * Denotes p value < 0.05 from t1 pre-operative control

3.4.5 DISCUSSION

These data demonstrate that there is a significant increase in neutrophil and monocyte activation following cardiac surgery with CPB that was not seen in patients undergoing off-pump cardiac surgery. However, despite this inflammatory cell activation there was no significant change in the circulating levels of the pro-inflammatory cytokines, TNF α or IL-1 β following CPB. In contrast the predominant and consistent response to CPB was the induction of soluble anti-inflammatory mediators (sTNFR, IL-1RA, IL-10 and IL-6), a response that was also observed in patients undergoing cardiac surgery without CPB.

Cardiac surgery with CPB has long been associated with production of inflammatory cytokines and the induction of a systemic inflammatory response that significantly contributes to several adverse postoperative outcomes, including cardiac, renal, pulmonary and neurological complications, bleeding, and even multiple organ dysfunction. Major contributing factors in the initiation of such a response are believed to be the ischaemia-reperfusion injury suffered by the heart and the lungs during the period of aortic cross clamp, the contact of blood with the non-endothelial surface of the bypass circuit and oxygenator, endotoxemia and the activation of complement. In order to overcome such adverse effects, techniques of 'off-pump' coronary artery bypass (OPCAB) surgery have been developed that avoid both the global myocardial ischaemia and the use of extracorporeal circuit. With the recent development of effective devices for target vessel exposure and stabilization, OPCAB via median sternotomy has gained widespread use as an alternative technique to conventional on-pump CABG and may result in fewer episodes of post-operative arrhythmia and pulmonary and neurological complications. Indeed, several recent reports have suggested that there is an improved clinical outcome in patients undergoing CABG when CPB is avoided (Al Ruzzeh et al., 2003; Angelini et al., 2002; Deuse et al., 2003; Lee et al., 2003; Potger et al., 2002). However, other studies have failed to demonstrate appreciable clinical benefits of OPCAB techniques over conventional CABG with CPB (Khan et al., 2004; Salamon et al., 2003) and concerns over the quality of anastomosis and graft patency as a result of the increased surgical experience and skill required to perform OPCAB surgery and reduced long term efficacy have brought into question the true benefits of OPCAB.

This present study was designed to investigate the contribution of CPB and potential benefits of OPCAB surgery with respect to leucocyte activation and the observed alteration in circulating cytokine profile in patients undergoing cardiac surgery.

3.4.5.1 Leucocyte activation

The activation of neutrophils, adherence to activated endothelium and accumulation of neutrophils in the lungs and myocardium appear to play an important role in the pathogenesis of the inflammatory response and enhanced risk of organ failure after cardiac surgery. Increases in the surface expression of CD11b is a common method of measuring neutrophil activation and has previously been shown to increase as a result of cardiac surgery (Asimakopoulos et al., 2000b; Rinder et al., 1992). The use of CPB is often held responsible for this leucocyte activation during cardiac surgery through the contact of blood constituents with the non-endothelial surface of the extracorporeal circuit and through the activation of complement. In support of this concept is the demonstration that blood circulating through a simulated extracorporeal circuit also induces an upregulation of both neutrophil and monocytes CD11b expression (Finn et al., 1996; Rinder and Fitch, 1996).

In the study presented in this thesis increased neutrophil CD11b and monocytes nuclear content of NF κ B p65 were not observed in the OPCAB patients suggesting that the use of cardiopulmonary bypass is a major contributing factor. Whether the artificial surface of the extracorporeal circuit and the oxygenator or the global myocardial ischaemia and subsequent reperfusion are responsible is unclear. Although the time course of events suggest that the latter is unlikely to be responsible for the observed increase in neutrophil CD11b expression.

Neutrophil CD11b is contained within the secretory vesicles (~20% of the total cell CD11b) and the gelatinase-containing and the secondary granules (75%, (Mollinedo et al., 1997; Sengelov et al., 1993). CD11b is mobilised to the cell surface first from the secretory vesicles upon weak stimulation. Stronger stimulation will result in CD11b incorporation in the cell membrane next from the gelatinase-containing granules and then finally from the specific granules (Sengelov et al., 1993). The contents of these CD11b-containing granules are generally mobilised before the contents of the azurophil/primary granules and thus CD11b surface expression is an early marker of neutrophil activation and may be followed by release of cytotoxic contents of the other granules.

In monocytes CD11b is contained within rapidly mobilised intracellular secretory granules in the cytoplasm (Calafat et al., 1993) and its surface expression has been shown to be upregulated during cardiac surgery with CPB. Consistent with the delayed

activation and recruitment of monocytes to sites of inflammation compared to neutrophils, CD11b expression on the monocytes has been shown to steadily increase during the course of surgery peaking some 2-4 hours after the termination of CPB (Rinder et al., 1992). Given this data, the lack of effect of cardiac surgery with CPB on monocyte CD11b expression in this current study may simply reflect the inappropriate timing of sample collection for the purpose of such measurements. Activation of pro-inflammatory pathway did however appear to occur in monocytes following CPB as demonstrated by the increased nuclear content of NF κ B p65. This effect appears to be dependent on the use of CPB. The stimuli for such transcription factor activation has not been determined in this setting but could be due to a number of factors such as endotoxin, thrombin, reactive oxygen species, complement C3a and C5a (Pan, 1998), β_2 integrins binding to either ICAM-1, iC3b or fibronectin (Sitrin et al., 1998) or cytokines. Such NF κ B activation in monocytes has been recently demonstrated in a simulated bypass circuit with nuclear NF κ B DNA binding activity rapidly increased after initiation of blood circulation. Such NF κ B activation has been shown to be responsible for the monocyte tissue factor expression with a subsequent increase in thrombin generation and pro-coagulant activity of blood (Morgan et al., 2003). That study not only suggested that the CPB circuit itself and not ischaemia-reperfusion was responsible for the activation of NF κ B in monocytes, but also demonstrated an essential role for this transcription factor in initiating adverse events related to such procedures. However, that was in a simulated bypass circuit in which the pro-inflammatory response appears to dominate in the absences of the phased anti-inflammatory response that is observed *in vivo* (McBride et al., 1995). In this respect the presence of anti-inflammatory factors such as IL-10 or IL-4 may interfere with NF κ B transactivation and inhibit the expression of inflammatory genes. Indeed the presence of such an anti-inflammatory response *in vivo* may explain the failure of some studies to detect increased monocyte tissue factor expression in patients undergoing cardiac surgery with CPB. With this in mind it is important not to draw too many unsubstantiated conclusions as to the importance of nuclear NF κ B p65 in the monocytes of the patients undergoing CPB since target gene transcription has not been measured. As such, nuclear NF κ B p65 in these cells can only be used as a measure of cell activation and the ability of such procedures to initiate inflammatory signalling pathways.

Unfortunately the methods used in this study to analyse NF κ B nuclear translocation was not well tolerated by all of the samples and poor cell morphology prevented analysis of

some of the slides. Other techniques, such as DNA binding assays or, better still, methods not requiring differential cell separation, for instance, flow cytometric analysis of whole blood samples (Hasegawa et al., 2003) will be required in order to gain a better understanding of the time course of monocyte NF κ B activation under such circumstances.

Nevertheless, this data suggests that the use of CPB is responsible for the activation of neutrophil (increased CD11b) and monocytes (NF κ B).

3.4.5.2 Circulation cytokines

Two randomized studies that have compared the kinetics of circulating TNF α levels in CABG versus OPCAB have yielded some what conflicting results. In the study from Schulz et al significant increases in the circulating levels of TNF α were observed in both OPCAB and CPB groups of patients (Schulze et al., 2000b) and throughout the study period levels were significantly greater in the CABG group. In contrast, in the study from Matata et al, no changes in TNF α levels were observed in OPCAB patients and in the CABG patients there was a trend towards increased TNF α in the early post-operative hours, which reached statistical significance only after 48 hours (Matata et al., 2000).

Given the significant leucocyte activation in the patients undergoing cardiac surgery with CPB, in particular the induction of NF κ B activity in the monocytes, it was surprising that no consistent change in circulating levels of pro-inflammatory cytokines were observed in either study N $^{\circ}$ 2 or N $^{\circ}$ 3 which covered an extended period of observation with frequent sampling. Confirming the observations from study N $^{\circ}$ 2 circulating levels of TNF α were increased to varying extents in seven of the patients undergoing CPB and IL-1 β was increased in three, two of which also demonstrated increased TNF α . In the OPCAB group 5 patients demonstrated increased TNF α levels, three of which also had increased IL-1 β levels, although circulating concentrations were not of the magnitude observed in some of the CPB patients. This data suggests that cardiac surgery, regardless of the technique used (CPB or OPCAB) is capable of initiating some degree of an inflammatory response in some patients, the magnitude and prevalence of which appears to increase with the use of CPB. The reason for the increased susceptibility of some patients for increases in certain markers of a systemic inflammatory response is unclear. Age, smoking habits, diabetes, hypercholestromia,

hypertension, NYHA functional class, time on bypass or pre-operative cytokine profile of patients did not appear to be different between responders and non-responders.

All of the patients included in this study experienced an uneventful post-operative course without significant clinical complications, implying that these increases in post-operative circulating pro-inflammatory cytokines and apparent increased leucocyte activation are clinically inconsequential. This is maybe due to a sizeable anti-inflammatory armoury capable of counteracting the pro-inflammatory response. Indeed this was suggested from the results of study N°1 in which these CABG patients and also the heart failure patients appeared to have a predominance of anti-inflammatory factors in their circulation immediately prior to surgery. In addition, cardiac surgery, both OPCAB and CABG induced a further increase in circulating anti-inflammatory cytokines (IL-6, IL-10), soluble receptors and receptor antagonist, the magnitude of which were comparable for the two operative techniques. Importantly, the molar ratio of TNF α to its soluble receptors was maintained throughout the peri- and post-operative period and that for IL-RA to IL-1 β was significantly increased, thus giving further credibility to the notion of the predominant anti-inflammatory response to cardiac surgery.

It is interesting to observe that the anti-inflammatory response was similar in the OPCAB and CPB patients suggesting that this response is a consequence of factors other than the use of cardiopulmonary bypass and also is not simply a compensatory mechanism to counteract increased circulating pro-inflammatory cytokines.

3.4.6 CONCLUSION

In conclusion this study has demonstrated that cardiac surgery appears to be able to initiate an inflammatory response in some patients, the magnitude of which increases with the use of CPB possible as a result of increased leucocyte activation. The magnitude of the anti-inflammatory response was independent of the use of CPB, occurred in the early post-operative period (2-5 hours) and was apparently capable of inhibiting the adverse consequences of the pro-inflammatory response.

3.5 GENERAL DISCUSSION

The data presented in this chapter demonstrate that the increased pro-inflammatory response observed in the circulation of the patients studied was accompanied by a substantial anti-inflammatory response apparently maintaining a balance in favour of

the latter. Under such circumstances certain biological consequences of increased $\text{TNF}\alpha$ or $\text{IL-1}\beta$ may be reduced or inhibited and thus caution should be taken when speculating as to the role of increased pro-inflammatory cytokines in the circulation of these patients.

Chapter Four: THE EFFECT OF INFLAMMATORY CYTOKINES ON NF κ B ACTIVITY IN CULTURED HUMAN CELLS

4.1 INTRODUCTION

The role of vascular cells during the inflammatory process is critical. Multiple cytokines and growth factors are present at sites of inflammation and in the circulation that can potentially influence the nature of the inflammatory response. Through the activation of transcription factors such as NF κ B, cells in the vessel wall (endothelial cells (EC) and smooth muscle cells (SMC)) must integrate the signals generated by these multiple factors to effectively regulate the immuno-inflammatory response through the expression of adhesion molecules, cytokines, chemokines, matrix metalloproteinases, and growth factors.

In addition to the manifestation of an inflammatory response in the myocardium and the vasculature during heart failure and in response to cardiac surgery, it is now becoming increasingly recognised there is also a significant pulmonary component to these pathologies in which pulmonary epithelial cells likely play a crucial role.

4.1.1 HEART FAILURE AND PULMONARY INFLAMMATION

It is known that when the left ventricle is dysfunctional, as in chronic heart failure, increased pulmonary venous pressure becomes the leading cause for damage of the alveolar-capillary interface that impairs the pulmonary gas transfer at the alveolar epithelial-vascular endothelial junction and contributes to the patient's symptoms and physical limitation. In addition, deterioration in gas conductance through the alveolar-capillary interface is a powerful independent predictor of a worse prognosis in stable heart failure (Guazzi et al., 2002). Chronic microvascular pulmonary pressure elevation observed during heart failure can induce pressure injury/pulmonary oedema resulting in adaptive structural changes that serve to thicken the alveolar-capillary barrier. Such pulmonary remodelling requires recruitment and activation of inflammatory cells at the alveolar-capillary barrier, which may also serve to potentiate alveolar-capillary barrier damage. De Pasquale et al found that there was an increase in neutrophil number and myeloperoxidase in the bronchoalveolar lavage fluid in rat model of heart failure (De Pasquale et al., 2003). These cells with their potentially harmful secretory products such as enzymes, reactive oxygen species, MMP and pro-inflammatory cytokines are thought to play an important role in epithelial cell activation and injury and in the early phase of lung architecture remodelling. In addition, the bi-directional protein movement across the alveolar-capillary barrier that is increased in heart failure can also potentially

activate resident cells (epithelial cells, macrophages) and contribute to this pathophysiological defect.

4.1.2 CARDIAC SURGERY AND PULMONARY INFLAMMATION

There is a large body of evidence demonstrating a significant pulmonary injury following cardiac surgery with cardiopulmonary bypass. Indeed pulmonary dysfunction following CPB is the most common clinical manifestation of bypass-induced damage. CPB can lead to changes in the bronchoalveolar architecture by inducing atelectasis (Magnusson et al., 1997), which facilitates production of pro-inflammatory cytokines by alveolar macrophages (Kisala et al., 1993) resulting in distal airway inflammation. Increased concentrations of IL-8 are found in the lungs following CPB (McBride et al., 1996) leading to the accumulation of activated neutrophils in the lungs (Kotani et al., 2000) which release the toxic contents of their intracellular granules and reactive oxygen species leading to lung injury. Increased expression of $\text{TNF}\alpha$, IL-8 and IL-6 have also been demonstrated in the leucocytes in the lungs of patients following CPB and were greater than those observed in the leucocytes in the systemic circulation (Kotani et al., 2000).

4.1.3 THE ROLE OF EPITHELIAL CELLS IN PULMONARY INFLAMMATION

Increasing evidence suggests that the airway epithelium is more than simply a passive, physical barrier but that it also plays an important role in the regulation of inflammatory processes in the lungs and airways. Under such circumstances of pulmonary inflammatory responses the epithelial cells are exposed to inflammatory mediators and inflammatory cells in the alveolar space just as the endothelial cells are exposed to such factors in the circulation. Studies have demonstrated that airway epithelial cells are able to act as immune effector cells by secreting pro-inflammatory cytokines and chemotactic factors such as IL- 1β , $\text{TNF}\alpha$, IL-6, IL-8, RANTES, GM-CSF and MCP-1 (Manni et al., 1996; Marini et al., 1992; Stadnyk, 1994; Standiford et al., 1990, 1991). The secretion of such pro-inflammatory factors in concert with the ability of epithelial cells to express a variety of surface molecules including ICAM-1 increases the inflammatory situation within the lung and facilitates the activation and adhesion of inflammatory cells (Tosi et al., 1992). The adhesion of activated leucocytes and the secretion of toxic substances and reactive species significantly contribute to the epithelial damage and subsequent lung injury that is observed in pathologies such as asthma and acute lung injury following cardiac surgery (Bloemen et al., 1993).

4.1.4 CONTROL OF ADHESION MOLECULE EXPRESSION

The recruitment of leucocytes from the circulation into the extravascular space is critical for inflammatory responses and repair of injured tissue and is reliant on the regulated expression of adhesion molecules such as E-selectin and ICAM-1.

4.1.4.1 E-selectin

The gene encoding E-selectin (or CD62E) is transcriptionally silent in quiescent endothelial cells but it is rapidly and transiently transcribed when the cells are activated by pro-inflammatory stimuli such as IL-1 β , TNF α or LPS. E-selectin interacts with sialylated Lewis X glycoprotein on the surface of leucocytes to mediate the 'leucocyte rolling' (Brandley et al., 1990).

Studies of the E-selectin promoter have identified several positive regulatory domains (PD) necessary for TNF α responsiveness. Three of these elements PD1, PDIII and PDIV contain NF κ B recognition sequences and one element PDII contains an AP-1 binding sequence. Upon activation NF κ B is believed to associate with the chromatin architectural protein, high mobility group protein 1 (Y) (HMG1(Y)) at possibly three functional site in the E-selectin promoter (Read et al., 1997).

4.1.4.2 ICAM-1

The adhesion molecule ICAM-1 (CD54) is a member of the immunoglobulin supergene family that is expressed in haematopoietic and non-haematopoietic cells (Springer, 1990). ICAM-1 mediates adhesive interactions by binding to two members of the β_2 integrin family CD11a/CD18 and CD11b/CD18 (Diamond et al., 1990; Staunton et al., 1988). ICAM-1 expression is essential in the transendothelial migration of leucocytes and in the activation of T-cells where it has been shown to be co-stimulatory in activation of T-cell receptor complex (Zuckerman et al., 1998).

The induction of the ICAM-1 transcription occurs rapidly indicating the utilisation of latent transcription factors. Indeed the 5'-flanking region of the ICAM-1 gene has been cloned and shown to possess potent constitutive and inducible promoter activity. Upstream of each initiation site is a consensus TATA element that binds the general transcription factor IID (TFIID). The ICAM-1 promoter also contains a number of binding sites for inducible transcription factors. The TNF α and IL-1 β responses are mediated by the co-operation between the transcription factors C/EBP and NF κ B. The

IFN γ response is mediated by STAT binding to the IFN γ response element (IRE). The H $_2$ O $_2$ response is mediated by the antioxidant response elements, which bind the transcription factors AP-1 and Ets (Roebuck and Finnegan, 1999).

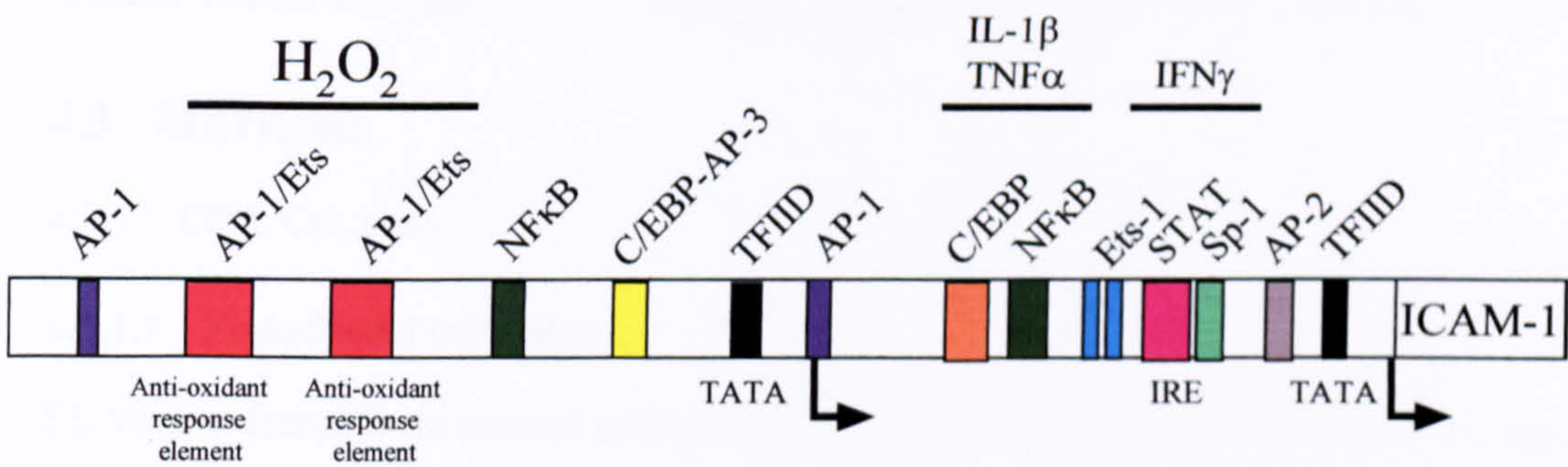


Figure 4.1. Structure and regulation of the ICAM-1 promoter.

4.1.4.3 Role of NF-κB in the Regulation of Adhesion Molecule Expression

Using a degradation resistant IκBα Lockyer et al demonstrated the essential requirement NFκB activity in TNFα-induced E-selectin and ICAM-1 expression in cultured human endothelial cells (Lockyer et al., 1998). Such an important role of NFκB in the induction of these adhesion molecules was confirmed by Denk et al with the use of dominant negative IKKβ (kinase inactive) and degradation resistant mutant of IκB proteins (Denk et al., 2001). That group demonstrated that TNFα -induced expression of E-selectin and ICAM-1 on cultured human endothelial cells was inhibited when NFκB activation was prevented. Further support for the dominant role of NFκB in adhesion molecule expression comes from the observation that endothelial cells expressing constitutively active IKKβ showed pronounced expression of adhesion molecules (E-selectin, ICAM-1 and VCAM-1) in the absence of additional pro-inflammatory stimuli.

* * *

Thus, in cells such as EC, vascular SMC and pulmonary epithelial cells that are exposed to increased pro-inflammatory cytokines during systemic and pulmonary inflammatory disorders the potential activation of NFκB may critically determine the expression of adhesion molecules and therefore control inflammatory cell activation and recruitment.

4.2 AIMS

To determine the influence of defined pro-inflammatory factors (IL-1 β , TNF α) on NF κ B activity and adhesion molecule expression in human endothelial cells, vascular smooth muscle cells and pulmonary epithelial cells in culture.

4.3 METHODS

4.3.1 CELL CULTURE

4.3.1.1 Endothelial cell culture

EC were cultured from unused portions of pulmonary arteries (PA) from heart and lung donors.

4.3.1.1.1 Isolation and propagation

The tissue was removed from the HBSS collection medium and placed in a sterile petri dish in a tissue culture hood. The PA was opened using scissors and laid flat on the dish luminal surface upwards. EC were removed from the luminal surface of the vessel by light scraping with a size 11 scalpel blade taking care not to remove any underlying smooth muscle cells. The harvested EC were transferred into M199 medium in a centrifuge tube. To maximise the endothelial cell yield this process was repeated until the whole lumen surface of the vessel had been scraped. The harvested cells were then centrifuged at 365 x g for 5 minutes. The supernatant was discarded and the cells were resuspended in 3 ml M199 growth medium (Sigma-Aldrich) supplemented with penicillin (100 units/ml), streptomycin (100 μ g/ml), L-glutamine (2mM), endothelial cell growth factor (Bovine Brain Extract 25 μ g/ml), heparin (15 units/ml, Sigma-Aldrich), 20% HI-FBS and HEPES (25mM, pH 7.4, Sigma-Aldrich). The cell suspension was seeded into 60mm plastic tissue culture dish pre-coated with 0.5% gelatine. Cells were maintained in a humidified atmosphere of 5% CO₂ /95% air at 37°C. Cells were fed every 2-3 days with fresh medium. When confluent, cells were propagated by trypsinisation and transferred to larger gelatine-coated tissue culture vessels (never reseeding the cells at less than 1/3rd their previous density).

4.3.1.1.2 Endothelial cell characterisation

EC were characterised visually by their cobble-stone appearance when confluent and positive expression of CD31 on their surface. E-selectin is an endothelial cell-specific adhesion molecule thus induction of E-selectin expression on these cells also served to

characterise them as EC. Ability to induce E-selectin expression on these cells was lost with increasing propagation demonstrating the de-differentiating nature of prolonged culturing process. For this reason EC were always used at the lowest passage possible (between 3 and 8) and inability to induce E-selectin expression was considered a contraindication for using individual cell isolates.

4.3.1.2 Smooth muscle cell culture

SMC were cultured from unused pieces of aorta from heart and heart/lung donors after the removal of EC as described above (section 4.3.1.1.1).

4.3.1.2.1 Isolation and propagation

After removal of the EC the inner most layer of media smooth muscle was easily peeled away from the remaining media and adventitia. This smooth muscle tissue was finely minced using scissors and scalpel blades and transferred to a centrifuge tube containing collagenase 1A (1mg/ml, Roche Diagnostics) and elastase (0.5mg/ml, Roche Diagnostics) solution in DMEM and incubated for 3-4 hours at 37°C with continuous shaking. At 1 hour intervals during the incubation the tissue was run through a 5ml serological pipette several times to aid cell dispersion. After 3-4 hours DMEM was added to the tissue/cell suspension to dilute the enzymes and the entire contents of the centrifuge tube was strained through a 100µm cell strainer to separate undigested tissue from cells. The resulting cell suspension was pelleted by centrifugation at 365 x g for 5 minutes. The supernatant was discarded, the cells resuspended in DMEM growth medium containing penicillin (100 units/ml), streptomycin (100µg/ml), L-glutamine (2mM), 15% HI-FBS and HEPES (25mM, pH 7.4) and transferred to tissue culture flask. Cells were maintained in a humidified atmosphere of 5% CO₂ /95% air at 37°C. Cells were fed every 2-3 day with fresh growth medium. When confluent the cells were propagated by trypsinisation.

4.3.1.2.2 Smooth muscle cell characterisation

SMC were characterised visually, under phase contrast, by their 'hill and valley' morphology. Cells were also characterised by positive staining for smooth muscle specific α -actin (mouse monoclonal, clone 1A4, 1/200 dilution, Sigma-Aldrich) and negative staining using antibodies against endothelial cells (mouse monoclonal, clone EN4, 1/100, Monosan) and fibroblast surface protein (mouse monoclonal, clone 1B10, 0.4µg/ml, Sigma-Aldrich) using immuno-fluorescent staining techniques. Cultures

containing contaminating fibroblasts were discarded. In addition, all Western blot analysis experiments using SMC used smooth muscle (SM)-specific α -actin counter staining for normalisation.

4.3.1.3 Primary airway epithelial cell culture

Primary human epithelial cells were cultured from trachea and bronchi trimmings from lung and heart / lung transplant donors

4.3.1.3.1 Isolation and propagation

The tissue was removed from the HBSS collection medium and placed in a sterile petri dish in a tissue culture hood. The tissue was cleaned of surrounding tissue, cut into small pieces (3 x 3 mm) and placed epithelial surface down approximately 1cm apart in tissue culture vessels. The tissue was covered with a small amount of medium (Nutrient mixture Ham's F12 (Invitrogen) containing hydrocortisone (1 μ M), epidermal growth factor (5ng/ml), insulin (10 μ g/ml), retinoic acid (10nM), transferrin (0.5 μ g/ml), 3,3',5'-triiodo-L-thyronine (2 μ g/ml), sodium bicarbonate (NaHCO₃, 1.5mg/ml), bovine pituitary extract (52 μ g/ml), penicillin (100 units/ml), streptomycin (100 μ g/ml), amphotericin B (250 ng/ml), ciprofloxacin (20 μ g/ml), gentamycin (50 μ g/ml) (all supplements were purchased from Sigma-Aldrich) and left at 37°C in a humidified atmosphere of 5% CO₂ /95% air. The cultures were fed every 3 days with fresh medium.

4.3.1.3.2 Epithelial cell characterisation

Epithelial cells were characterised by the presence of positive staining for cytokeratin (mouse monoclonal MNF116, DakoCytomation) using immunocytochemistry staining techniques (peroxidase or immunofluorescence). Cultures containing cytokeratin negative cells were discarded.

4.3.1.4 A549 cell culture

The A549 cell line was initiated in 1972 by D.J.Giard et al through explant culture of lung carcinomatous tissue from a 58 year-old Caucasian male (Giard et al., 1973). The culture, in passage 68, was deposited into American Type Culture Collection (ATCC) in 1976. The A549 cell line exhibits many properties of type II pulmonary epithelial cells and has proved to be a useful model to study various airway epithelial cell functions including inflammatory processes.

4.3.1.4.1 Propagation

A549 cells were grown in 75cm² tissue culture flasks in DMEM supplemented with 10% HI-FBS, penicillin (100 units/ml), streptomycin (100µg/ml), L-glutamine (2mM) and HEPES (25mM, pH 7.4). Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ /95% air. Cells were fed every 2-3 day with fresh growth medium and propagated by trypsinisation when confluent.

4.3.2 NFκB ACTIVATION

NFκB activation in EC, SMC and pulmonary epithelial cells was monitored by analysis of cellular levels of IκBα, the association of NFκB p65 with IκBα, nuclear levels of NFκB p65, NFκB DNA binding activity, NFκB-dependant adhesion molecule expression.

4.3.2.1 IκBα depletion

Cells in 24 well plates, 80-90% confluent (primary epithelial cells in 6 well plates) were used for these experiments and each treatment was performed in duplicate.

Cells were incubated with DMEM containing no additives overnight prior to the start of the experiment and all experiments were performed in this medium. After cytokine treatment, total cell lysis preparations were prepared according to methods section 2.3.1.1. IκBα levels in total cell lysis preparations were determined by SDS-PAGE (section 2.3.2), Western Blotting (section 2.3.3) and immunostaining (section 2.3.4) using rabbit anti-IκBα polyclonal antibody (C-21, Santa Cruz) at 0.2 µg/ml followed by goat anti-rabbit IgG- HRP-conjugated secondary antibody (DakoCytomation) at 0.3 µg/ml. Following immunostaining for IκBα the membranes were stripped of antibodies (section 2.3.5) and, in the case of endothelial and epithelial cell proteins, were re-stained for β-tubulin using mouse anti-human β-tubulin monoclonal antibody (clone TUB2.1, Sigma-Aldrich) at 1.8 µg/ml. In the case of smooth muscle cell proteins the membranes were re-stained for smooth muscle specific α-actin using mouse anti-human smooth muscle α-actin monoclonal antibody (clone 1A4, Sigma-Aldrich) at 1/1000 dilution. In both cases the secondary antibody used was a rabbit anti-mouse IgG–HRP-conjugate used at 1.3µg/ml. Following all immunostaining the membranes were counter stained with Ponceau S to visualise all the protein bands.

Optical density analysis of protein bands was determined by laser densitometry and results expressed as a percent of control band (protein from control treated cells) and normalised to β -tubulin or SM α -Actin levels.

4.3.2.2 NF κ B/I κ B α association

Cells, at 80-90% confluence, were treated with cytokines at the indicated concentrations and time, after which NF κ B p65 was immunoprecipitated from the cells according to section 2.3.1.2. The resulting protein sample was subjected to SDS-PAGE, Western blot analysis and immunostaining for I κ B α . The presence of immunoreactive I κ B α band indicates the association of this protein with NF κ B p65 in the cell at the end of cytokine exposure.

4.3.2.3 Nuclear NF κ B by Western blotting

Cells in 10cm dishes, at 80-90% confluence, were used for these experiments and each treatment was performed in duplicate.

Cells were incubated with DMEM containing no additives overnight prior to the start of the experiment and all experiments were performed in this medium. After cytokine treatment nuclear proteins were isolated according to methods section 2.3.1.3. NF κ B p65 levels in the isolated nuclear protein samples were determined by SDS-PAGE (section 2.3.2), Western Blotting (section 2.3.3) and immunostaining (section 2.3.4) using rabbit anti-human NF κ B p65 polyclonal antibody (C-20, Santa Cruz) at 0.4 μ g/ml followed by goat anti-rabbit IgG-HRP-conjugated secondary antibody at 0.3 μ g/ml.

Optical density analysis of protein bands was determined by laser densitometry and results expressed as a percent of control band (protein from control treated cells).

4.3.2.4 Nuclear NF κ B by immunoperoxidase staining

Cells in 8 well, 0.5% gelatine coated SUPER CELL™ chamber slides (VWR International), at no more than 70% confluence were used for this procedure. Following experimental treatment cells were washed in PBS and the plastic chambers were removed from the slides. Cells were stained for NF κ B with specific mouse anti-human NF κ B p65 monoclonal antibody, clone F-6 (Santa Cruz) at 6.7 μ g/ml overnight at 4 °C using the protocol described in methods section 2.5.

4.3.2.5 NFκB/DNA binding activity

Nuclear proteins isolated for Western blot analysis of nuclear NFκB were used for DNA binding analysis according to section 2.4.

4.3.2.6 NFκB-driven gene transcription

NFκB driven gene transcription was assessed by monitoring the resynthesis of IκBα protein by Western Blot analysis and the expression of NFκB dependent cell surface adhesion molecules. On EC the expression of E-selectin was measured and on SMC and epithelial cells ICAM-1 expression was determined.

4.3.2.6.1 E-selectin expression

E-selectin expression was measured by immunostaining of cell suspensions and flow cytometry as described in section 2.6 using mouse monoclonal anti-E-selectin (clone BBIG-4E (5D11) 2μg/ml, R&D Systems) primary antibody. Mouse Anti-human endothelial cell monoclonal antibody (EN4, 1/100 dilution, Monosan) was used as a positive control and MOPC 21 (2μg/ml) was used as an isotype matched negative control. FITC-conjugated F(ab')₂ fragment of an affinity isolated goat anti-mouse IgG secondary antibody was used at 33 μg/ml.

4.3.2.6.2 ICAM-1 expression

ICAM-1 expression on SMC and epithelial cells was measured by immunostaining of cell suspensions and flow cytometry as described in section 2.6 using mouse monoclonal anti-ICAM-1 (clone 6.5B5, 3.8μg/ml, Dako Cytomation) primary antibody. Mouse Anti-human HLA Class I monoclonal antibody (W6/32, 1/100 dilution) was used as a positive control and MOPC 21 (3.8μg/ml) was used as an isotype matched negative control. FITC-conjugated F(ab')₂ fragment of an affinity isolated goat anti-mouse IgG secondary antibody was used at 33 μg/ml.

4.3.3 DATA ANALYSIS

All data is expression as mean ± standard error of the mean (sem). Significance was determined using one way analysis of variance followed by post hoc t-test (Bonferroni) where required.

4.4 EXPERIMENTAL PROTOCOL FOR CHARACTERISATION OF NFκB ACTIVATION

Using the methods described above the time course of events in the NFκB activation pathway were examined following cellular stimulation with IL-1β and TNFα. The profile of stimuli concentration response curves was also established, as was the effect of pharmacological manipulation of the 26S proteasome pathway with the tripeptide aldehyde inhibitor MG132 (cbz-Leu-Leu-Leucinal) (Lee and Goldberg, 1998).

4.5 RESULTS

4.5.1 ENDOTHELIAL CELLS

4.5.1.1 IκBα levels

The time course of cytokine-induced IκBα depletion in cultured PA EC was studied over 120 minutes. Treatment of these cells for 15 minutes with IL-1β (1ng/ml) or TNFα (10ng/ml) caused IκBα levels to fall to 23.2 ± 5.5 and $9.3 \pm 1.7\%$ of that in untreated cells, respectively. Cytokine-induced IκBα depletion was transient and was followed by the resynthesis of this protein with detectable levels observed after 60 minutes treatment. After 120 minutes of IL-1β or TNFα treatment IκBα protein levels were significantly greater than those observed at 30 minutes ($75.7 \pm 23.0\%$ ($p=0.016$, $n=4$) and $61.4 \pm 9.7\%$ ($p=0.003$, $n=5$) of that in untreated cells respectively, Figure 4.2).

The effects of both these cytokines on IκBα levels in PAEC were dose dependent. Reduced levels of IκBα were detectable by Western blot analysis after 30 minutes treatment with 0.2pg/ml IL-1β ($67.2 \pm 5.2\%$ of control levels, $n=7$) or 10pg/ml TNFα ($59.6 \pm 3.2\%$ of control, $n=5$). Thirty minutes treatment with IL-1β (0.2pg/ml – 2000pg/ml) resulted in a progressive reduction in IκBα levels to maximum effect with 2ng/ml ($9.7 \pm 1.8\%$ of control, Figure 4.3a and c). For TNFα, a maximum effect was observed after 30 minutes treatment with 10ng/ml ($14.6 \pm 7.5\%$ of control, $n=5$, Figure 4.3 c and d).

Representative Western blots in Figure 4.4a demonstrate the appearance of higher molecular weight IκBα immunoreactive proteins, likely corresponding to the phosphorylated and ubiquitin modified forms, following treatment with IL-1β or TNFα in the presence of MG132 (10μM), the 26S proteasome inhibitor. These higher molecular weight IκBα proteins remained present throughout the 120 minute time

course studied. Optical density analysis of the lower molecular weight I κ B α species (phosphorylated and non-phosphorylated) reveals that the profile of IL-1 β (1ng/ml) and TNF α (10ng/ml)-induced changes in cellular levels of I κ B α was altered by the presence of MG132 with no maximum depletion being achieved over the 120 minute time course. Over a 30 minute time course I κ B α depletion induced by IL-1 β (0.02ng/ml, 48.1 ± 10.9 , n=5) was significantly inhibited by the presence of MG132 (10 μ M, $115.5 \pm 19.3\%$ of control, p=0.015, n=5, Figure 4.4d). Similarly I κ B α depletion induced by TNF α (0.1ng/ml, $30.2 \pm 7.5\%$ of control, n=6) was also inhibited by the presence of MG132 (10 μ M, $62.7 \pm 11.1\%$ of control, p=0.038, n=6, Figure 4.4d).

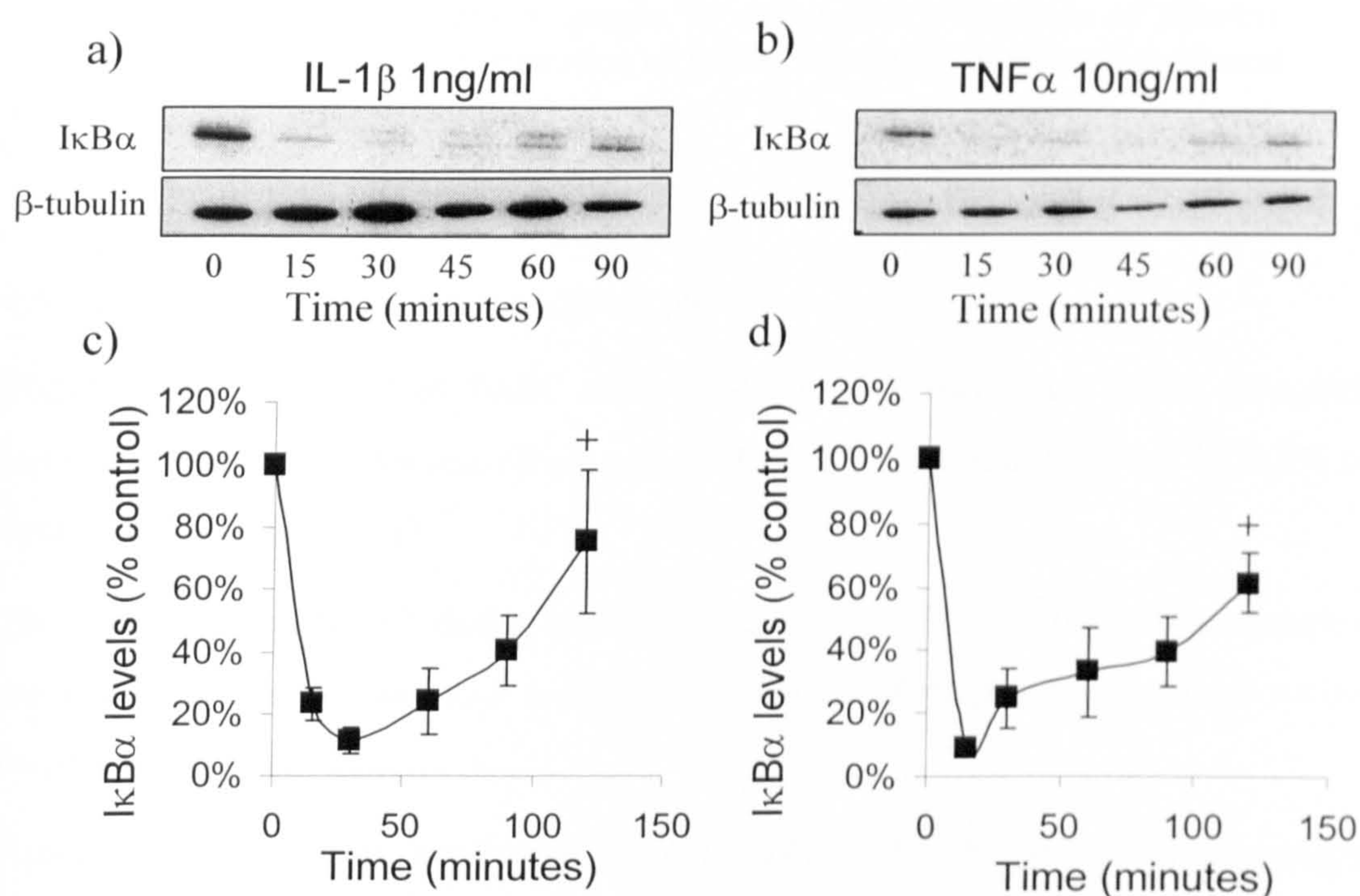


Figure 4.2. Time course of I κ B α depletion and resynthesis in human EC induced by pro-inflammatory cytokines. Representative Western blot and summary graph of I κ B α levels following IL-1 β (1ng/ml, a and c) and TNF α (10ng/ml, b and d) treatment. ⁺Denotes p<0.05 from I κ B α levels after 30 minutes of cytokine treatment.

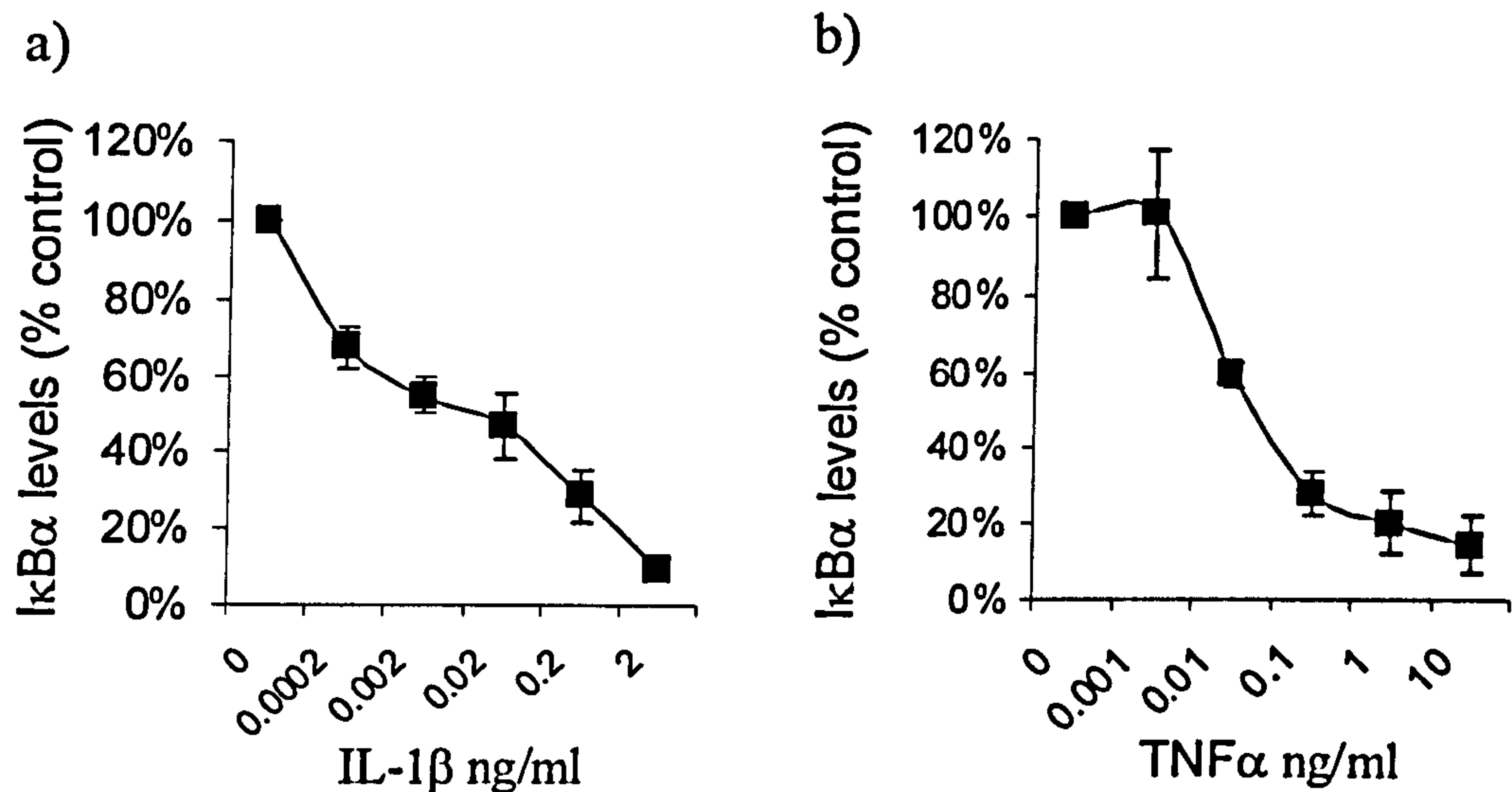


Figure 4.3. Dose dependant effects of pro-inflammatory cytokines on human EC IκBα levels. Summary graphs of densitometric analysis of Western blots of total cell lysis preparation of PAEC following 30 minutes treatment with IL-1β (a) and TNFα (b).

4.5.1.2 Nuclear translocation of NFκB p65

Sixty minutes treatment of PAEC with either IL-1β (1ng/ml) or TNFα (10ng/ml) induced an increase in nuclear NFκB p65 (2633.4 ± 1811.5 and $2712.4 \pm 1920.0\%$ of control levels respectively).

The presence of MG132 during IL-1β (1ng/ml) and TNFα (10ng/ml) stimulation resulted in 42.0 ± 18.4 and $62.9 \pm 8.1\%$ inhibition of NFκB p65 nuclear translocation respectively (Figure 4.5).

Analysis of the cellular localisation of NFκB p65 in EC by immunocytochemistry illustrates nicely the accumulation of NFκB p65 (brown staining) in the nuclei of cells following cytokine treatment Figure 4.5a).

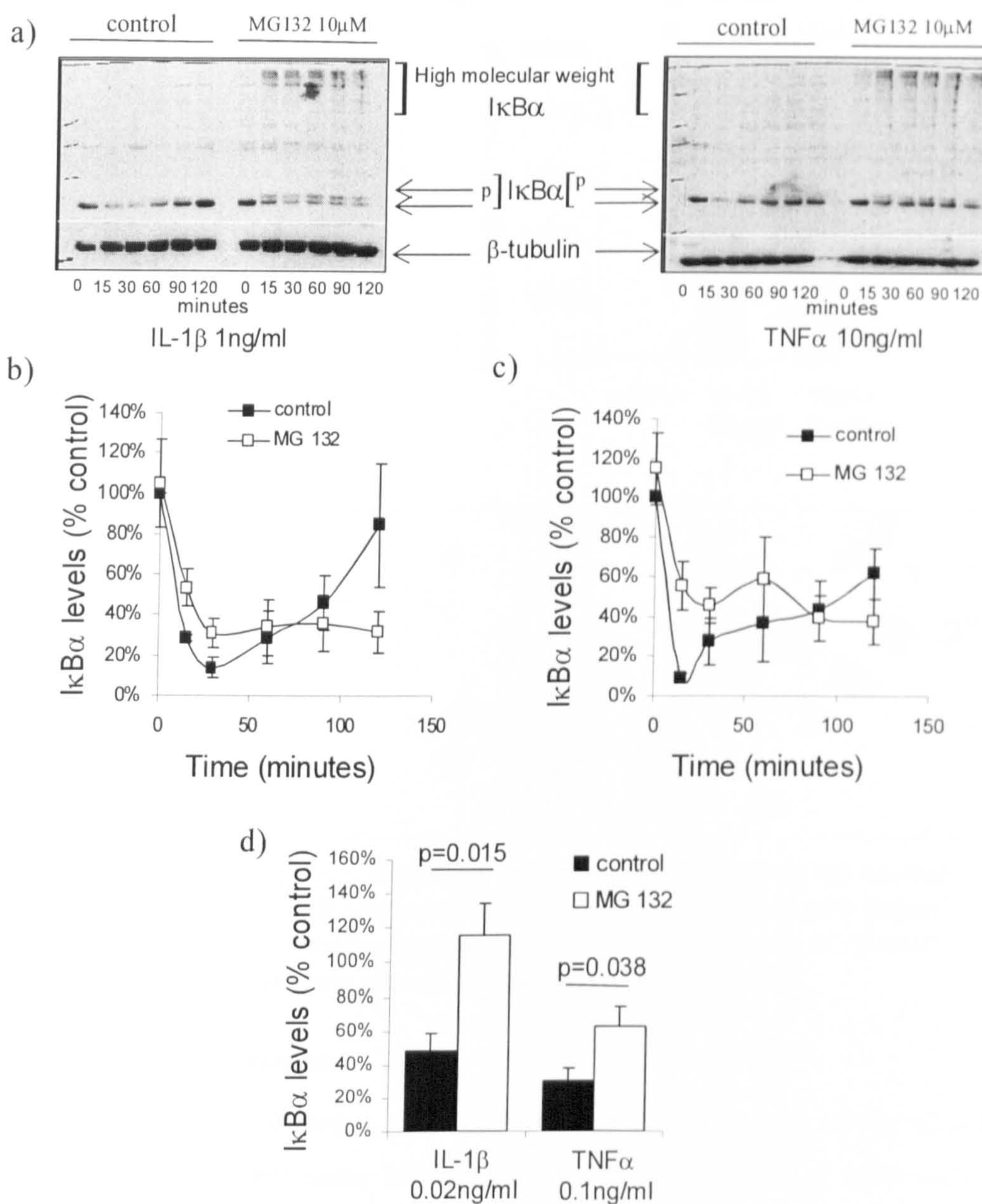


Figure 4.4. Effect of MG132 on cytokine-induced I κ B α depletion in PAEC. Representative Western blots and summary graphs showing the effects of MG132 (10 μ M) on the time course of IL-1 β (1 ng/ml, a and b) and TNF α (10ng/ml, a and c) -induced I κ B α depletion. d) Summary graph of the effects of MG132 on I κ B α depletion induced by 30 minutes incubation with submaximal concentrations of IL-1 β (0.02 ng/ml) and TNF α (0.1ng/ml).

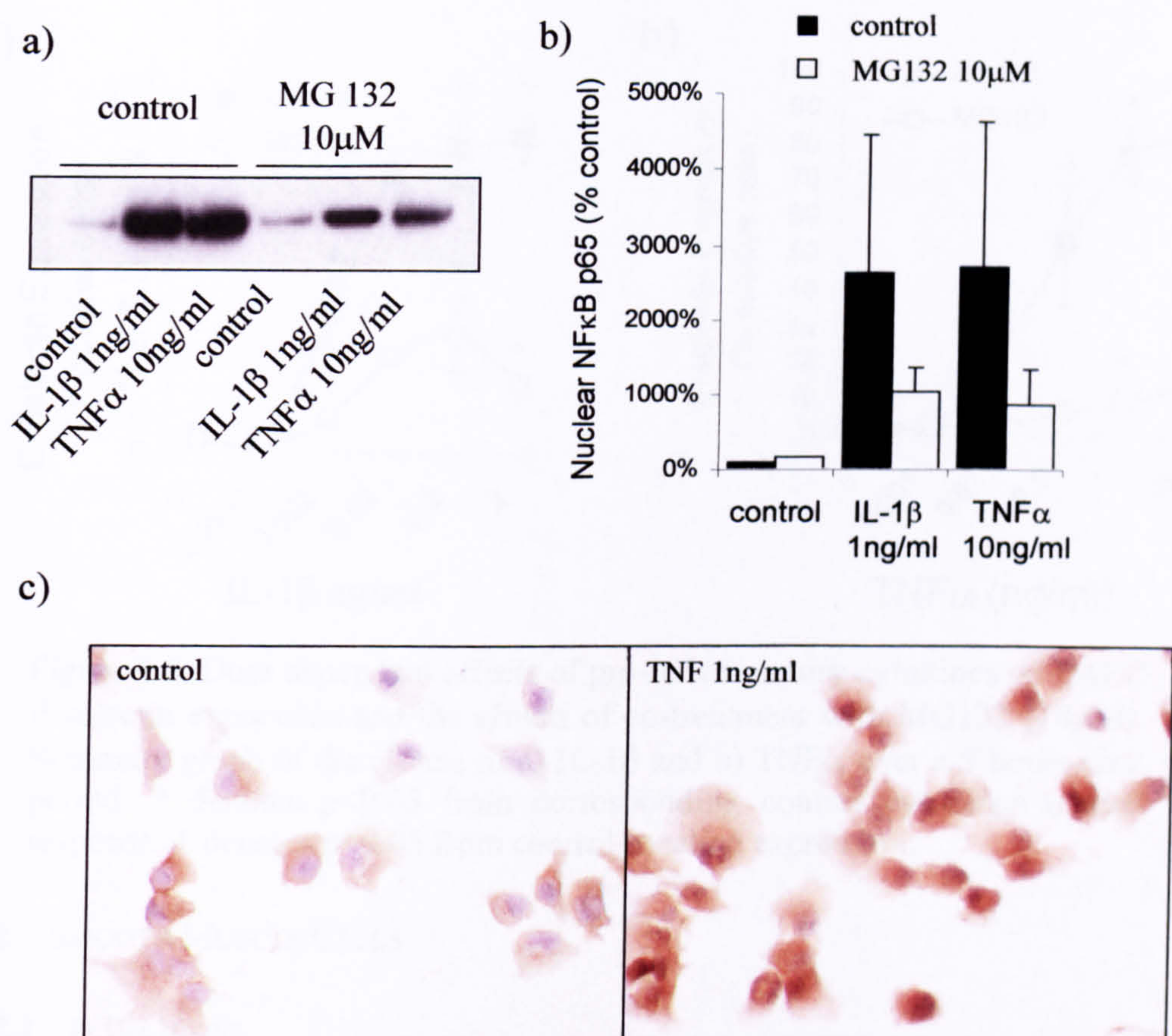


Figure 4.5. Effect of pro-inflammatory cytokines and MG132 on the nuclear levels of NFκB p65. a) Representative Western blots of NFκB p65 stained nuclear proteins, b) summary graph of optical density analysis of Western blots, c) NFκB p65 immunoperoxidase staining of treated PAEC.

4.5.1.3 E-selectin Expression

Flow cytometry analysis of surface E-selectin revealed low expression of this adhesion molecule on human PAEC under resting conditions ($3.45 \pm 0.43\%$ of cells were E-selectin positive). E-selectin expression on these cells was significantly increased, in a dose dependant manner, by 5 hours treatment with IL-1β (38.4 ± 9.53 , 76.6 ± 7.3 , 83.2 ± 9.8 and $86.3 \pm 6.2\%$ positive cells for 2, 20, 200 and 2000pg/ml respectively, $p < 0.05$, $n = 3$) and TNFα (52.8 ± 16.3 , 77.6 ± 7.5 , $82.0 \pm 5.4\%$ positive cells for 0.1, 1 and 10pg/ml, $p < 0.05$, Figure 4.6). The increase in E-selectin expression induced by the highest concentration of either of these pro-inflammatory cytokines was significantly inhibited by pre-treatment of the cells with MG132 (19.3 ± 2.9 for IL-1β 2ng/ml, $p < 0.05$ and 14.3 ± 1.6 for TNFα 10ng/ml, $p < 0.05$, Figure 4.6).

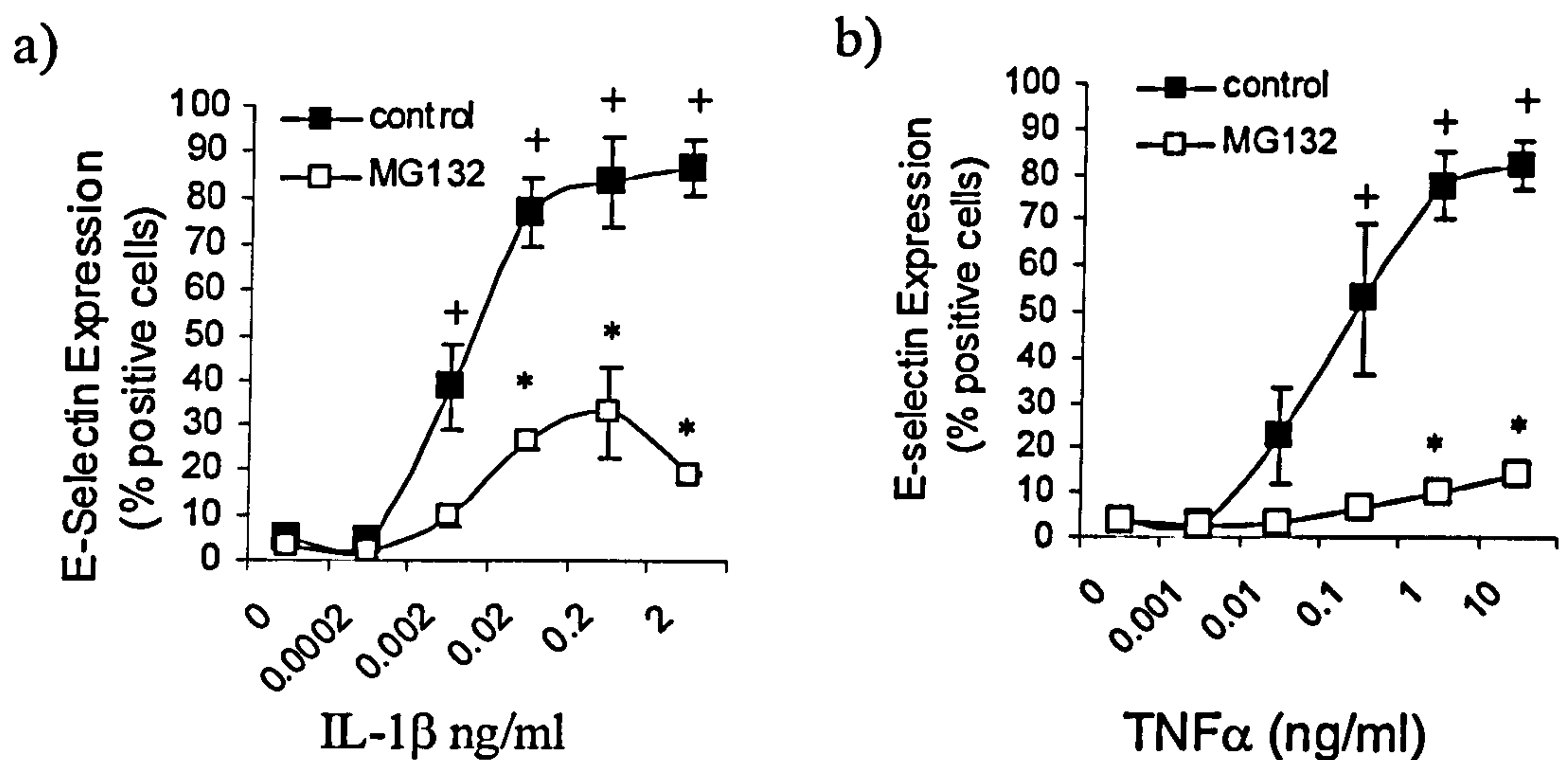


Figure 4.6. Dose dependant effects of pro-inflammatory cytokines on PAEC E-selectin expression and the effects of co-treatment with MG132 (10 μ M). Summary graph of the effects of a) IL-1 β and b) TNF α over a 5 hours time period. * denotes $p < 0.05$ from corresponding control cytokines treated response, + denotes $p < 0.05$ from control baseline expression.

4.5.2 SMOOTH MUSCLE CELLS

4.5.2.1 I κ B α levels

Western blot analysis of total protein samples from 1% SDS lysed SMC revealed a strong I κ B α immunoreactive band demonstrating the presence of I κ B α in resting cells. Exposure of the cells to IL-1 β (1ng/ml) for 5 minutes resulted in the appearance of a slower migrating I κ B α band. Longer exposure (30 minutes) caused a significant decrease in the amount of I κ B α in the cells ($22.6 \pm 4.5\%$ of that in resting cells compared to $109.4 \pm 5.2\%$ after 5 minutes IL-1 β treatment, $p < 0.05$, $n = 4-13$). Depletion of I κ B α was transient, with the reappearance of I κ B α following 60 minutes exposure to IL-1 β . Following 90 minutes IL-1 β exposure I κ B α levels were significantly increased compared to those observed at 30 minutes ($71.7 \pm 12.6\%$ of control, $p < 0.05$, $n = 9$) and continued to increase until levels returned to resting levels at 150 minutes ($100.1 \pm 21.3\%$, Figure 4.7).

Following TNF α (10ng/ml) treatment a similar time course of I κ B α depletion and resynthesis was observed with levels falling to $14.2 \pm 3.7\%$ of control levels after 30 minutes treatment ($n = 8$). Resynthesis of I κ B α was observed at 60 minutes ($50.0 \pm 14.6\%$ of control levels) and following 120 minutes treatment levels were significantly above those observed at 30 minutes ($74.1 \pm 19.1\%$ of control, $p < 0.05$, $n = 7$, Figure 4.7).

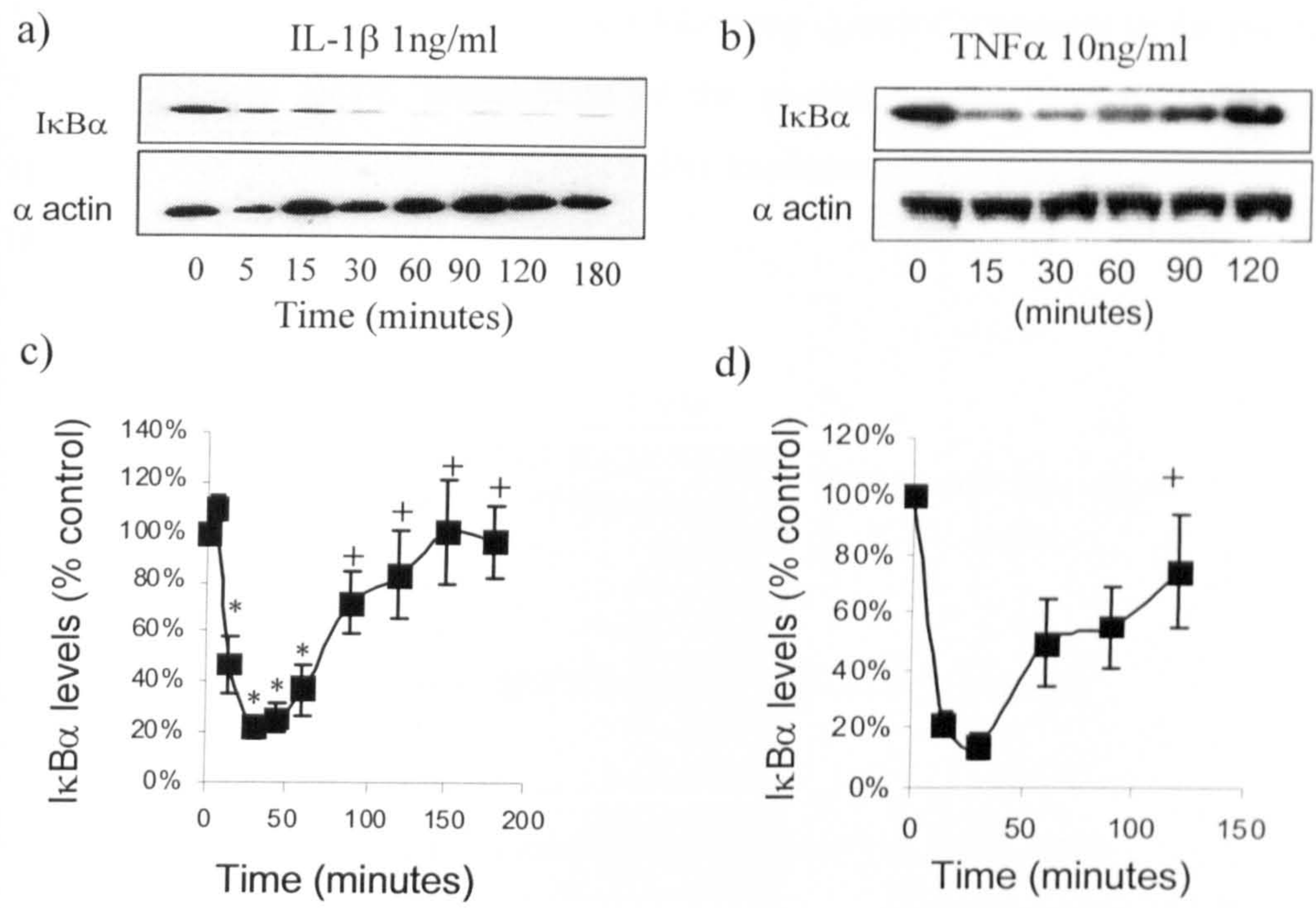


Figure 4.7. Time course of IκBα depletion and resynthesis in human vascular SMC induced by pro-inflammatory cytokines. Representative Western blot and summary graph of IκBα levels following IL-1β (1ng/ml) (a and c) and TNFα (10ng/ml, b and d). (* p<0.05 from 5 minutes IL-1β treatment, n=4-13, + p<0.05 from corresponding 30 minute time point).

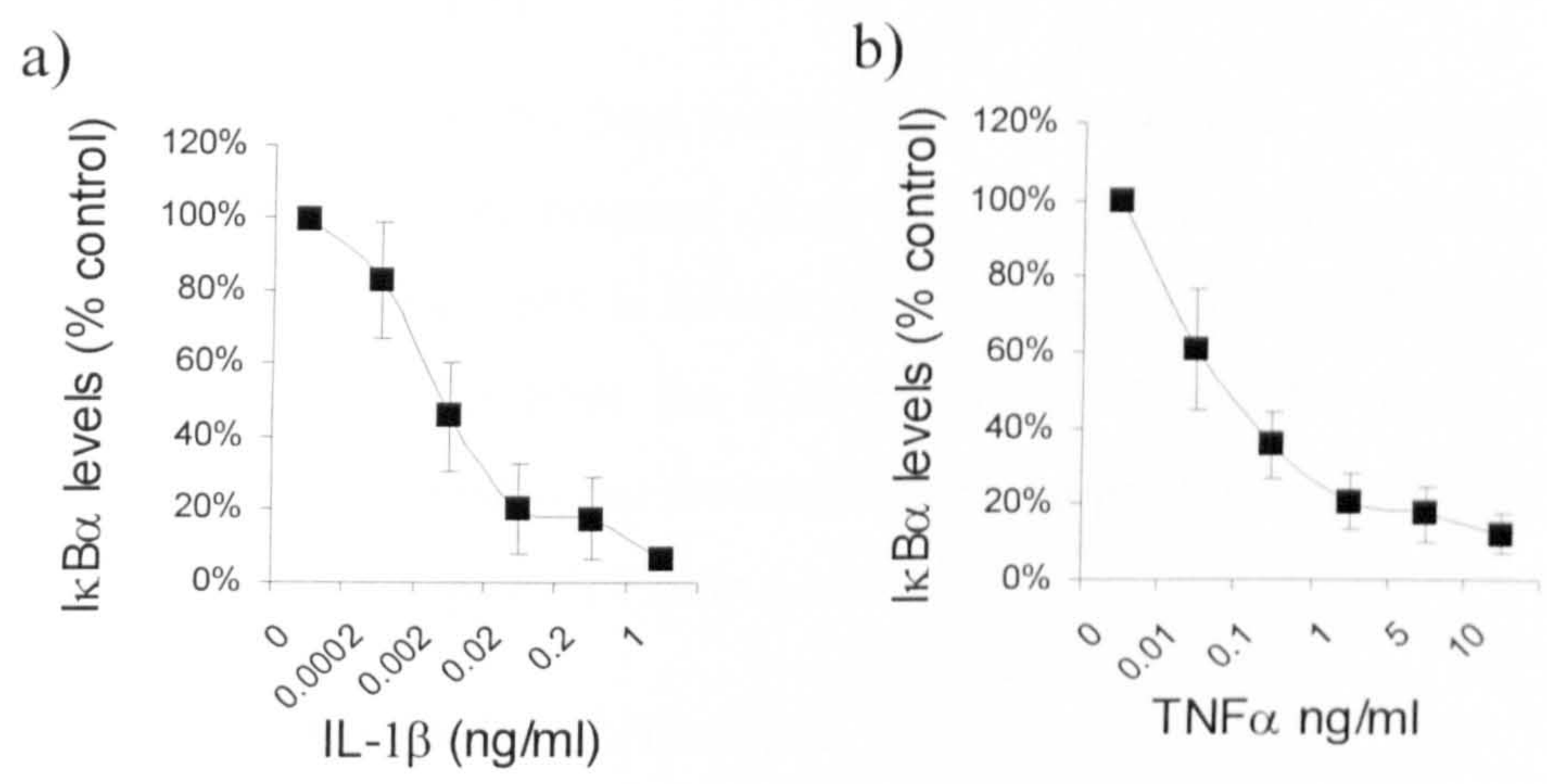


Figure 4.8. Dose dependant effects of pro-inflammatory cytokines on human SMC IκBα levels. Summary graphs of densitometric analysis of Western blots following 30 minutes treatment with IL-1β (a) and TNFα (b).

The effect of IL-1β and TNFα on human vascular SMC IκBα depletion over 30 minute time course was dose dependant with maximum depletion being reached in response to 1 and 10 ng/ml, respectively.

Western blot analysis of I κ B α proteins following cytokine treatment in the presence of MG132 resulted in the preservation of the phosphorylated form of I κ B α and the appearance of high molecular weight I κ B α immunoreactive proteins corresponding to ubiquitin conjugated I κ B α .

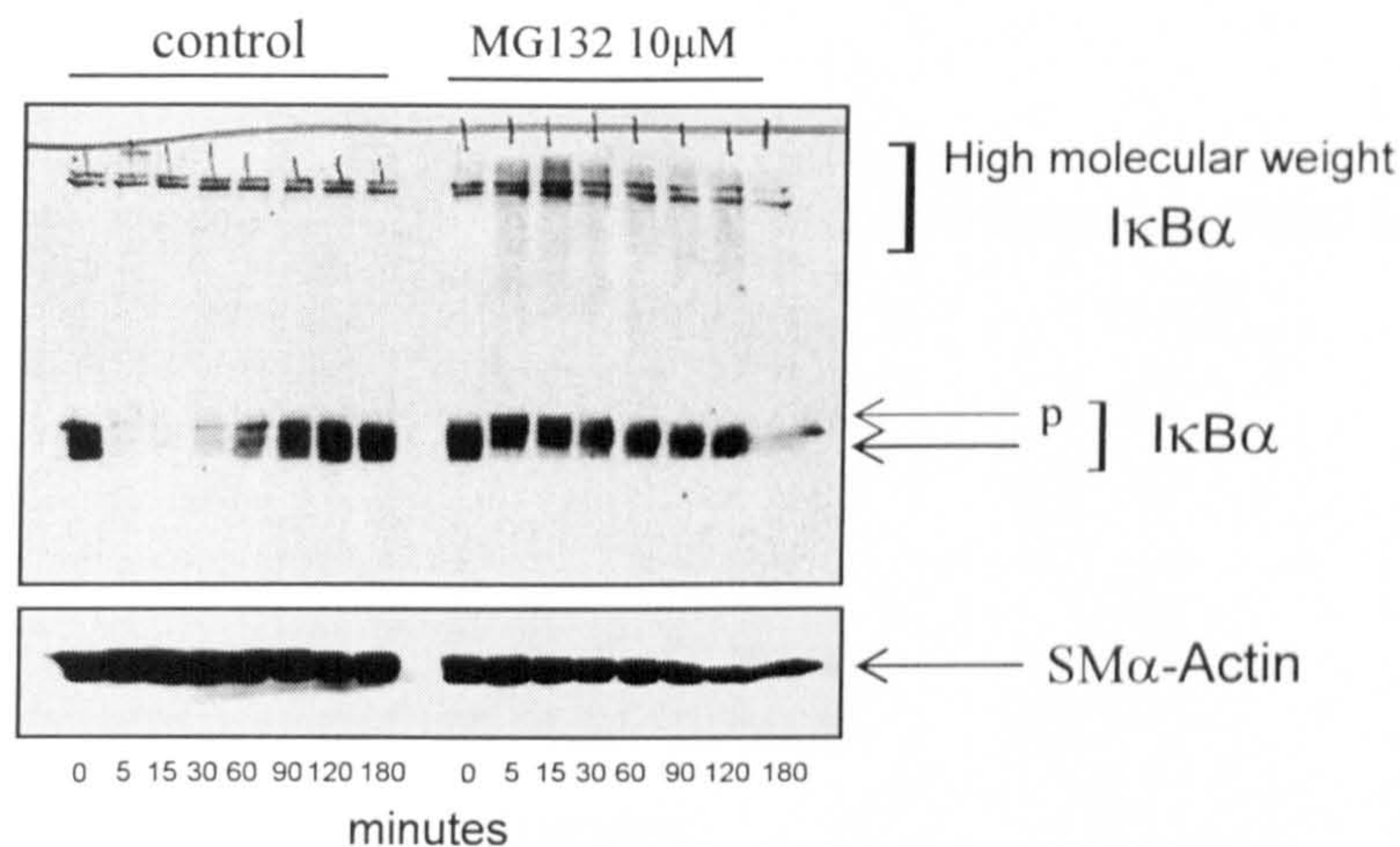


Figure 4.9. Effect of proteasome inhibition on the time course of IL-1 β (1ng/ml)-induced I κ B α depletion in human SMC. Representative Western blot.

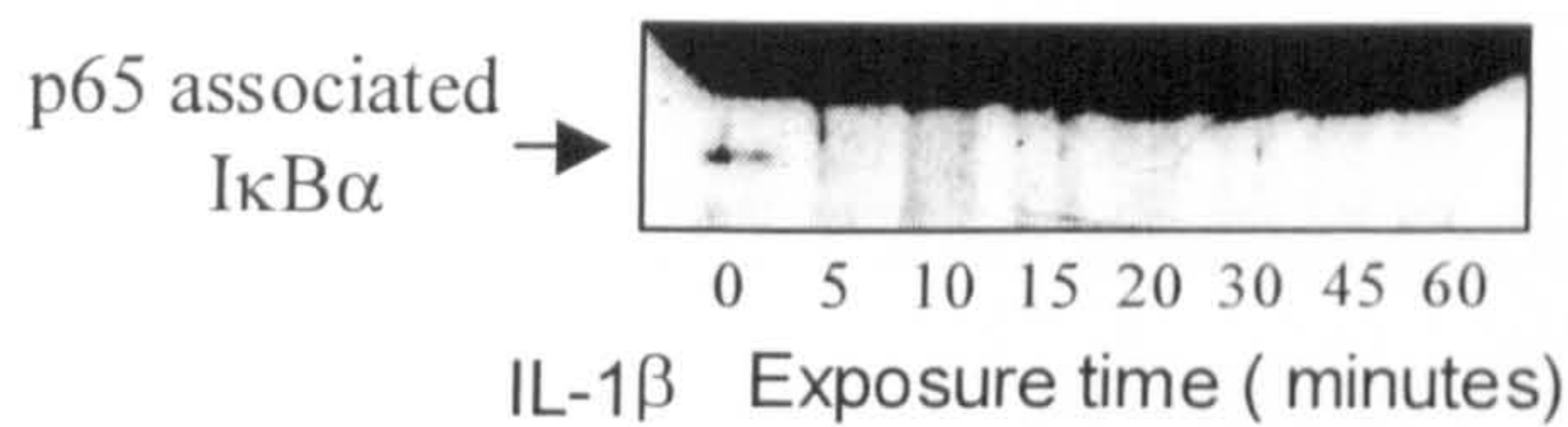
4.5.2.2 I κ B α /p65 association

Immunoprecipitating NF κ B p65 from resting SMC resulted in the co-precipitation of I κ B α , as demonstrated by the presence of an I κ B α immunoreactive protein of about 37kDa indicating that NF κ B p65 is bound to the inhibitor protein I κ B α . Associated with the depletion of I κ B α from the cells following 30 minutes IL-1 β (1ng/ml) treatment there was a decrease in the amount of I κ B α co-precipitating with NF κ B ($9.5 \pm 4.6\%$ of that in resting cells, $n=5$, Figure 4.10)

4.5.2.3 Nuclear Translocation of NF κ B p65

Separation of cytosolic and nuclear proteins revealed the presence of small amounts of NF κ B p65 subunit in the nuclei of resting cells. Associated with I κ B α depletion from the cytoplasm following 30 minutes IL-1 β (1 ng/ml) treatment, nuclear levels of p65 increased five-fold ($517.0 \pm 140.2\%$ of that in resting cells, $n=3$) and remained elevated for a further 90 minutes of IL-1 β treatment (Figure 4.11).

a)



b)

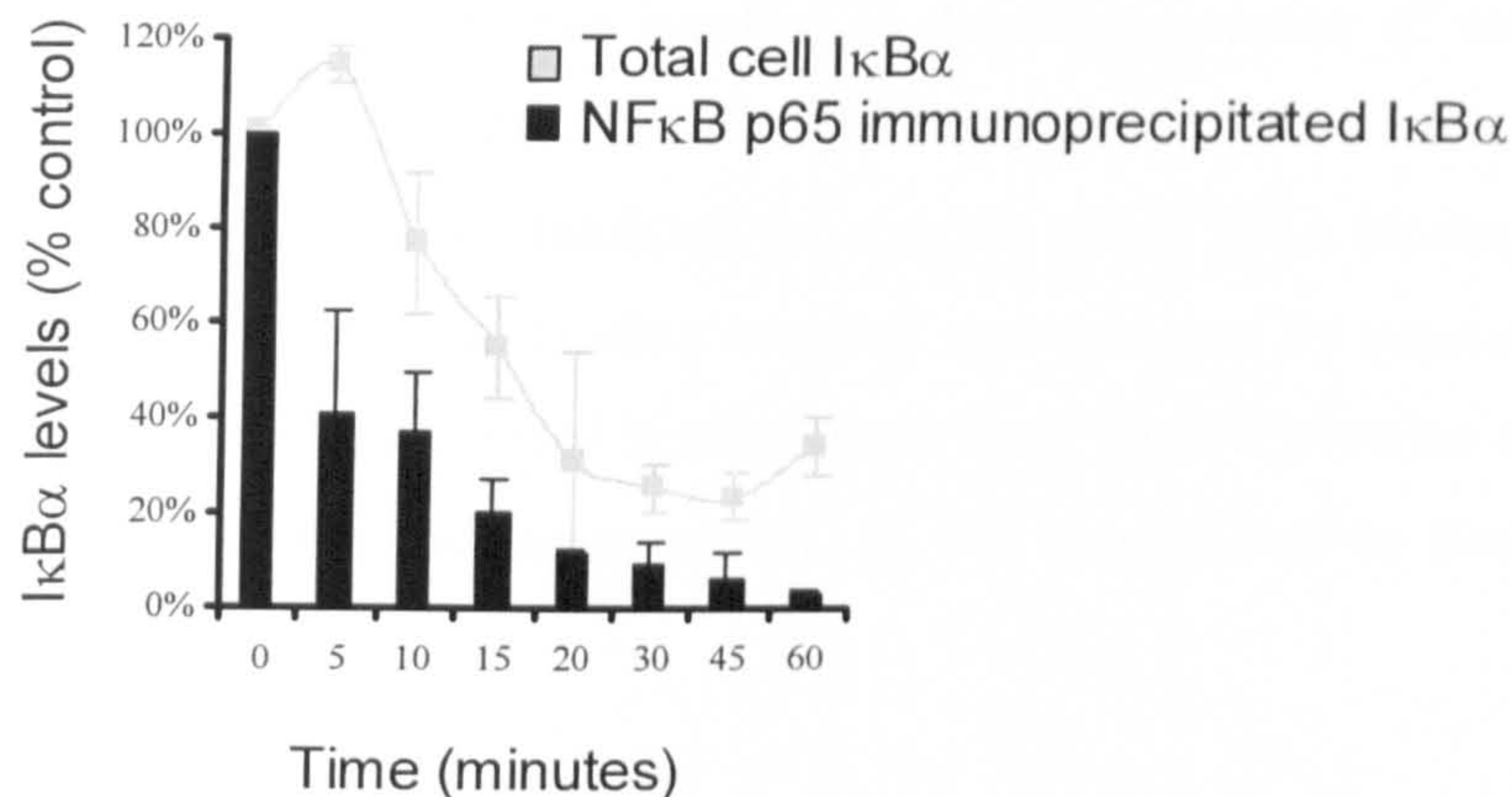
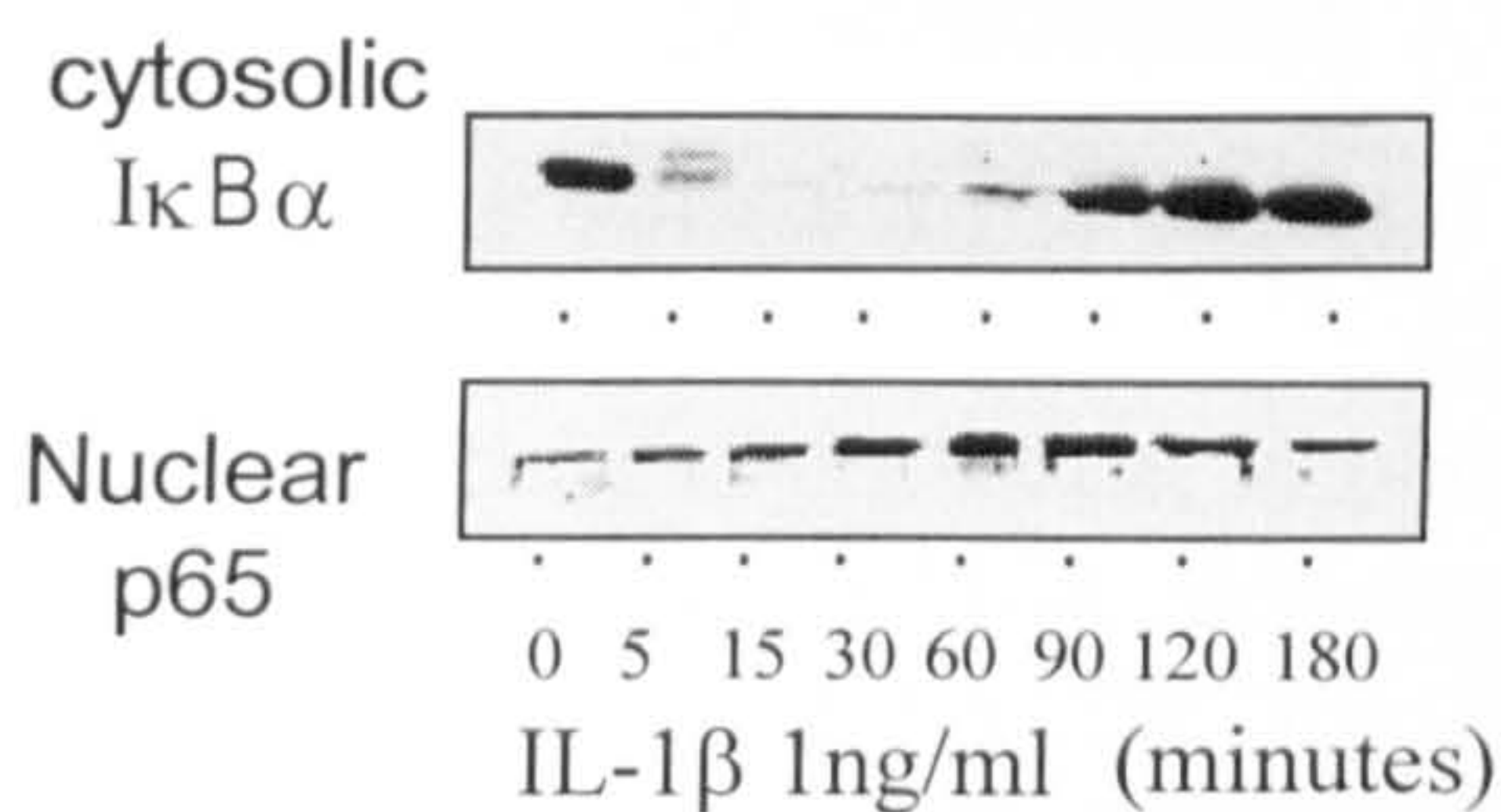


Figure 4.10. Time course of IL-1 β -induced dissociation of I κ B α from NF κ B p65. a) Western blot analysis of I κ B α co-precipitated with NF κ B p65 from human vascular SMC. b) Graph of optical density analysis of I κ B α immunoreactive proteins from NF κ B p65 immunoprecipitations with a graph of levels of total cell I κ B α superimposed (n=5).

a)



b)

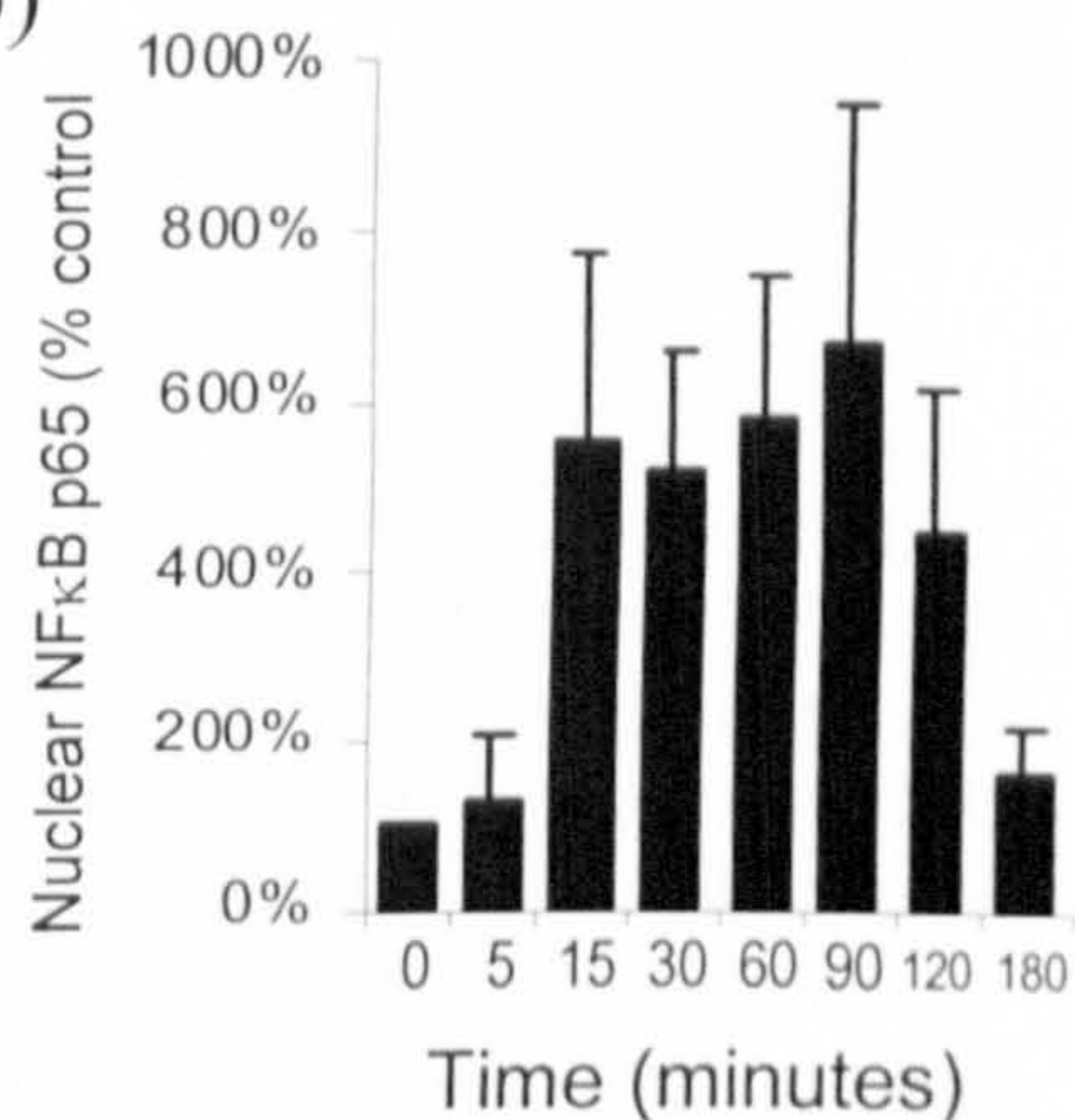
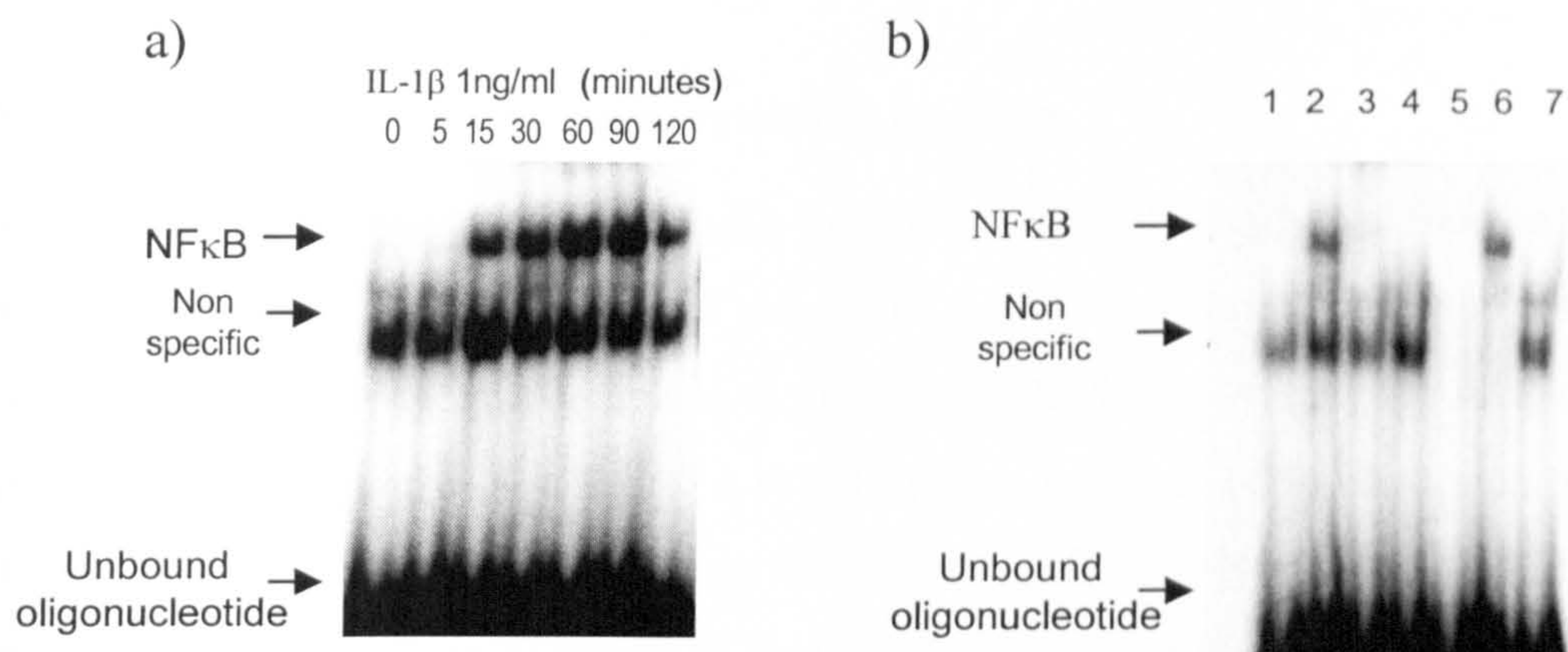


Figure 4.11. Time dependent activation of NF κ B in human vascular SMC by IL-1 β (1ng/ml). Representative Western blot of cytosolic I κ B α levels and nuclear NF κ B p65. b) Summary graph of nuclear NF κ B p65 (n=3).

4.5.2.4 NF κ B /DNA binding

IL-1 β treatment also increased the binding of nuclear proteins to the κ B consensus DNA as demonstrated by EMSA using 32 P labelled double-stranded oligonucleotide containing a specific κ B consensus sequence. Specificity of the reaction is supported by experiments where 100-fold excess cold competitive oligonucleotide caused the disappearance of all protein/DNA complexes whilst addition of unlabeled mutant κ B oligonucleotide to the binding reaction resulted in the disappearance of non-specific binding activity. Furthermore, inclusion of a purified fragment of I κ B α protein (amino acids 1-317) in the reaction mixture inhibited the specific NF κ B DNA binding activity. The molecular identity of the DNA binding complex was analyzed by supershift assay using specific antibodies. The increased binding capacity of nuclear proteins to specific κ B consensus DNA, following treatment with IL-1 β was supershifted by both the p50



and p65 antibodies (Figure 4.12).

Figure 4.12. Time dependent activation of NF κ B in human aortic SMC by IL-1 β 1ng/ml. a). EMSA demonstrating increased binding activity of nuclear proteins to specific κ B consensus DNA. b) Analysis of the specificity and molecular identity of the DNA/protein complexes induced by IL-1 β treatment. Lane 1. Nuclear proteins from untreated human aortic SMC. Lanes 2-7, nuclear proteins from cells treated with IL-1 β , 1ng/ml for 30 minutes. Lane 3, addition of anti-p50 antibody in the binding reaction. Lane 4, addition of p65 antibody in the binding reaction. Lane 5, addition of 100-fold excess cold κ B oligonucleotide, Lane 6 contains 100-fold excess cold mutant oligonucleotide and Lane 7 contains purified I- κ B α protein corresponding to amino acid sequence 1-317.

4.5.2.5 ICAM-1 expression

Under resting conditions 7.9 ± 1.5 % of aortic SMC were ICAM-1 positive. ICAM-1 expression on these cells was increased in a dose dependant fashion following 18 hours treatment with IL-1 β or TNF α with a significant increase above control levels observed at 0.02 ng/ml ($42.8 \pm 1.6\%$, $p < 0.05$) and 1.0 ng/ml ($42.8 \pm 7.9\%$, $p < 0.05$), respectively (Figure 4.13 a and b).

Inhibition of 26S proteasome activity significantly inhibited ICAM-1 expression induced by high concentrations of IL-1 β and TNF α . IL-1 β (1ng/ml)-induced increase in ICAM-1 expression ($67.2 \pm 3.3\%$ positive cells) was reduced to $30.7 \pm 14.6\%$ positive cells ($p = 0.029$) following pre-treatment with MG132. TNF α (10ng/ml)-induced ICAM-1 expression ($61.0 \pm 5.7\%$ positive cells) was reduced to $13.0 \pm 1.5\%$ in the presence of MG132 ($p = 0.008$ Figure 4.13c).

4.5.3 AIRWAY EPITHELIAL CELLS

4.5.3.1 Effect of pro-inflammatory cytokines on I κ B α

The time course of cytokine-induced I κ B α depletion in A549 cells was similar to that observed in EC and SMC. Treatment of these cells for 15 minutes with IL-1 β (1ng/ml) or TNF α (10ng/ml) caused I κ B α levels to fall to 25.3 ± 5.5 and $16.7 \pm 4.7\%$ of that in untreated cells, respectively (Figure 4.14). Cytokine-induced I κ B α depletion was followed by the resynthesis of this protein with detectable levels observed visually from Western blot analysis after 60 minutes of IL-1 β or TNF α treatment. After 90 minutes of IL-1 β (1ng/ml) treatment or 120 minutes for TNF α treatment I κ B α levels were significantly greater than those observed at 30 minutes ($70.4 \pm 12.0\%$ of control, $p = 0.006$, $n = 12$ and $78.5 \pm 22.7\%$ of control levels, $p = 0.048$, $n = 5$ respectively, Figure 4.14).

The effects of both of these cytokines on I κ B α levels in A549 cells was dose dependent. Reduced levels of I κ B α were detectable by Western blot analysis after 30 minutes treatment with 2.0 pg/ml IL-1 β or 10pg/ml TNF α . Thirty minutes treatment with IL-1 β (2.0 pg/ml – 1000pg/ml) resulted in a progressive reduction in I κ B α levels. Maximum effect was achieved with 200pg/ml ($10.1 \pm 2.8\%$ of control, $n = 4$). Progressive reduction in I κ B α levels was also observed when A549 cells were treated with increasing

concentrations of TNF α for 30 minutes with the maximum effect observed with 1ng/ml (8.3 $\pm$ 2.7 % of control, n=5, Figure 4.15).

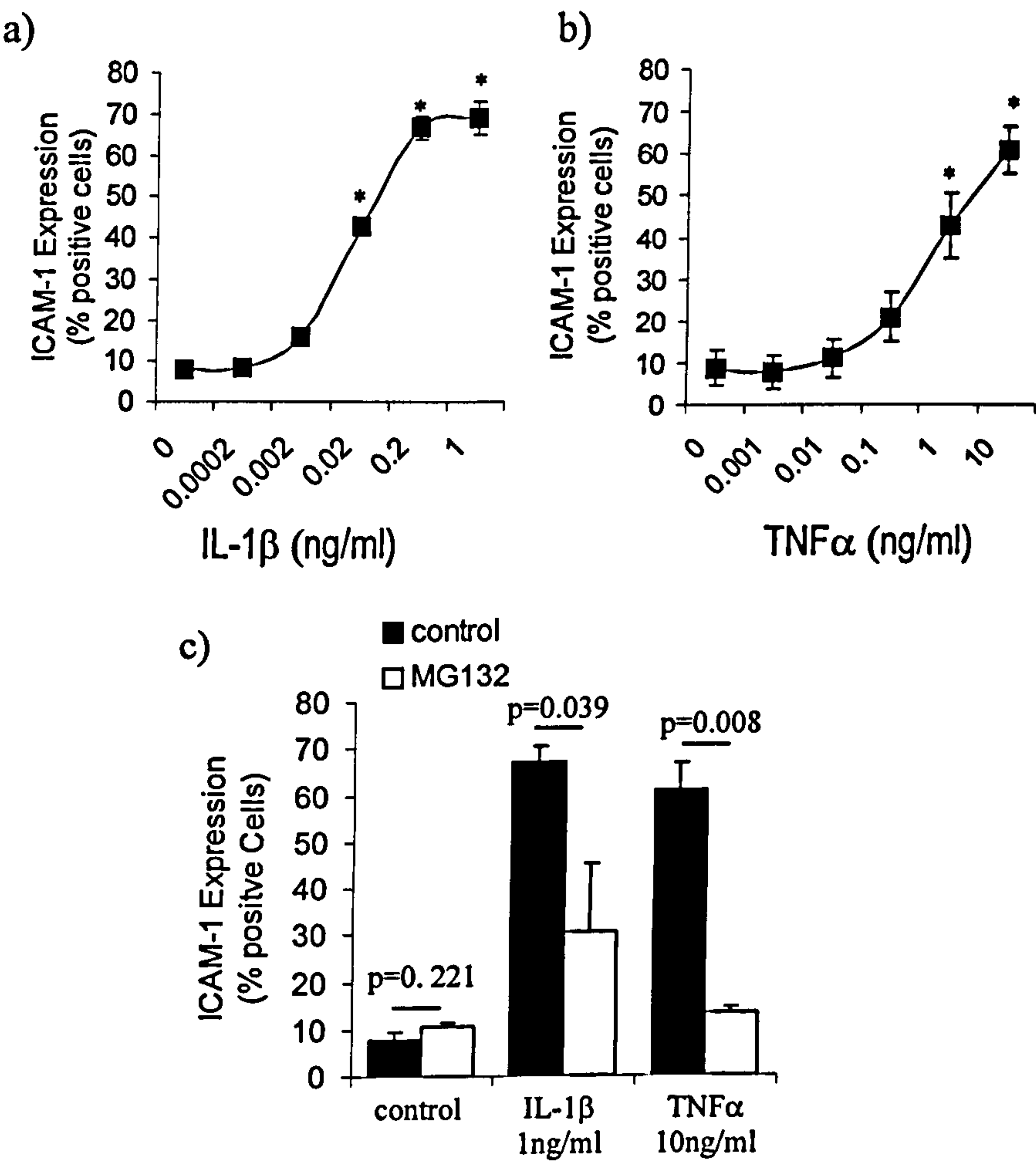


Figure 4.13. Effect of pro-inflammatory cytokines and MG132 on human SMC ICAM-1 expression. Summary graphs of the dose-dependant effects of 18 hours treatment with a) IL-1 β and b) TNF α . c) Summary graph of the effects of MG132 treatment on IL-1 β (1ng/ml) and TNF α (10ng/ml)-induced ICAM-1 expression. * denotes p< 0.001 from control levels.

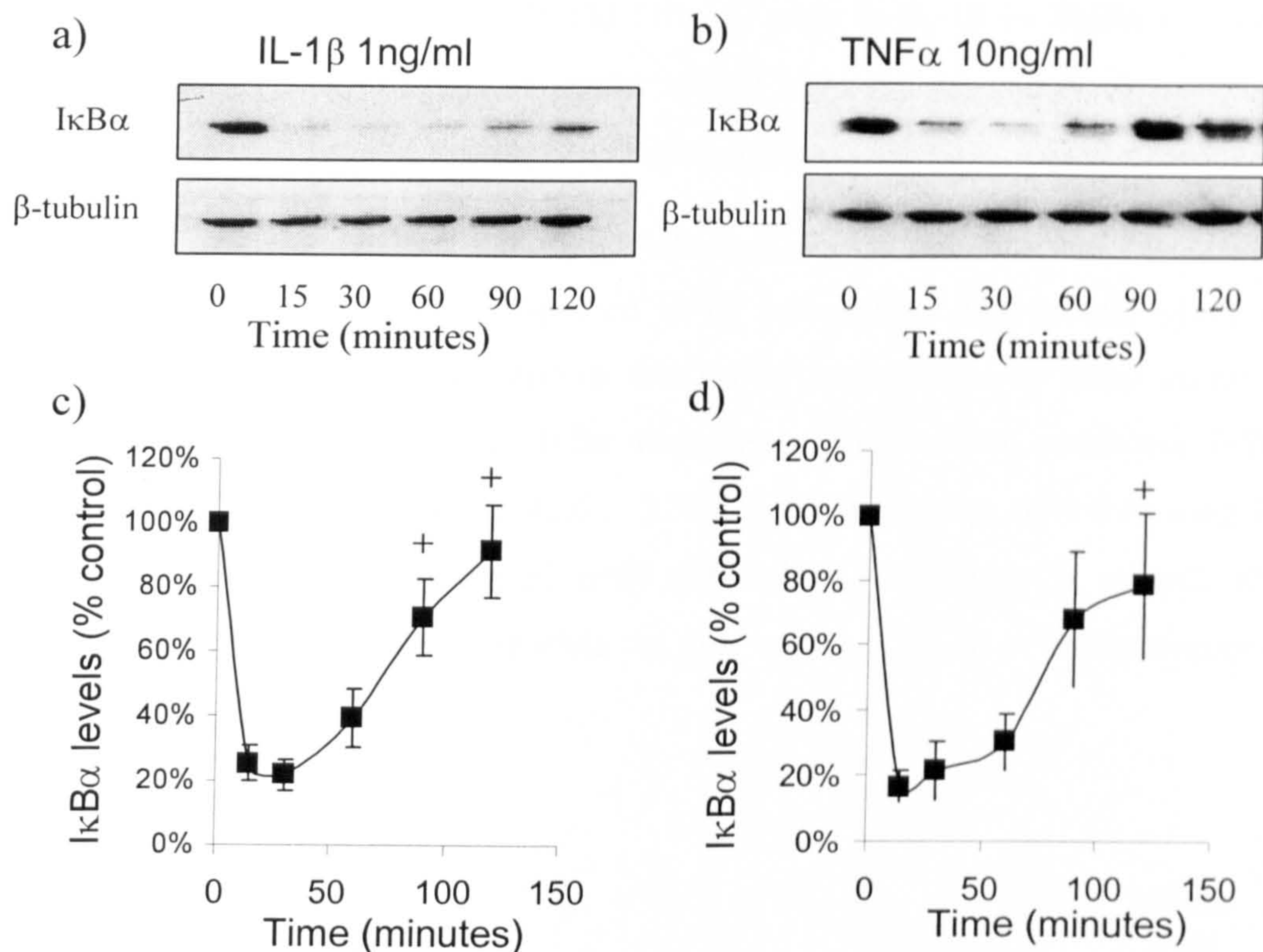


Figure 4.14. Time course of IκBα depletion and resynthesis in A549 cells induced by pro-inflammatory cytokines. Representative Western blots and summary graph of IκBα levels following IL-1β (1ng/ml) (a and c) and TNFα (10ng/ml, b and d) treatment. ⁺Denotes p<0.05 from IκBα levels after 30 minutes of cytokine treatment.

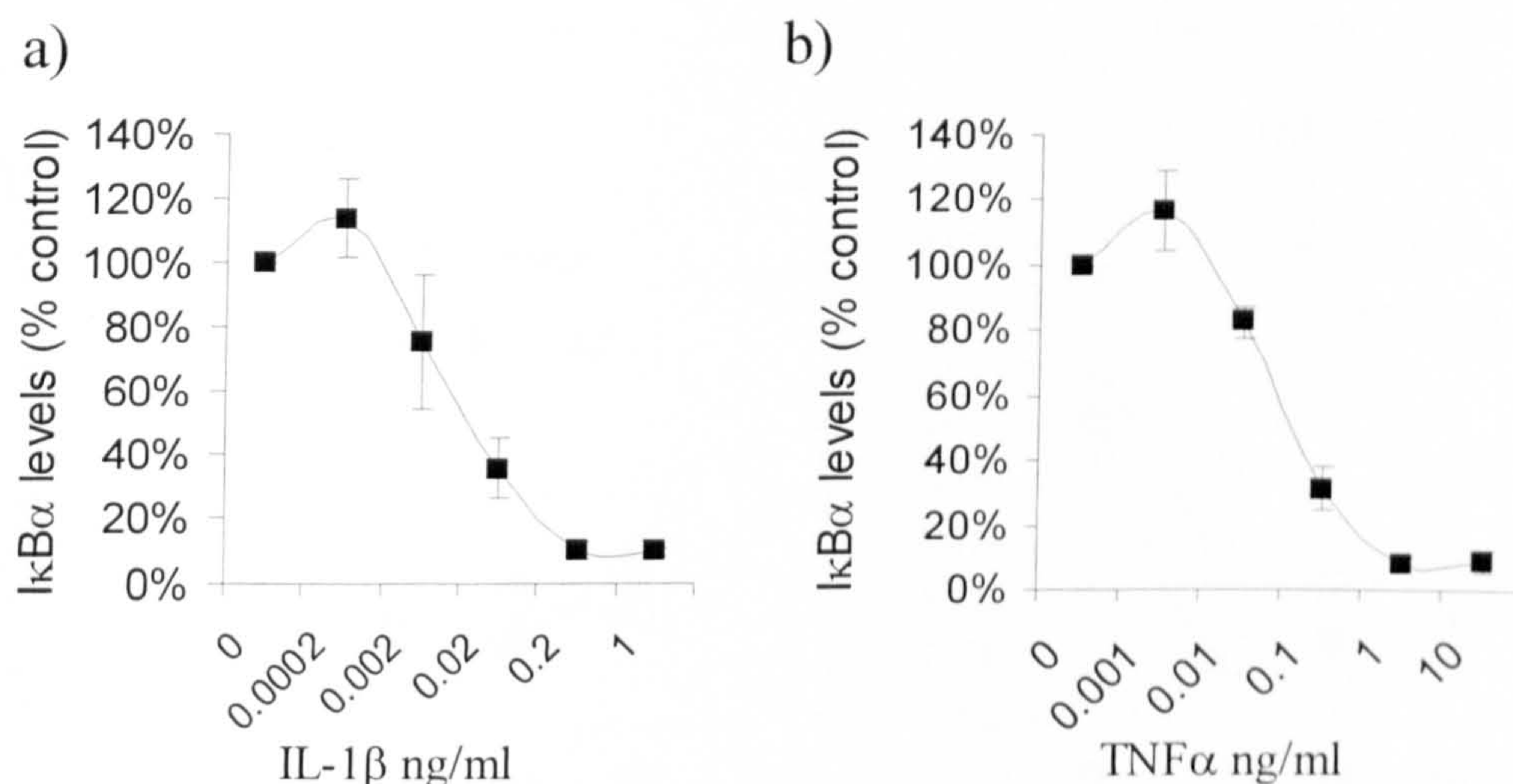


Figure 4.15. Dose dependant effects of pro-inflammatory cytokines on A549 cell IκBα levels. Summary graphs of densitometric analysis of total cell protein Western blots for IκBα following 30 minutes treatment with increasing concentrations of IL-1β (a) and TNFα (b).

Pre treatment of A549 cells with MG132 (10 μ M) prior to IL-1 β or TNF α treatment resulted in the appearance of high molecular weight I κ B α species and the preservation of the phosphorylated form of this protein (Figure 4.16).

In primary airway epithelial cells the time course of I κ B α depletion was slower than that observed in A549 cells and appeared to be incomplete. The protein synthesis inhibitor cycloheximide was included in this set of experiments to avoid potential masking of I κ B α depletion due to I κ B α resynthesis. Under these conditions I κ B α protein levels fell to 40.8 $\pm$ 8.8 and 42.6 $\pm$ 2.3% of that in resting cells following 60 minutes treatment with IL-1 β (2ng/ml, n=6) and TNF α (10ng/ml, n=3), respectively. I κ B α levels were not reduced further when the time course of IL-1 β or TNF α treatment was extended (Figure 4.17).

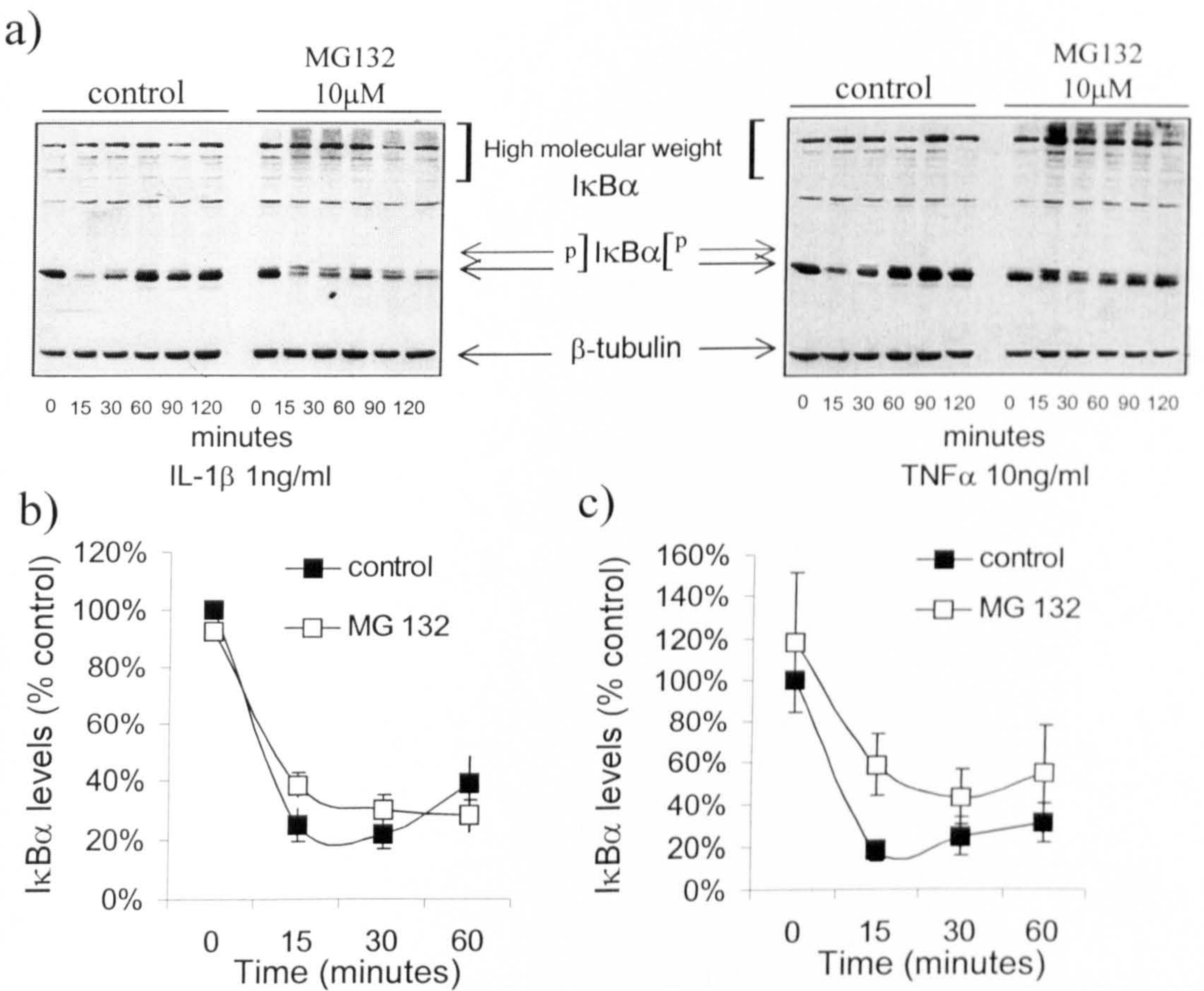


Figure 4.16. Effect of MG132 on cytokine-induced I κ B α depletion in A549 cells. Representative Western blots of total cell I κ B α proteins and summary graphs showing the effects of MG132 (10 μ M) on the time course of a) IL-1 β (1 ng/ml a and b) TNF α (10ng/ml a and c) -induced I κ B α depletion.

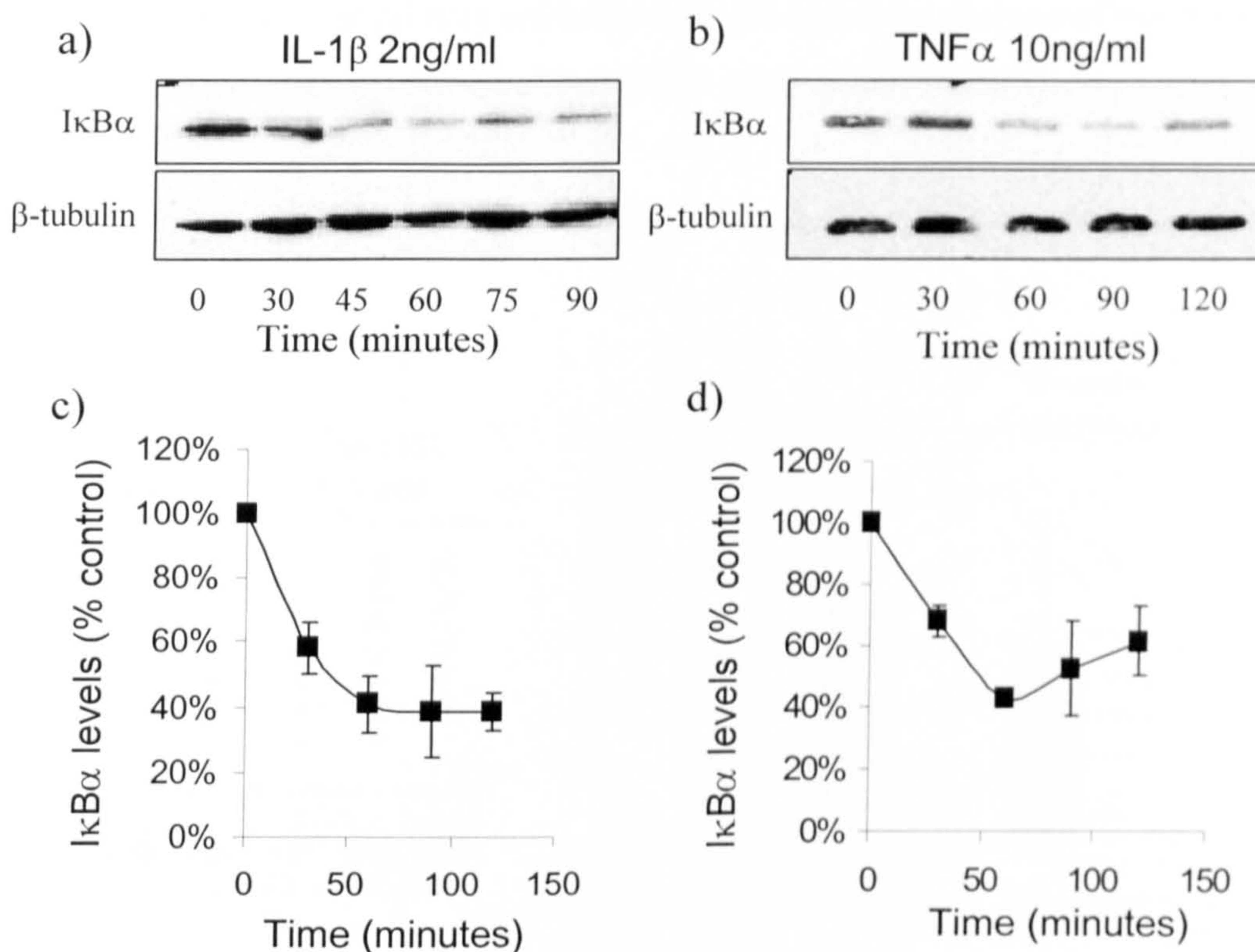


Figure 4.17. Time course of pro-inflammatory cytokine-induced IκBα depletion in primary human airway epithelial cells. Representative Western blot and summary graph of total cell IκBα protein levels following IL-1β (2ng/ml, a and c) and TNFα (10ng/ml, b and d) treatment in the presence of 10μM cycloheximide.

4.5.3.2 Effect of pro-inflammatory cytokines on NFκB p65 nuclear translocation

Treatment of A549 cells for 60 minutes with IL-1β (0.2ng/ml) caused nuclear NFκB p65 levels to increase to $2621.0 \pm 1874.8\%$ of control levels. Pre-treatment with MG132 reduced IL-1β-induced nuclear accumulation of NFκB p65 to $311.6 \pm 62.0\%$ of resting levels. Similarly 60 minutes of TNFα (1ng/ml) treatment resulted in a $2069.3 \pm 1530.0\%$ increase in nuclear p65 above control levels which was reduced to $251.2 \pm 107.6\%$ by pre-treatment with MG132 (10μM, Figure 4.18).

In primary human airway epithelial cells both IL-1β (2ng/ml) and TNFα (10ng/ml) induced an increase in nuclear expression of NFκB p65 following 120 minutes of treatment ($322.8 \pm 57.7\%$ (n=9) and $309.0 \pm 55.3\%$ (n=4) of control levels, respectively, Figure 4.19).

The limited availability of primary epithelial cells did not allow analysis of the effect of proteasome inhibition on cytokine-induced NFκB nuclear accumulation.

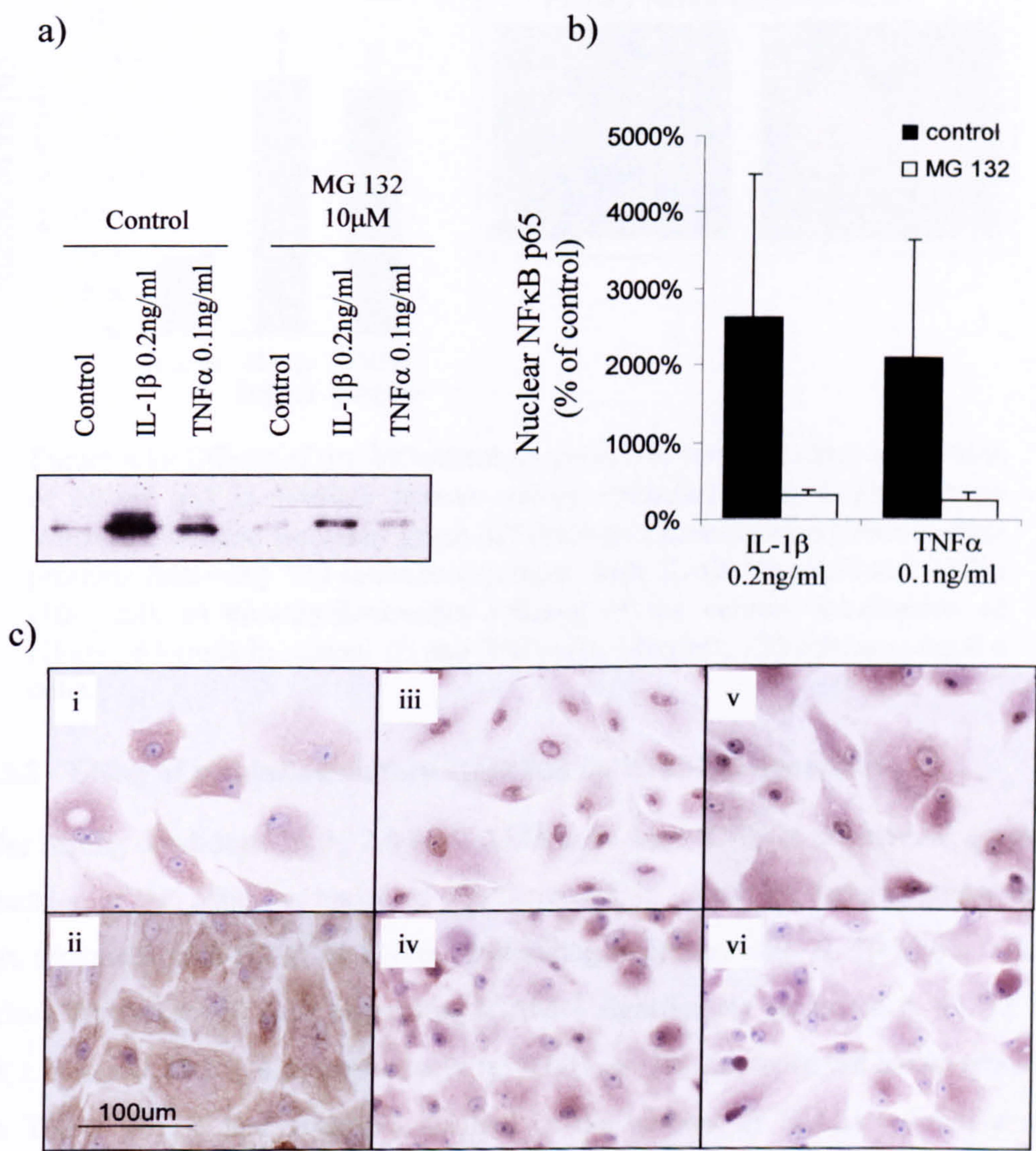


Figure 3.18 Effects of MG132 (10μM) on pro-inflammatory cytokine induced nuclear translocation of NFκB p65 in A549 cells. Representative Western blot (a) and summary graph (b) of NFκB p65 staining of nuclear proteins following 60 minutes cytokine treatment in the presence of MG132. c) Immunoperoxidase staining of NFκB p65 (brown) in A549 cells following 60 minutes treatment with i) control iii) IL-1β 1ng/ml, iv) IL-1β + MG132 v) TNFα 10ng/ml, vi) TNFα + MG132. ii) cytokeratin positive control staining.

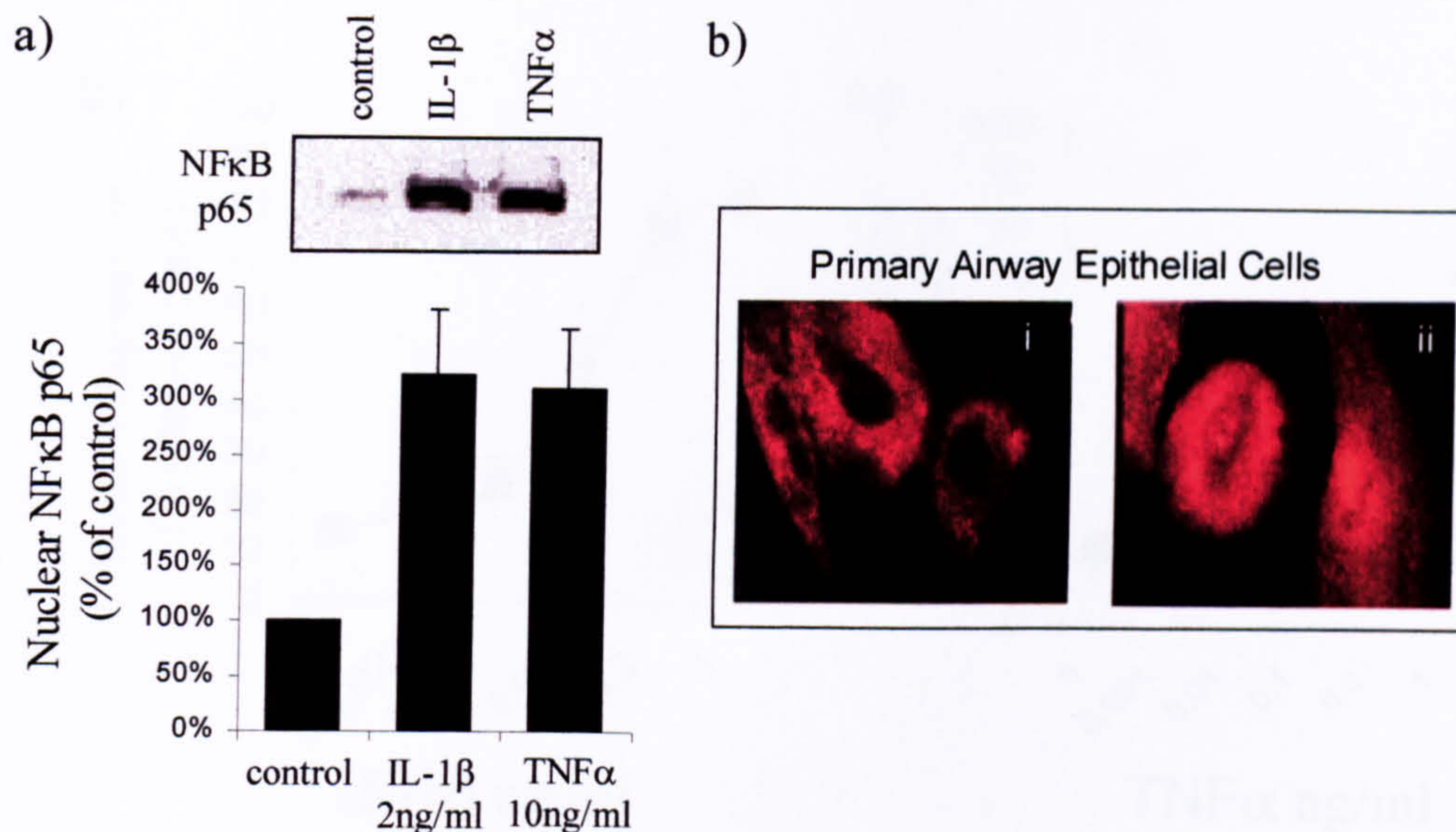


Figure 4.19 Effects of pro-inflammatory cytokines on the nuclear expression of NFκB p65 in primary human airway epithelial cells. Representative Western blots and summary graph of NFκB p65 staining of isolated nuclear proteins following 120 minutes treatment with IL-1β (2ng/ml) and TNFα (10ng/ml). b) immunofluorescent staining of the cellular localisation of NFκB p65 (red) in control (i) and TNFα (ii, 10ng/ml, 120 minutes) treated cells.

4.5.3.3 Effect of pro-inflammatory cytokines on ICAM-1 expression

Under resting conditions 16.9 ± 2.0 % of A549 cells were ICAM-1 positive. The surface expression of this adhesion molecule was increased, in a dose dependant manner by 18 hours treatment with IL-1β or TNFα. Following treatment with IL-1β (0.02, 0.2 and 1ng/ml) the percent of cells expressing ICAM-1 significantly increased to 47.7 ± 6.6 , 77.3 ± 6.3 , and 83.2 ± 4.5 , respectively ($p < 0.001$, $n = 7$). Likewise, 18 hours treatment with TNFα (0.5, 1 and 10ng/ml) resulted in the percent of ICAM-1 positive cells increasing to 43.6 ± 8.9 ($p = 0.002$, $n = 6$), 51.5 ± 6.6 ($p < 0.001$, $n = 10$) and 75.3 ± 4.7 , $p < 0.001$, $n = 9$), respectively (Figure 4.20 a and b).

A549 cell ICAM-1 expression induced by even the highest concentration of IL-1β (1ng/ml) or TNFα (10ng/ml) was significantly inhibited by the presence of MG132 (10μM) in the culture medium with the percent of positive cells reduced to 39.0 ± 5.8 ($p = 0.039$, $n = 3$) and 41.7 ± 7.7 % ($p = 0.019$, $n = 7$), respectively (Figure 4.20c).

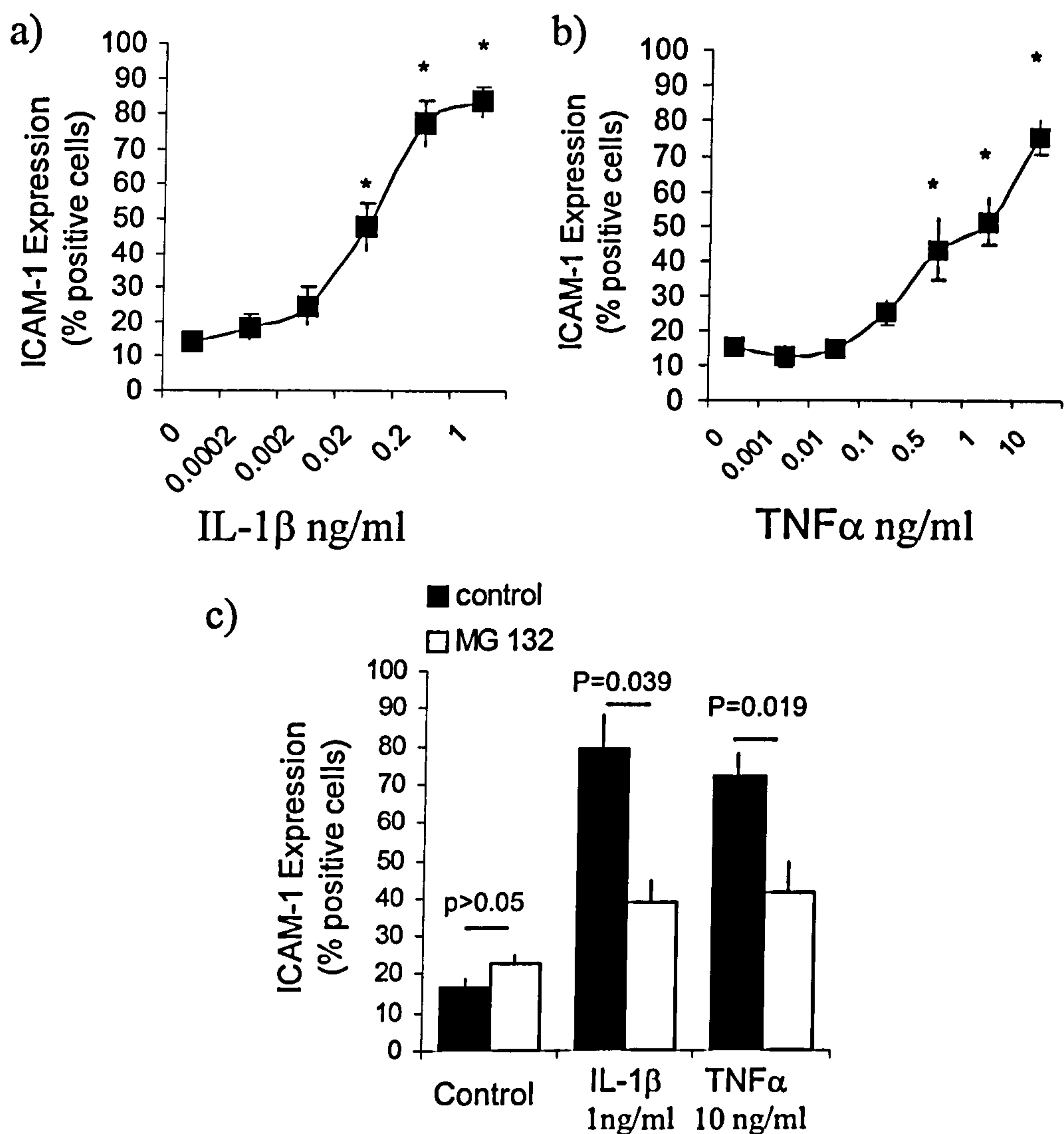


Figure 4.20. Dose dependant effect of pro-inflammatory cytokines on A549 cell ICAM-1 expression and the effects of co-treatment with MG132 (10 μ M). Summary graph of the effects of a) IL-1 β and b) TNF α over a 18 hours time period. * denotes $p < 0.05$ from control baseline expression. c) Effect of MG132 on ICAM-1 expression on A549 cells.

In contrast to the effects of IL-1 β and TNF α , A549 cell ICAM-1 expression induced by IFN γ was not inhibited by co-treatment with MG132. This is consistent with the lack of effect of this cytokine on NF κ B activation in these cells (NF κ B p65 nuclear translocation, Figure 4.21).

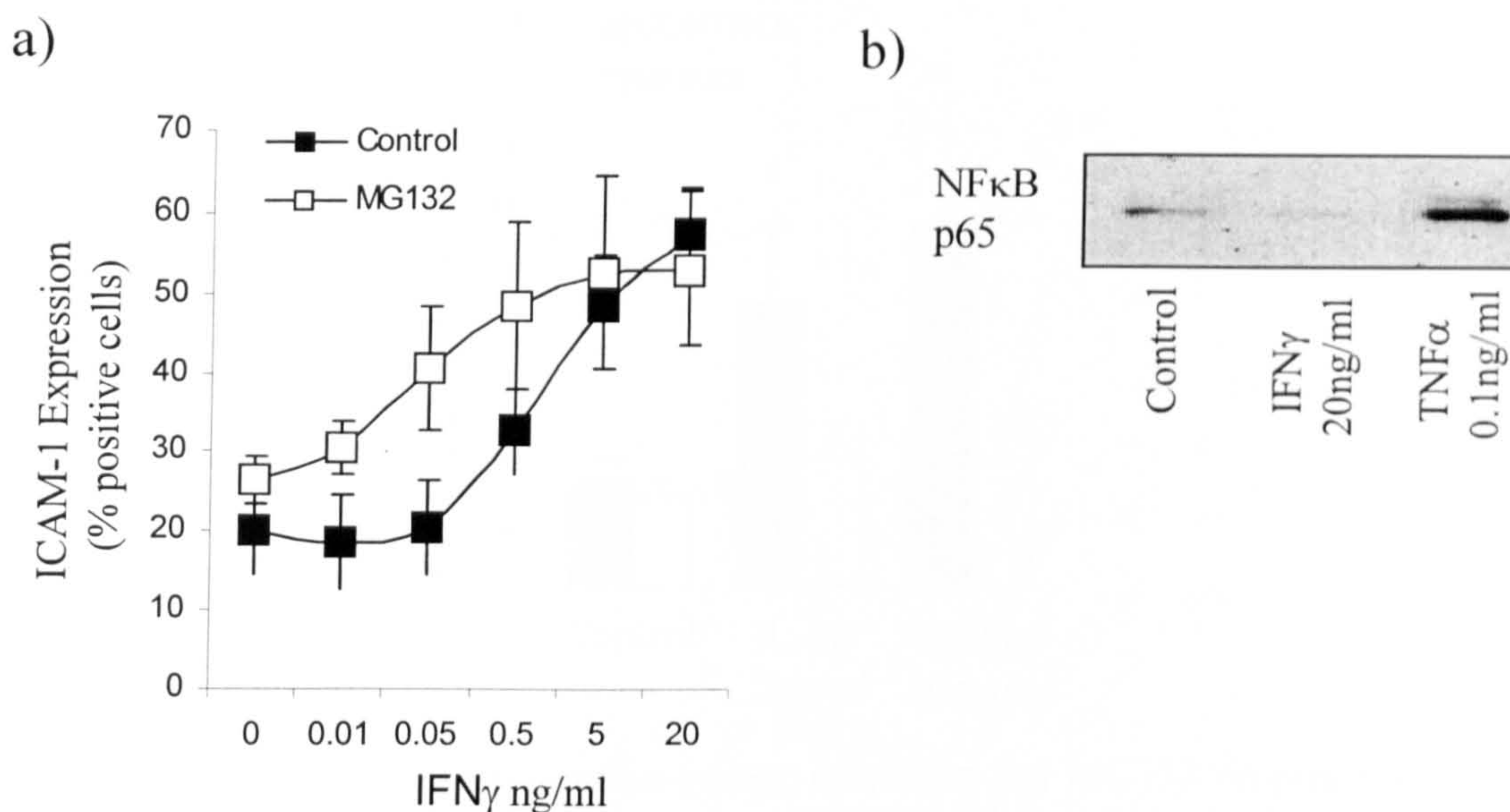


Figure 4.21. Effect of MG132 on IFN γ -induced ICAM-1 expression on A549 cells. .a) Summary graph of IFN γ -induced ICAM-1 expression following 18 hours treatment in the presence of MG132 (10 μ M). b) Effect of IFN γ on nuclear NF κ B p65 following 60 minute treatment.

Under resting conditions the percent of primary airway epithelial cells expressing surface ICAM-1 was 8.5 ± 1.7 (n=5). Treatment of these cells for 24 hours with IL-1 β (2ng/ml) or TNF α (10ng/ml) caused the percent of ICAM-1 expressing cells to significantly increase to 22.9 ± 6.3 % (p=0.044, n=4) and 29.3 ± 9.6 % (p=0.047, n=4) respectively. As with A549 cells, proteasome inhibition with MG132 reduced ICAM-1 expression induced by IL-1 β and TNF α by 48.2 ± 5.7 and 37.0 ± 7.7 % respectively (Figure 4.22).

4.6 DISCUSSION

These results have demonstrated that NF κ B activity can be rapidly induced in cultured human vascular endothelial and smooth muscle cells and pulmonary epithelial cells by the pro-inflammatory cytokines, TNF α and IL-1 β .

In all cell types examined these cytokines caused the rapid depletion of the inhibitor I κ B α with accompanying nuclear translocation of NF κ B p65 protein. In addition, these events were followed by the rapid resynthesis of I κ B α , and the slower induction of adhesion molecule expression.

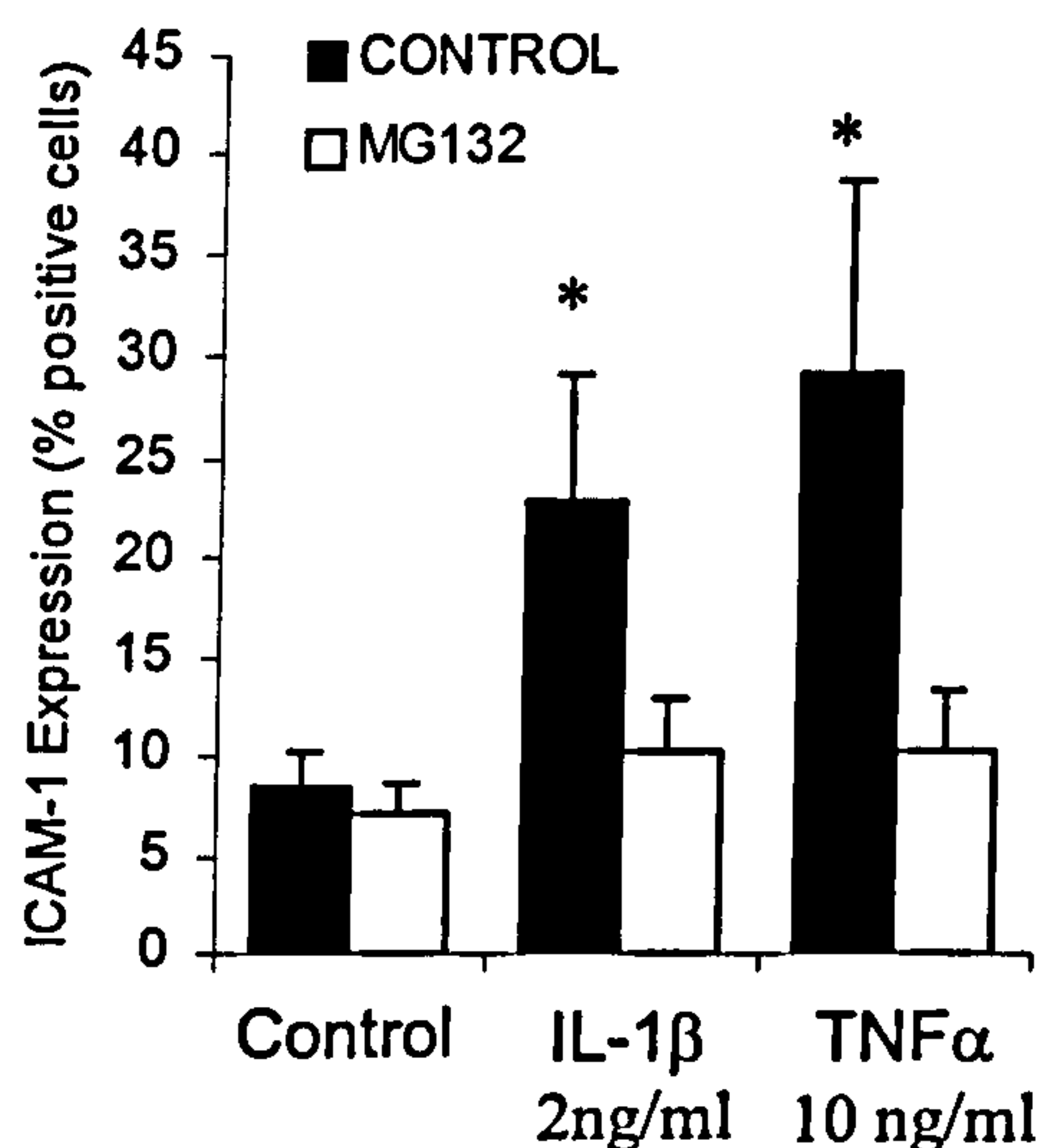


Figure 4.22. Effect of pro-inflammatory cytokines and MG132 on primary epithelial cell ICAM-1. Summary graph of the effects of IL-1 β (2ng/ml), TNF α (10ng/ml) following 24 hours stimulation alone and in the presence of MG132 (10 μ M). * denotes $p < 0.05$ from control cells.

4.6.1 I κ B α DEPLETION AND NF κ B NUCLEAR ACCUMULATION

The time course of I κ B α depletion in response to IL-1 β and TNF α in the EC, SMC and the A549 cells was rapid with maximum effects (80- 90% of I κ B α protein degraded) observed within 15 – 30 minutes of cytokine treatment. Furthermore, high levels of nuclear NF κ B p65 protein were achieved within 60 minutes of treatment. The primary epithelial cells appeared to be much less sensitive to cytokine stimulation than the other cell types and required a longer cytokine exposure to achieve reproducible results between cultures. In addition, in these cells I κ B α depletion experiments had to be performed in the presence of the protein synthesis inhibitor, cycloheximide, to prevent I κ B α resynthesis from masking the depletion of this protein. These primary epithelial cells required around 60 minutes cytokine treatment for maximum I κ B α degradation to occur which only reached around 60%. In addition, 120 minutes cytokine treatment was required when analysing nuclear accumulation of NF κ B p65. This observation might reflect the heterogeneity of the population of primary airway epithelial cells that were all grown directly from tissue and not passaged. This culture method may preserve the cells in a more native, differentiated state compared to cells dissociated from tissues and cultivated in culture.

Signal-induced degradation of I κ B α in many cells and in response to diverse stimuli has been shown to proceed via the phosphorylation and ubiquitin dependent proteasome

pathway. Indeed I κ B α mutants carrying serine to alanine substitutions at positions 32 and 36 significantly reduce I κ B α phosphorylation, ubiquitination and degradation and reduces signal-induced NF κ B-driven reporter activity (Brown et al., 1995; DiDonato et al., 1996; Kabouridis et al., 2002; Park et al., 2001a; Purcell et al., 2001; Rodriguez et al., 1996; Roff et al., 1996; Traenckner et al., 1995). In addition I κ B α mutants carrying a lysine to alanine substitution at amino acid residues 21 and 22 do not prevent I κ B α phosphorylation but significantly reduce signal induced ubiquitination and degradation, preserving the phosphorylated form of I κ B α and inhibiting stimuli-induced NF κ B reporter activity in a manner similar to pharmacological inhibition of the 26S proteasome (Baldi et al., 1996; DiDonato et al., 1996; Rodriguez et al., 1996).

Many peptide aldehyde compounds have now been designed that can inhibit the activity of the proteasome. By virtue of their ability to inhibit I κ B degradation, these compounds stabilise the phosphorylated form that remains bound to NF κ B and therefore the subsequent nuclear translocation of the NF κ B dimer is prevented (Traenckner et al., 1994). Such compounds have become a widely used tool in NF κ B research.

4.6.1.1 Effect of proteasome inhibition

In all the cell types studied in this chapter pre-treatment with the proteasome inhibitor MG132 before addition of pro-inflammatory cytokines (IL-1 β or TNF α) inhibited the depletion of I κ B α from the cells, as determined by Western blot analysis of whole cell lysates. In the presence of proteasome inhibition cytokine treatment was associated with the appearance of a distinct I κ B α protein band of slightly higher molecular weight than that in resting cells, likely corresponding to the phosphorylated form of this protein that remained preserved throughout the 120 minute time course. In addition there was an increase in I κ B α staining in the high molecular region of the blot in cells treated with proteasome inhibitor and cytokine. This staining most likely corresponds to ubiquitin-conjugated I κ B α proteins that are preserved due to the inhibition of proteasome-mediated degradation. These results demonstrate the phosphorylation and ubiquitination of I κ B α in response to stimulation with IL-1 β and TNF α that precedes the degradation of this inhibitor protein. In addition these results reinforce the essential requirement for these events in the activation of NF κ B in response to IL-1 β and TNF α by the

demonstration that such treatment was associated with reduced NFκB p65 nuclear accumulation.

4.6.2 IκBα RESYNTHESIS

Induction of the IκBα gene and IκBα resynthesis following stimuli-induced degradation has been shown to be mediated by NFκB. Scott et al first demonstrated that overexpression of NFκB p65 increased IκBα protein expression (Scott et al., 1993). Subsequently the 5' flanking region of the transcriptional start site of the human IκBα gene was cloned, sequenced and shown to contain three NFκB binding sites that are required for successful gene induction (Ito et al., 1994; Le Bail et al., 1993). Saccini et al classified the IκBα gene as having a constitutively and immediately accessible (CIA) promoter, meaning that it is immediately accessible to the NFκB dimer once it translocates to the nucleus and does not require additional signals. As such NFκB can thus rapidly direct transcriptional activation of the IκBα gene (Saccani et al., 2001). The rapid resynthesis of IκBα following cytokine-induced degradation is therefore a good measure of efficient NFκB driven gene transcription and full NFκB activation.

In this study the initial IκBα depletion induced by both IL-1β and TNFα was followed by the reappearance of IκBα proteins. The resynthesis of IκBα was rapid with new protein observed as early as 60 minutes after the addition of cytokines with levels returning to those found in unstimulated, resting cells within about 120 minutes. This data demonstrates that these pro-inflammatory cytokines are able to induce the rapid and effective activation of NFκB in human endothelial, vascular smooth muscle and pulmonary airway epithelial cells.

4.6.3 ADHESION MOLECULE EXPRESSION

In addition to IκBα, NFκB can induce the expression of a large number of genes involved in inflammation. In this study the inducible expression of E-selectin and ICAM-1, adhesion molecules relevant to vascular and pulmonary inflammation, were measured to indicate NFκB dependant inflammatory gene induction. The expression of these genes occurs at a much delayed time course compared to the IκBα gene suggesting that additional signals, co-factors and accessory molecules are required for the induction of transcriptional activity.

In EC both IL-1 β and TNF α -induced a concentration dependant increase in E-selectin expression over a five hour time period. Co-treatment of the cells with MG132 to inhibit I κ B α depletion and NF κ B activation dramatically inhibited the observed increase in E-selectin expression. These data indicate that NF κ B activation is an essential requirement for the successful induction of the E-selectin gene and that IL-1 β and TNF α can both induce sufficient signalling in human vascular EC to rapidly and potently induce NF κ B-dependant inflammatory gene induction.

In the smooth muscle and epithelial cells ICAM-1 induction was increased in a dose dependant manner by both IL-1 β and TNF α over an 18 hours time course. In the primary epithelial cells a longer time course of 24 hours was required to induce detectable ICAM-1 expression. Similar to the induction of E-selectin, MG132 pre-treatment significantly inhibited ICAM-1 expression induced by both these cytokines demonstrating the essential requirement for NF κ B. IFN γ also induced ICAM-1 expression in A549 cells in a manner that was not inhibited by MG132. IFN γ did not induce I κ B α depletion or nuclear p65 accumulation ($114.1 \pm 21.2\%$ of control levels) in these cells and thus IFN γ -induced ICAM-1 expression is independent on NF κ B activation. These data demonstrate that MG132 is not having a non-specific effect on ICAM-1 induction and supports the indispensable role of NF κ B activation in conferring IL-1 β and TNF α -induced ICAM-1 induction in pulmonary epithelial cells and in vascular smooth muscle cells.

4.7 CONCLUSION

Taken together these data demonstrate that IL-1 β and TNF α can induce a rapid and potent activation of NF κ B in vascular and airway cells. Through the multistep process of I κ B α phosphorylation, ubiquitination and degradation these cytokines induce the translocation of NF κ B p65 to the nucleus where it can bind to the promoter region of target genes, in either an immediate or a delayed fashion, to induce gene transcription. During systemic and pulmonary inflammatory responses the increased expression of pro-inflammatory cytokines in the circulation and in the lungs may therefore directly activate these cells. This will lead to the increased production of inflammatory cytokines, chemokines, adhesion molecules and enzymes, many of which are, at least in part regulated by NF κ B. In this manner these cells form a pivotal part of the inflammatory process and can direct the course of the inflammatory response.

However under conditions of systemic inflammation IL-1 β and TNF α do not exist in isolation and other factors may also be present in the circulation that can induce alternative signalling pathways or interfere with the pathways utilised by IL-1 β and TNF α and thus may potentiate or inhibit target gene transcription.

**Chapter Five: BIOACTIVITY OF PATIENTS' PLASMA TO
INFLUENCE NF κ B ACTIVITY IN ENDOTHELIAL CELLS –
EFFECT OF HEART FAILURE AND CARDIAC SURGERY**

5.1 INTRODUCTION

The data presented in chapter 3 clearly demonstrate the variability of the systemic inflammatory response, with regard to circulating cytokine concentrations and leucocyte activation in patients with heart failure and in response to cardiac surgery. However, the complex interactions that exist between the multiple pro- and anti- inflammatory factors and the redundancy that exists within the cytokine network makes predicting the functional inflammatory consequences of changes in the levels of individual mediator virtually impossible.

The data presented in chapter 4 of this thesis demonstrates the potent NF κ B inducing activity of the pro-inflammatory cytokines IL-1 β and TNF α in cultured human cells. However, *in vivo* cells are unlikely to be exposed to these cytokines in isolation and the presence or absence of other soluble factor and the precise timing of events both in terms of intra- and extra-cellular, inhibitory and co-stimulatory will often determine the response of cells to the pro-inflammatory cytokines.

Increased expression of soluble TNF α receptors and interleukin-1 receptor antagonist can specifically block the interaction between TNF α /IL-1 β and their cellular receptors and thus can inhibit the initiation of signal transduction pathways such as NF κ B. T-helper (Th)₂-like cytokines and other anti-inflammatory factors such as IL-4, IL-13, IL-6 and IL-10 can, in addition to inducing the expression of soluble receptors (Dickensheets et al., 1997a; Joyce and Steer, 1996) and receptor antagonists (Jenkins et al., 1994) exert an effect by blocking specific events in the NF κ B signal transduction pathway activated by inflammatory mediators (Lentsch et al., 1997; Schottelius et al., 1999).

As well as inhibitory activities, specific combinations of cytokines and soluble factors can produce synergistic or additive effects on gene expression through actions on the same or co-operating signalling pathways thus affecting transactivation of the target gene.

The fact that NF κ B activity can be modulated by a host of soluble factors, both stimulatory and inhibitory and the central role of NF κ B in the inflammatory process offers a good opportunity to exploit this transcription factor pathway as a tool to assess net-inflammatory bioactivity in biological samples.

5.2 AIMS

To determine the potential net inflammatory consequences (bioactivity) of altered circulating cytokine profile in the plasma of the patients studied in chapter 3 (patients with heart failure and those undergoing CABG) using an *in vitro* cell culture bioassay.

5.3 STUDY DESIGN

Based on the characterisation of stimuli-induced NF κ B activity in cultured human endothelial cells (chapter 4) a bioassay system was developed to detect an inflammatory imbalance in patient plasma. Two specific events in the NF κ B activation pathway were monitored in human PAEC, in response to treatment with patient plasma, as a measure of inflammatory bioactivity, 1) I κ B α depletion and 2) increase surface expression of E-selectin.

This system was chosen for the bioassay for several reasons:

- Reproducible results can be obtained between endothelial cell isolates with regard to I κ B α depletion and E-selectin expression in response to pro-inflammatory cytokines (Chapter 4, section 4.5.1).
- The induction of E-selectin expression is highly dependent on the activation of NF κ B, unlike ICAM-1 expression in A549 cells, which can be induced in an NF κ B independent manner.
- E-selectin is expressed only on activated endothelial cells, thus baseline expression in resting cells is minimal allowing standardisation between experiments (baseline expression always set at 2% positive cells) and the ability to detect low levels of activation.
- With respect to E-selectin expression, the bioactivity of relatively large numbers of test samples can be analysed simultaneously.
- The anatomical location of endothelial cells means that the effect of plasma on their activation state may be clinically relevant.

One potential drawback of this assay is that cell availability is limited because with increasing time in culture endothelial cells lose their ability to express E-selectin. Thus comparisons between samples must be performed at the same time, on the same cell cultures, at a low passage.

5.4 METHODS

5.4.1 PATIENTS AND SAMPLES

The same patient groups and samples described in Chapter 3 (sections 3.2.4.1, 3.3.4.1, 3.4.3.1, 3.2.4.2, 3.3.4.4, 3.4.3.3) were used for this study.

5.4.2 ENDOTHELIAL CELL CULTURE

Chapter 4, section 4.3.1.1.

5.5 BIOASSAY

A bioassay system was established based on the characterisation of NF κ B activation and E-selectin expression induced on human pulmonary artery endothelial cells by IL-1 β and TNF α .

5.5.1 GENERAL

PAEC were grown to 80% confluence in 24 well plates for all bioassay experiments. Cells were left in DMEM containing no additive for 24 hours prior to the start of each experiment. This medium was used throughout the course of the bioassay experiments.

Cells were treated with medium (control), AB serum diluted with equal volumes of medium (AB serum control), cytokines (positive controls) or native patient plasma diluted with equal volumes of medium. Plasma from LPS treated blood was diluted as indicated in the specific results sections.

5.5.2 I κ B α BIOASSAY

All I κ B α bioassays were performed in the presence of cycloheximide (10 μ M) and on cells pre-treated for 30 minutes with cycloheximide (10 μ M) in order to inhibit protein synthesis and thus prevent the potential masking of I κ B α depletion by newly synthesised I κ B α protein.

Cells were incubated with samples or cytokines for 60 minutes at 37° C after which time they were washed in PBS and total cell lysis samples prepared using 1% SDS lysis buffer. I κ B α levels in the lysed cell preparations were quantified by Western blot analysis and laser densitometry as described in section 4.3.2.1 and the results expressed as a percent of I κ B α levels in control treated cells.

5.5.3 E-SELECTIN BIOASSAY

Cells were incubated with samples or cytokines for 5 hours at 37°C before they were analysed for cell surface E-selectin expression by flow cytometry as described in section 4.3.2.6.1.

5.5.4 NEUTRALISING CYTOKINE ACTIVITY

For cytokine neutralising studies, samples or cytokines were pre-incubated at 37°C with the neutralising antibody for 30 minutes before addition to the cells. All other aspects of the bioassay were as standard.

5.5.5 DATA ANALYSIS

Results involving patient samples did not pass normality and/or equal variance testing and thus the data cannot be assumed to be normally distributed. These results are therefore expressed as median values with ranges. Differences between groups were assessed using Mann-Whitney Rank Sum Test or Kruskal-Wallis One Way Analysis of Variance on Ranks followed by Dunn's post hoc test. Box plots represent median and 10th, 25th, 75th and 90th percentiles with circles below and above the whiskers representing 5th and 95th percentiles. For Bioassay data, at least three independent assays, using PAEC isolates from different sources, were performed for each patient sample and the mean response for each sample used for grouped comparisons.

All other data that passed normality and equal variance tests are expressed as mean $\pm$ sem. Differences between groups were assessed using ANOVA with post hoc t-test where required. Experiments were performed on at least three independent occasions using different PAEC isolates.

Correlations between parameters were analysed using Spearman's rank order correlation. p value < 0.05 was considered significant.

5.6 RESULTS

5.6.1 CONTROLS

For both bioassays the treatment of the PAEC with control AB serum had no significant effect of I κ B α levels (94.1 $\pm$ 21.0 % of control levels) or E-selectin expression (3.8 $\pm$ 0.4% positive cells (n=8) versus 3.3 $\pm$ 0.3% in control resting cells (n=19, p=0.391).

5.6.2 HEART FAILURE PATIENTS

5.6.2.1 Effect of patient plasma on PAEC IκBα levels

Compared to levels following treatment with AB serum IκBα proteins in PAEC were significantly reduced to 35.5 (32.8-72.8)% of that in control cells following treatment with Tx patient plasma (n=10, p<0.05) and to 42.0 (27.8-74.4)% following treatment with plasma from LVAD patients (n=10, p<0.05). There was no significant difference between the ability of either Tx or LVAD patient plasma to induce IκBα depletion (p=0.623, Figure 5.1).

To investigate the potential importance of TNFα in conferring this effect of the plasma the bioassay was performed in the presence of anti-TNFα neutralising antibody. Under conditions that significantly inhibited TNFα but not IL-1β-induced IκBα depletion in PAEC (Figure 5.2a), the presence of anti-TNFα neutralising antibodies prevented IκBα depletion in PAEC treated with plasma from only 2 Tx patients (from 69.6 to 134.3 and from 61.5 to 112.6 % of control IκBα levels). However, such treatment failed to alter the grouped data in this respect (Tx p=0.574 or LVAD p=0.694).

There was no significant correlation between the bioactivity of plasma to induce IκBα depletion and the plasma concentration of any single cytokine or molar ratio measured (p>0.05).

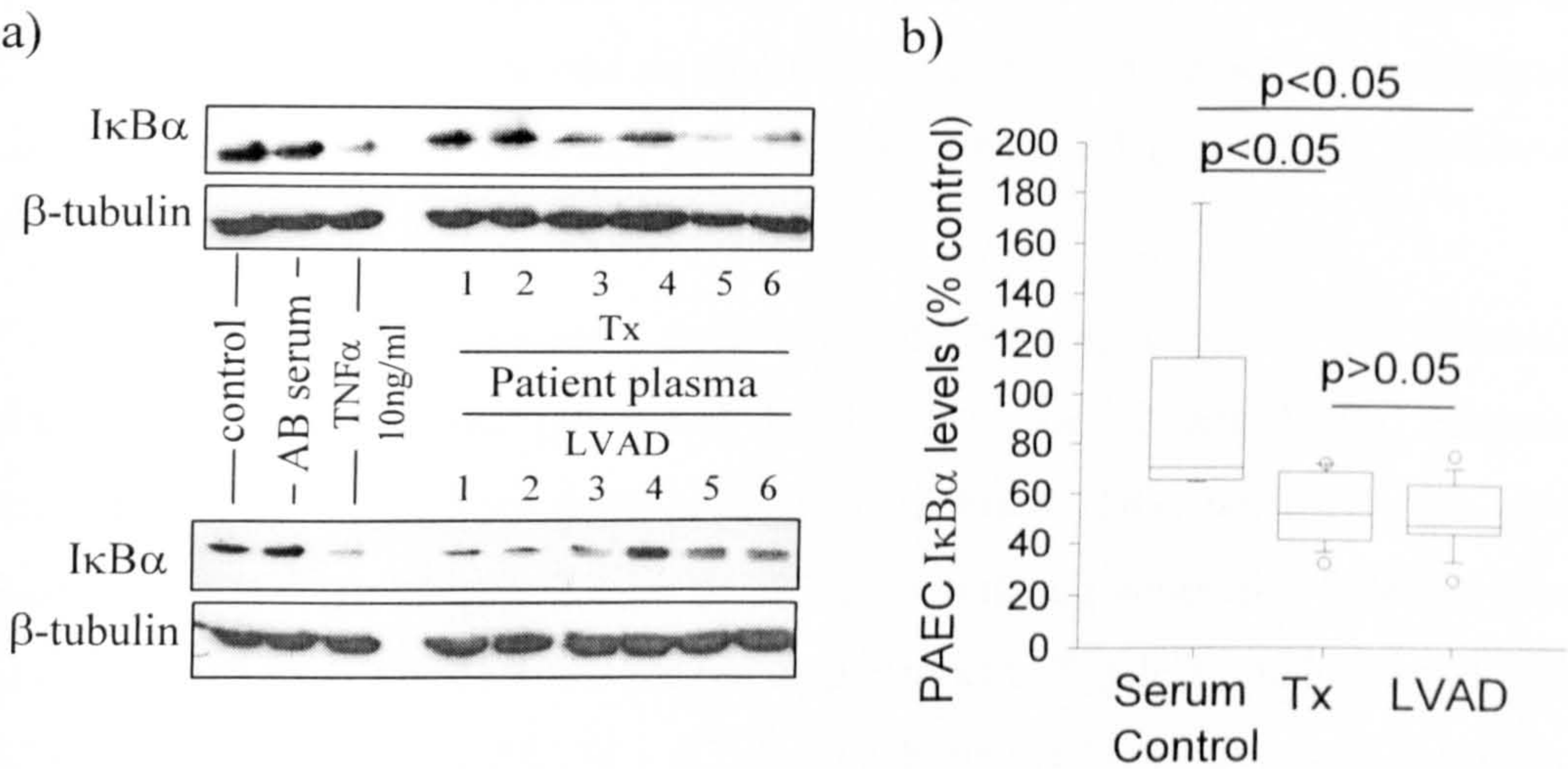


Figure 5.1. Effect of plasma from Tx and LVAD patients on IκBα levels in PAEC compared to the effect of control AB serum. a) Representative Western blots showing the bioactivity of 6 Tx and 6 LVAD plasma samples to induce PAEC IκBα degradation. b) Summary graph of the densitometric analysis of IκBα Western blots.

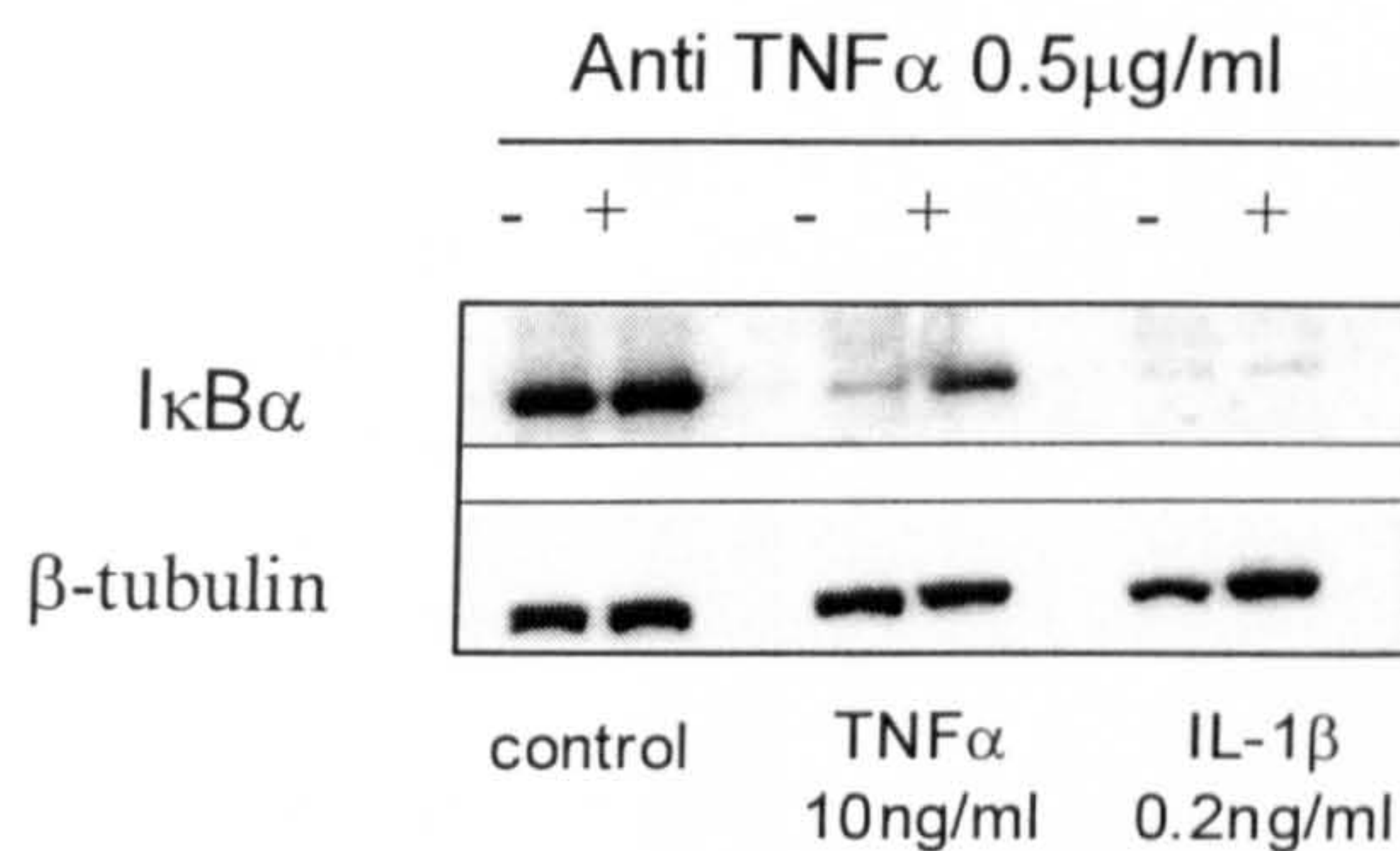


Figure 5.2. Effect of anti TNF α neutralising antibody on I κ B α depletion in PAEC. Western blot demonstrating the inhibitory effect of anti-TNF α antibodies on TNF α but not IL-1 β induced I κ B α depletion.

	control	Anti-TNF α	p value
Tx	61.5 (23.7 – 151.0)	56.4 (27.7 – 136.8)	0.574
LVAD	51.2 (20.9 – 116.7)	52.6 (27.9 – 122.9)	0.694

Table 5.1. Effect of anti-TNF α neutralising antibodies on I κ B α levels following treatment of PAEC with Tx and LVAD patient plasma (% of control PAEC I κ B α levels, median values with ranges).

5.6.2.2 Effect of patient plasma on endothelial cell E-selectin expression

Treatment of PAEC with Tx plasma caused a small but significant increase in E-selectin expression (5.6 (3.2-9.4)% positive cells, $p < 0.05$, $n = 15$) above control levels (2.9 (2.1 – 7.7) % positive cells). There was no significant increase in E-selectin expression above control levels following treatment with LVAD plasma (3.1 (2.3-9.8) % positive cells, $p > 0.05$, $n = 14$, Figure 5.3).

To investigate further the potential importance of TNF α in conferring this effect of the plasma the bioassay was performed in the presence of anti-TNF α neutralising antibodies. Under conditions that significantly inhibited TNF α but not IL-1 β -induced E-selectin expression (Figure 5.4a) anti-TNF α neutralising antibodies failed to alter the bioactivity of either Tx or LVAD plasma with respect to E-selectin induction (3.8 (2.7 – 5.5), $p = 0.970$, $n = 10$ and 2.2 (0.74 – 6.1), $p = 0.909$, $n = 14$) % positive cells, respectively, Figure 5.4b). In agreement with this result there was no significant correlation between TNF α levels or sTNFR/TNF α molar ratios and bioactivity to induce E-selectin in either group of patients.

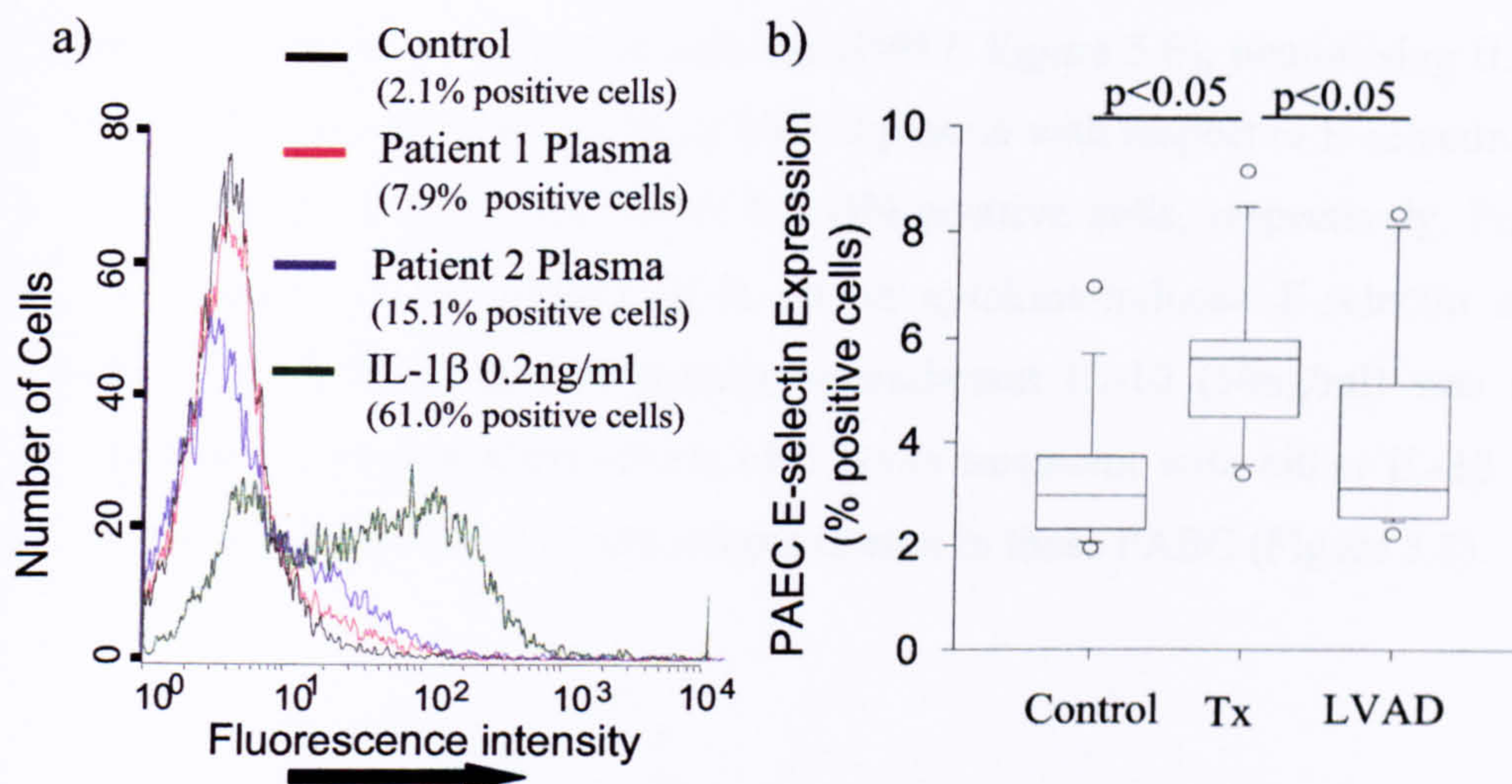


Figure 5.3. Effect of plasma from Tx and LVAD patients on E-selectin expression on PAEC compared to control expression. a) Representative E-selectin flow cytometry histogram showing the effect of plasma from two patients on the level of E-selectin expression on PAEC. b) Summary graph of grouped data.

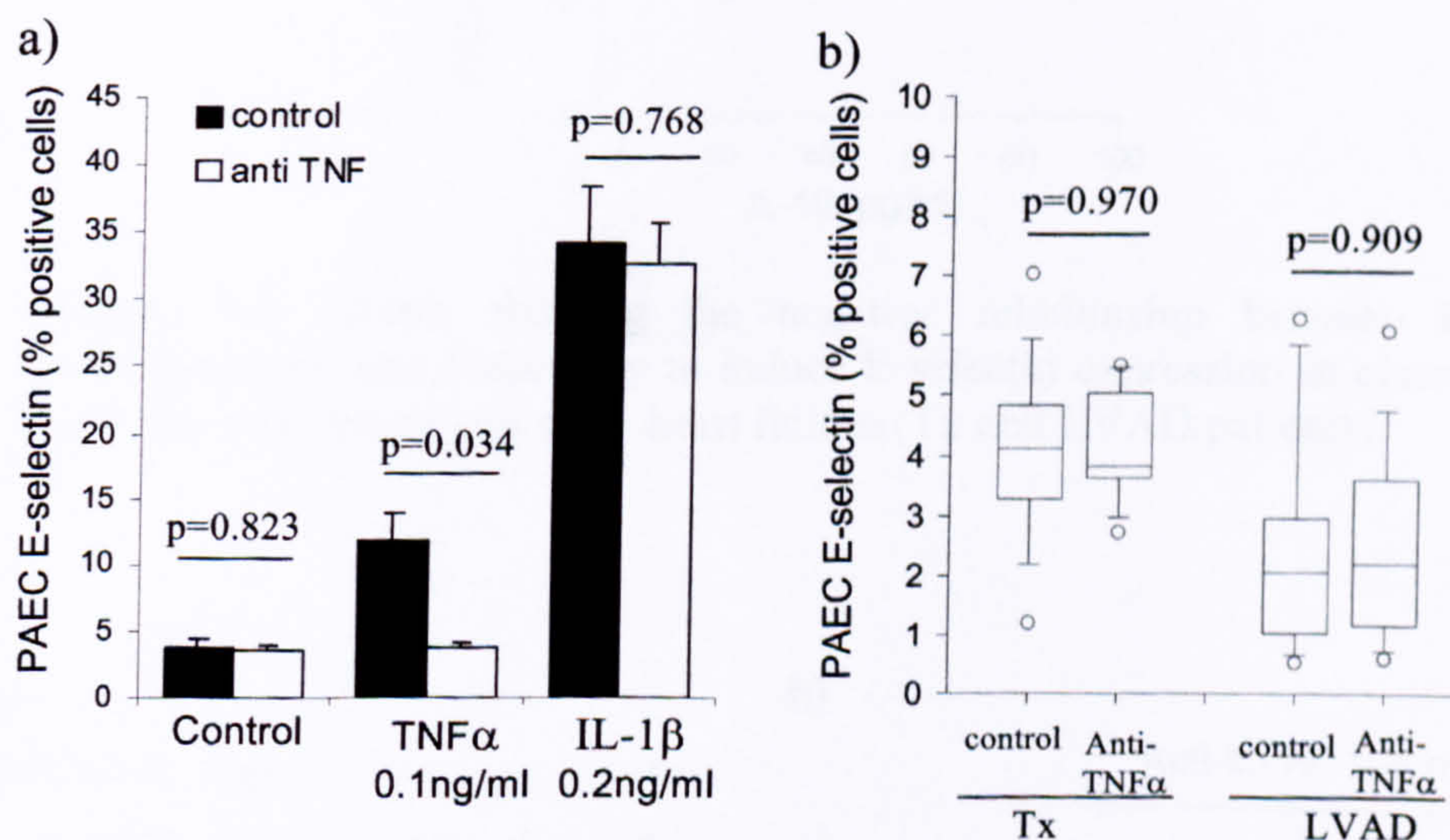


Figure 5.4 Effect of anti-TNF α neutralising antibody on E-selectin expression in PAEC. a) Graph demonstrating the inhibitory effect of anti-TNF α antibodies on TNF α but not IL- 1β -induced increase in E-selectin. b) Summary graph showing the lack of effect of TNF α neutralising antibody on E-selectin expression in PAEC following treatment with Tx and LVAD patient plasma.

There was a significant negative correlation between bioactivity to induce E-selectin expression and plasma IL-10 concentrations ($r = -0.664$, $p < 0.001$, Figure 5.5). To explore the influence of IL-10 on E-selectin expression the bioassay experiments were carried out in the presence of anti-IL-10 neutralising antibodies. Under conditions that

specifically attenuated IL-10 (50ng/ml)-induced signalling events (JAK1 and STAT1 phosphorylation) in a responsive cell line (U937, Figure 5.6), neutralising IL-10 failed to alter the bioactivity of either Tx or LVAD plasma with respect to E-selectin induction in PAEC (5.2 (2.9-6.8)% and 3.1 (1.8-6.1)% positive cells, respectively, Figure 5.7). Further analysis of the effects of IL-10 on cytokine-induced E-selectin expression revealed that, in this bioassay system, recombinant IL-10 (50ng/ml) was unable to modulate the dose dependant effects of 5 hours treatment with either IL-1 β or TNF α with respect to induction of E-selectin expression in these PAEC (Figure 5.8).

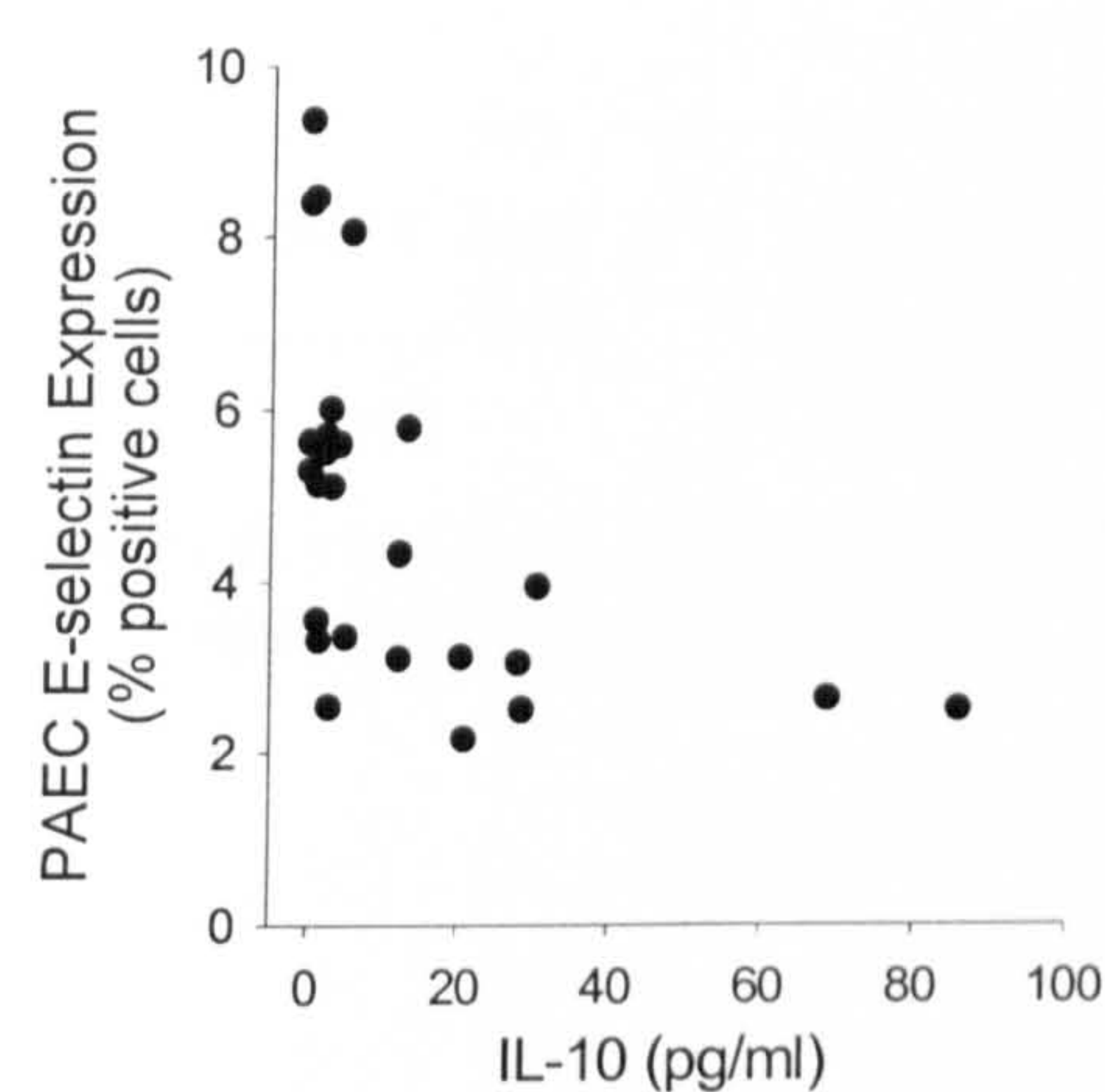


Figure 5.5. Graph showing the negative relationship between IL-10 concentrations and bioactivity to induce E-selectin expression in plasma of patients with severe end stage heart failure (Tx and LVAD patients).

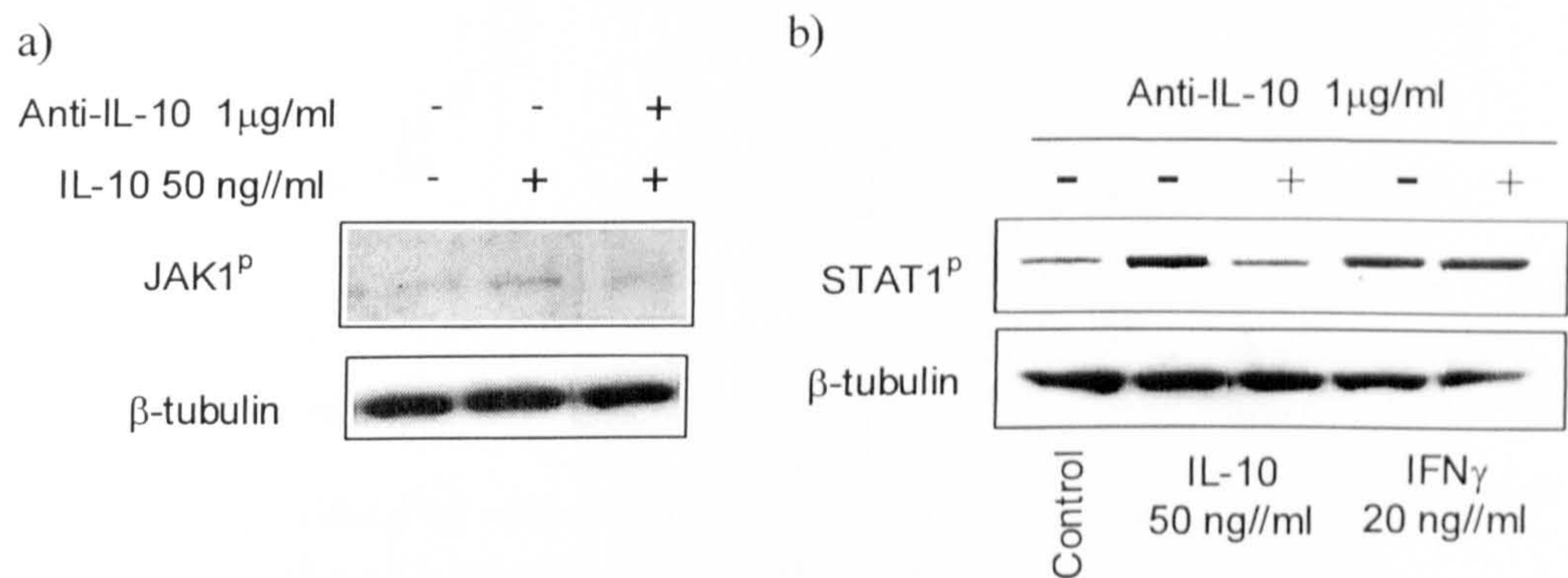


Figure 5.6 Western blot demonstrating the specific inhibitory activity of anti-IL-10 neutralising antibody on IL-10 but not IFN γ signalling events a) JAK1 phosphorylation b) STAT1 phosphorylation.

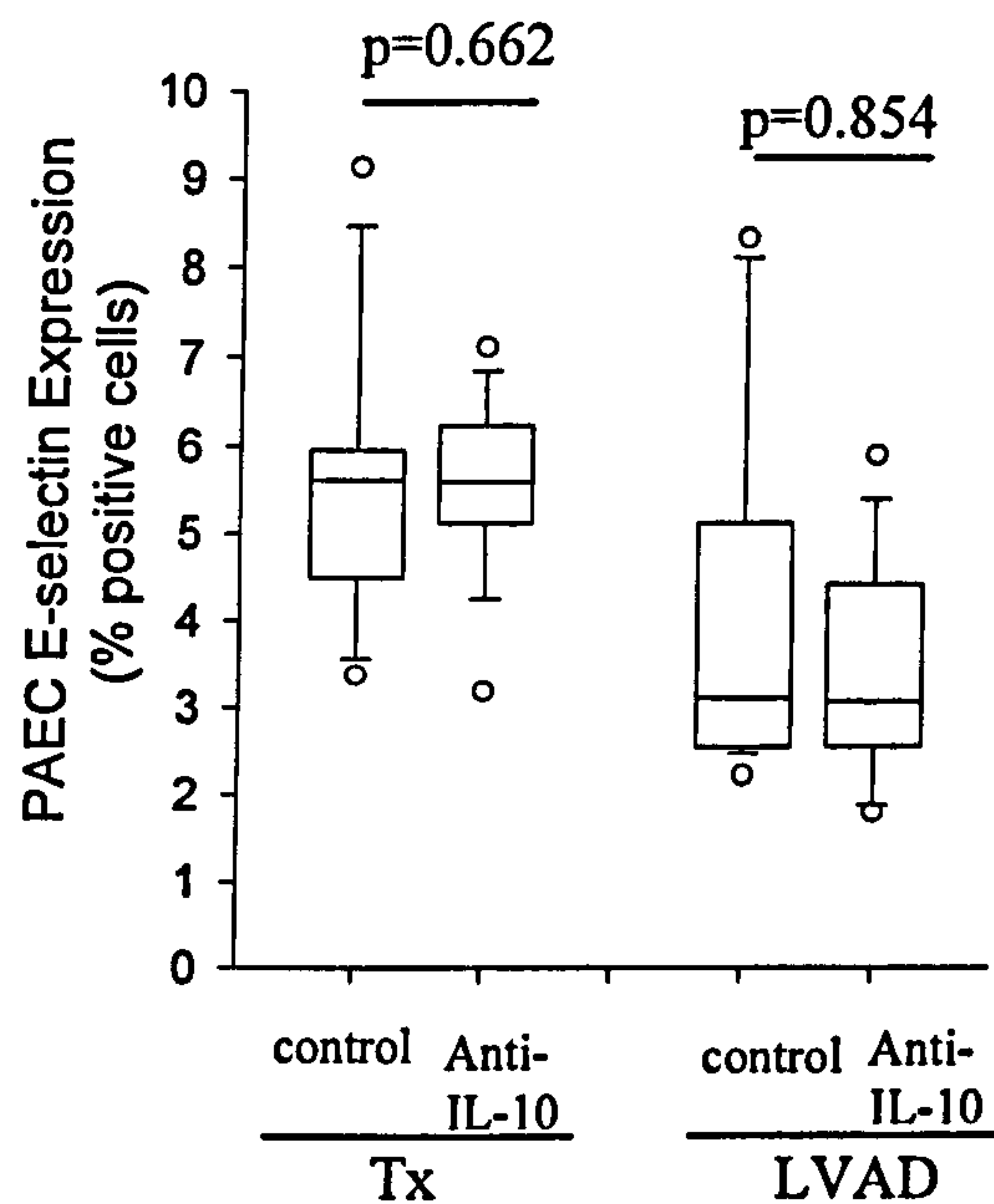


Figure 5.7 Effect of neutralising IL-10 activity with anti-IL-10 antibodies on the bioactivity of Tx and LVAD patient plasma to induce E-selectin expression.

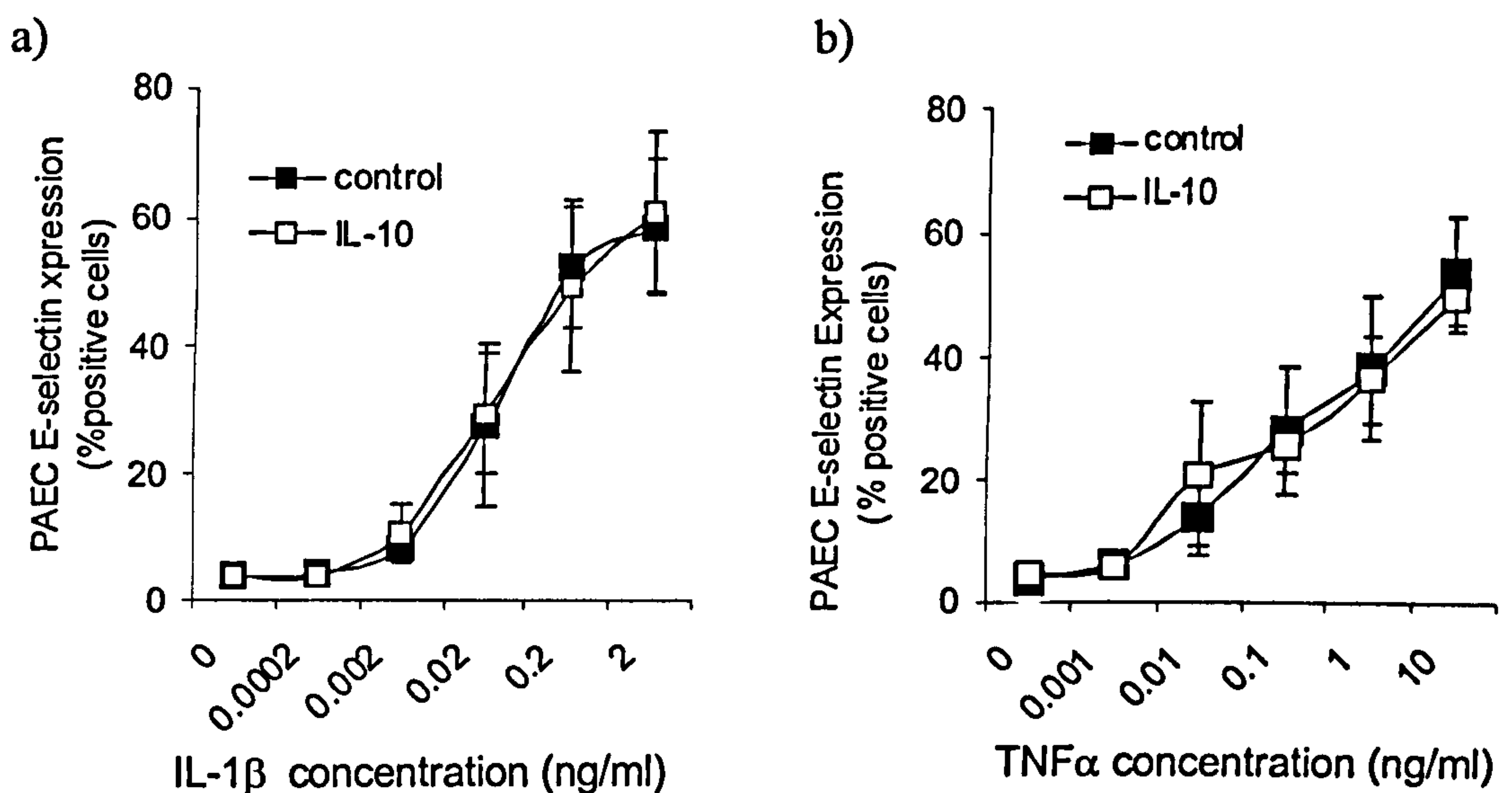


Figure 5.8. Effect of recombinant human IL-10 (50ng/ml) on a) IL-1 β and b) TNF α -induced E-selectin expression in human PAEC.

5.6.3 PATIENTS UNDERGOING CARDIAC SURGERY UTILISING CPB FOLLOWED BY *IN VITRO* LPS STIMULATION

5.6.3.1 Effect of patient plasma on I κ B α levels in PAEC

I κ B α levels in PAEC following treatment with native pre CPB plasma were variable (39.4 – 147.7 (median: 80.3) % of control levels, n=9) and not significantly different from those following treatment with post CPB native plasma (34.3 – 233.1 (median 67.8) % of control, n=9, p=0.860, Figure 5.9a and b).

In contrast, plasma (diluted 1/1000) from the LPS (1ng/ml)-stimulated whole blood collected before CPB stimulated PAEC and caused significant I κ B α depletion (median: 41.4 (25.0 – 80.3)% of control) when compared to plasma obtained from control incubated whole blood (median: 104.6 (44.6 – 186.1%, p<0.05, n=9, Figure 5.9c and d). Internal controls demonstrated that this effect of the plasma was not due to the presence of the stimulating LPS, since this concentration of LPS (1pg/ml after dilution) had no effect on I κ B α levels in cultured PAEC (104.1 $\pm$ 15.8% of control levels).

Treatment of PAEC with plasma (1/1000 dilution) from post CPB LPS-stimulated blood resulted in variable I κ B α depletion (50.5 (36.8 – 148.0) % of control) that was not different from that observed following treatment with plasma (1/1000 dilution) from control incubated blood (79.1 (10.9 – 204.1)% of control I κ B α levels, n=9, Figure 5.9c and d).

5.6.3.2 Effect of patient plasma on E-selectin expression on endothelial cells

In this study 4.8 $\pm$ 0.4 % of cells were E-selectin positive under basal conditions. Low levels of E-selectin induction were evident following incubation of PAEC with some pre and post CPB native plasma samples, however the grouped data did not significantly differ from basal levels (6.6 (5.0 – 11.8) and (4.3 (2.2 – 9.1) % cells respectively, p>0.05). Pre treatment of PAEC with MG132 significantly reduced the level of E-selectin expression following 5 hours treatment with pre- (3.1 (2.2 – 5.3) % positive cells, p=0.001)) and post (2.8 (2.1 – 6.7) % positive cells, p=0.04) CPB native plasma (Figure 5.10).

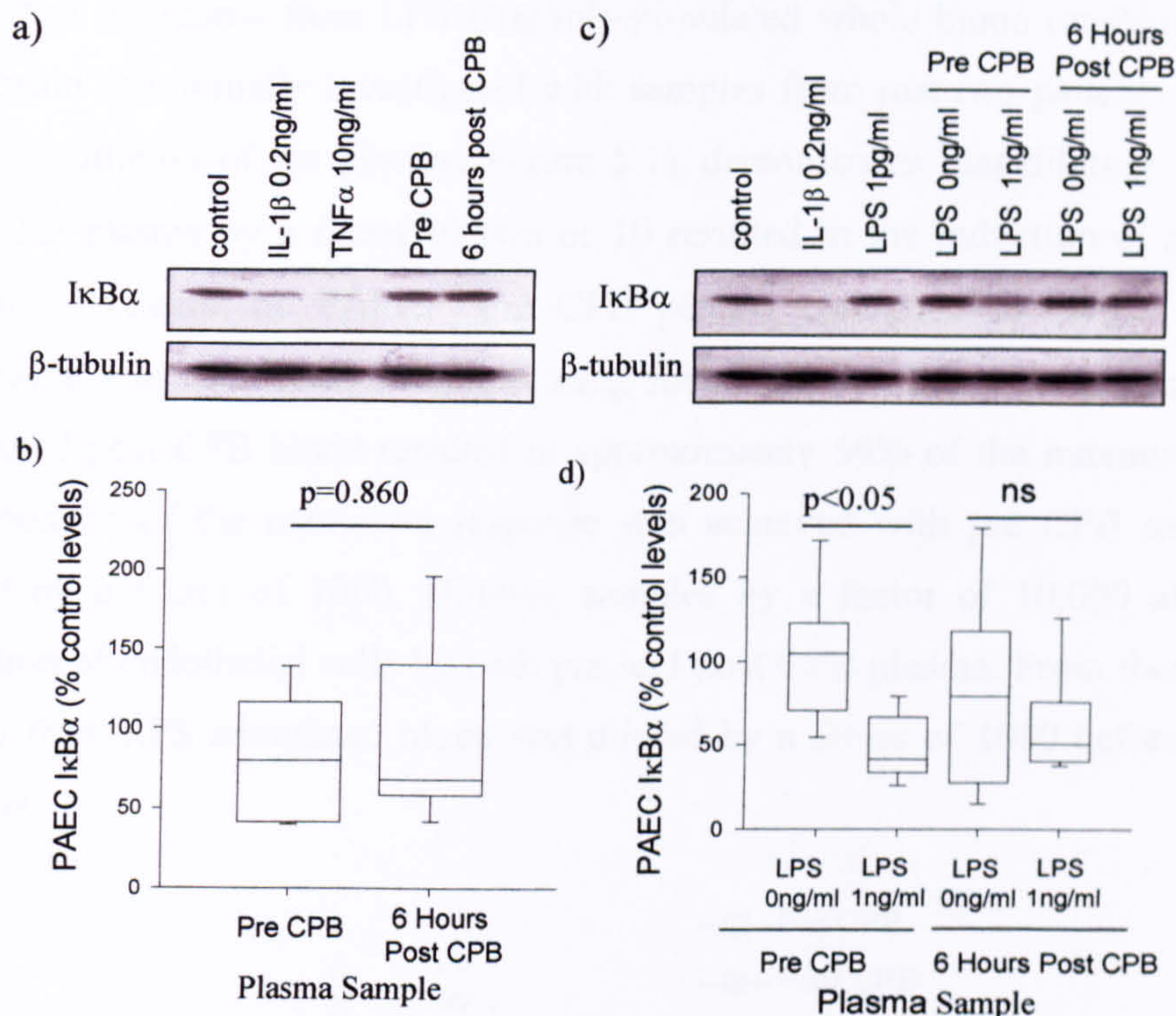


Figure 5.9. Bioactivity of plasma from patients undergoing cardiac surgery with CPB to induce I κ B α depletion. a) Representative Western blot and b) summary graph demonstrating the effect of pre and post CPB native plasma on PAEC I κ B α levels. c) Representative Western Blot and d) summary graph demonstrating the effect of plasma from pre and post CPB, LPS (0 and 1 ng/ml)-stimulated whole blood on PAEC I κ B α levels.

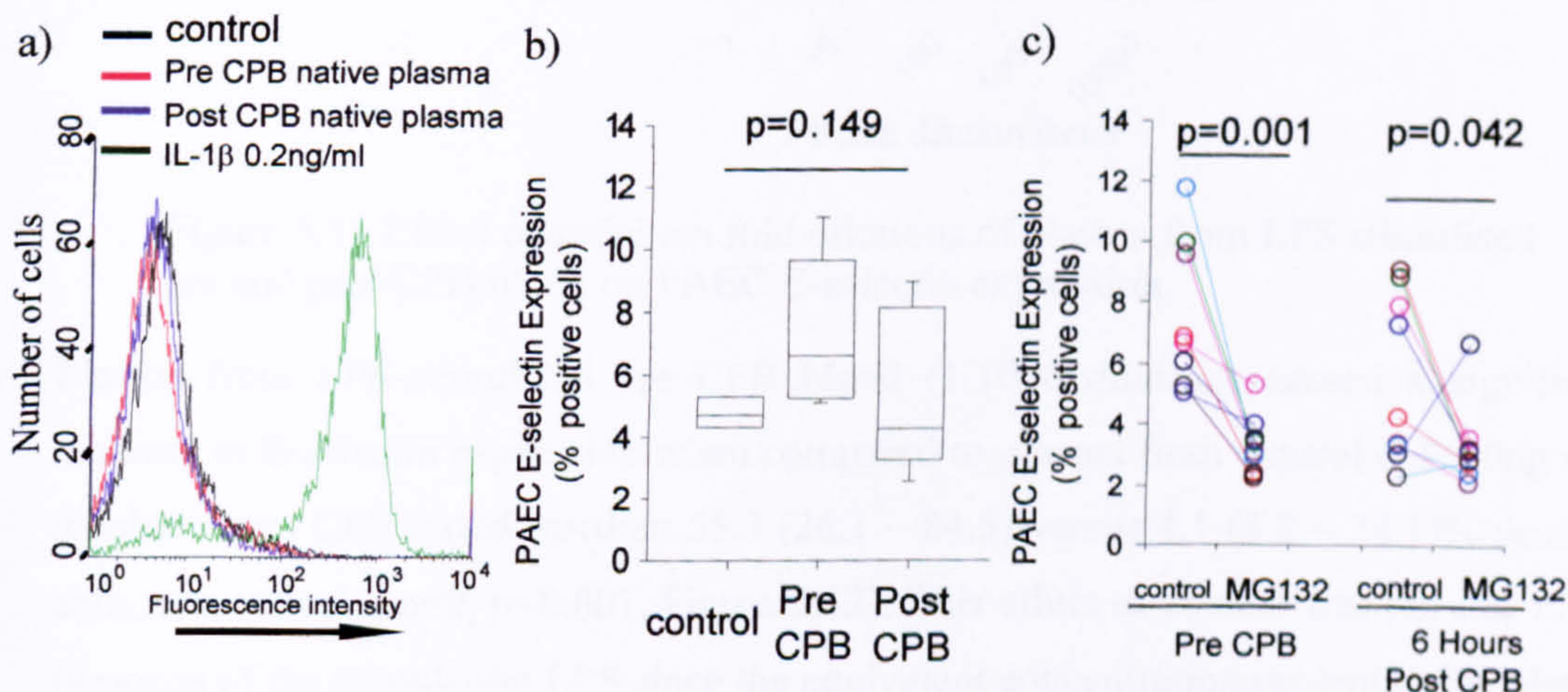


Figure 5.10. Bioactivity of native plasma from patients undergoing cardiac surgery with CPB to induce E-selectin on cultured PAEC. a) Representative E-selectin flow cytometry histogram showing levels of E-selectin expression following treatment of PAEC with native pre and post CPB plasma from one patient. b) Summary box plot of grouped data compared to control E-selectin levels. c) Individual data points for each patient demonstrating the effects of MG132 on PAEC E-selectin expression following treatment with the pre and post operative native plasma.

The effect of plasma from LPS (1ng/ml)-stimulated whole blood on PAEC E-selectin expression was initially investigated with samples from just two patients to determine optimum dilution of the plasma. Figure 5.11 demonstrates that diluting both pre and post CPB plasma by a factor of two or 10 resulted in the induction of maximum E-selectin expression on PAEC. Pre CPB plasma continued to induce a maximum response at 1 in 100 dilution whereas using such a dilution factor for plasma from LPS-stimulated post CPB blood resulted in approximately 50% of the maximum response. Fifty percent of the maximum response was achieved with pre CPB samples when diluted by a factor of 1000. Diluting samples by a factor of 10,000 abolished the activation of endothelial cells by both pre and post CPB plasma. From these results all plasma from LPS stimulated blood was diluted by a factor of 1000 before addition to the cells.

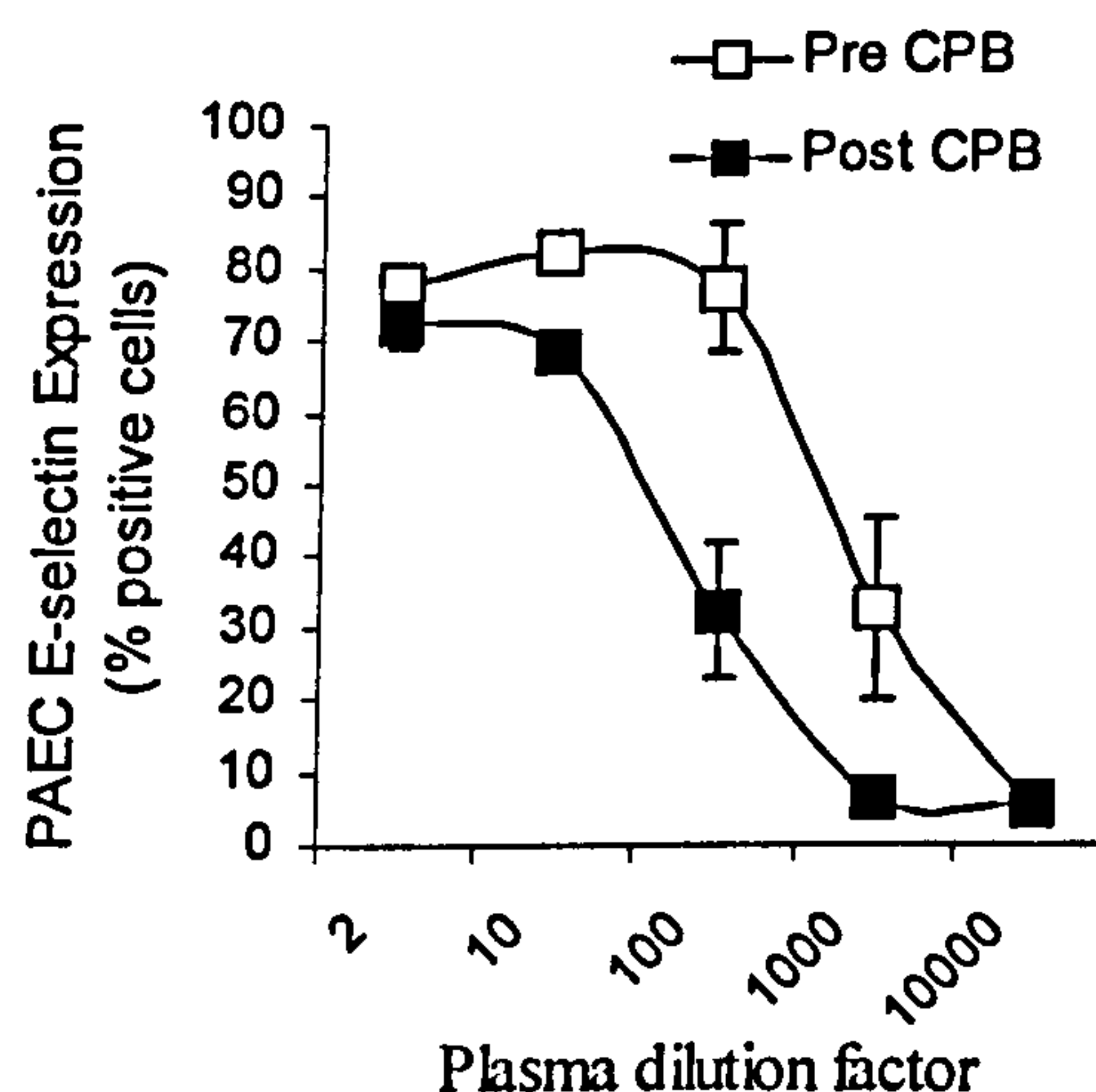


Figure 5.11 Effect of serial ten fold dilutions of plasma from LPS stimulated pre and post CPB blood on PAEC E-selectin expression.

Plasma from LPS-stimulated pre CPB blood (1/1000 dilution) caused a significant increase in E-selectin expression when compared to plasma from control (LPS 0ng/ml)-incubated pre CPB blood (median 55.3 (26.1 – 84.5) versus 4.1 (3.2 – 24.1)% positive cells, respectively, $n=9$, $p<0.001$, Figure 5.12). This effect of plasma was not due to the presence of the stimulating LPS since the equivalent concentration present in the plasma samples following dilution (1pg/ml) did not increase E-selectin expression above control levels (Figure 5.13). E-selectin expression induced by plasma from LPS-stimulated blood was significantly reduced after CPB (21.3 (4.1-60.7) % E-selectin positive cells, $p<0.001$, $n=9$, Figure 5.12) and was not significantly different from that

induced by plasma from control incubated post CPB blood (9.8 (3.0 – 29.6) % positive cells).

There was a significant correlation between the extent of PAEC I κ B α depletion and E-selectin expression following treatment with plasma from both pre ($r=0.73$, $p=0.02$) and post ($r=0.669$, $p=0.04$) CPB samples after *in vitro* LPS stimulation. Furthermore, the presence of MG132 during PAEC incubation with plasma from pre-CPB LPS-stimulated blood significantly reduced the levels of E-selectin expression 24.4 (4.9 – 55.1) % positive cells, $p=0.005$). E-selectin expression induced by plasma from LPS-stimulated post operative blood was also reduced by the presence of MG132 (median: 4.33 (2.7 – 38.7)% positive cells) however the low level induction by some samples under control conditions prevented this from reaching statistical significance ($p=0.092$).

The plasma concentration of both IL-1 β and TNF α alone correlated with the bioactivity of the plasma to induce I κ B α depletion ($p=0.0016$ and $p=0.005$) and E-selectin expression ($p<0.001$). In addition an increased ratio of IL-1RA/IL-1 β and sTNFR/TNF α appeared to confer a reduced plasma bioactivity to induce both I κ B α depletion ($p=0.01$ and $p=0.02$) and E-selectin expression ($p<0.001$).

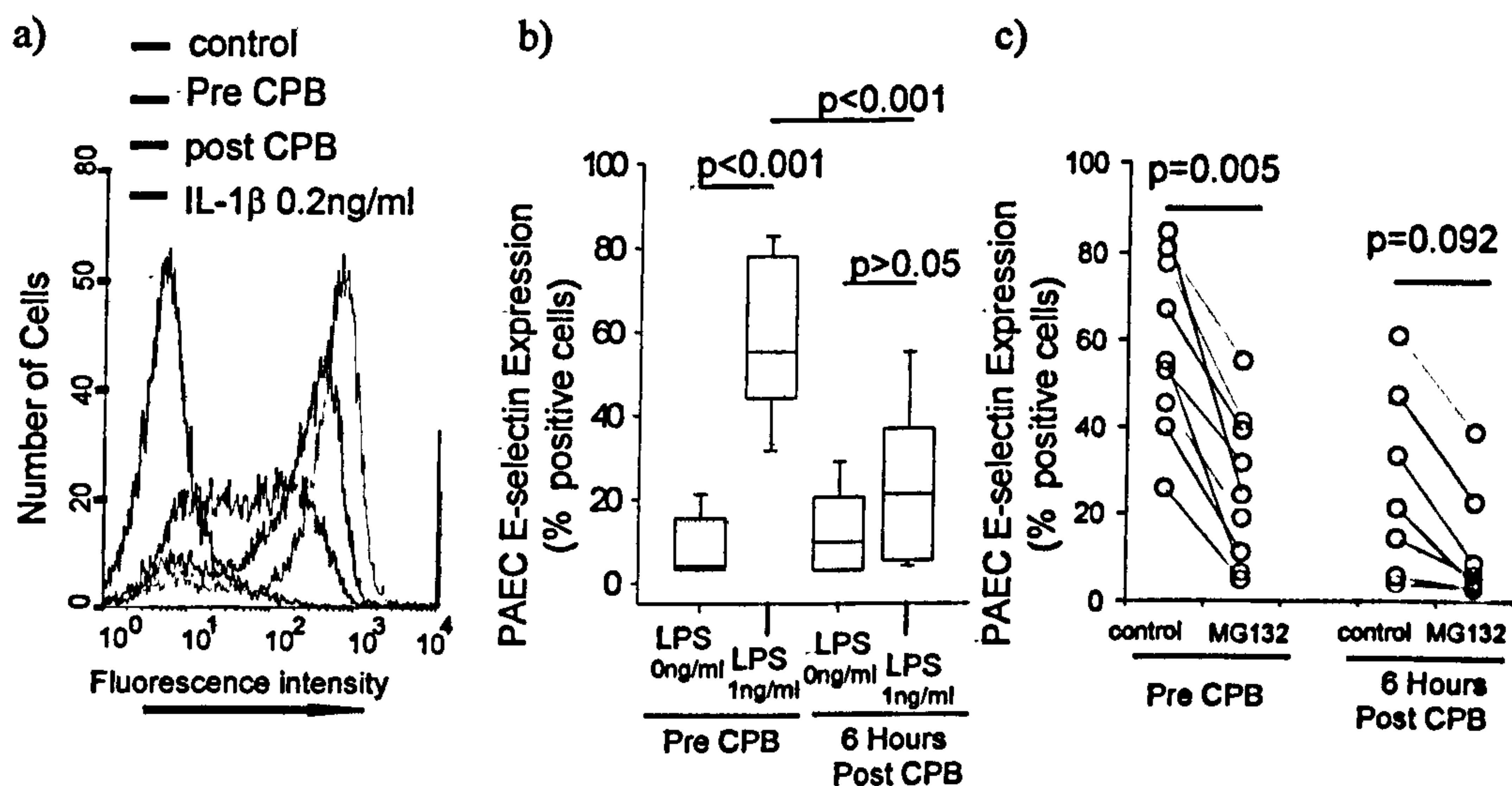


Figure 5.12. Bioactivity of pre and post CPB plasma to induce E-selectin expression following *in vitro* LPS (0 and 1 ng/ml) stimulation. a) Representative E-selectin flow cytometry histogram showing changes in PAEC E-selectin expression following treatment with plasma from LPS (1ng/ml)-stimulated whole blood from one patient, both before (pre CPB) and 6 hours after (post CPB) CPB. b) Summary box plot showing grouped data. c) Individual data points from each sample showing the effects of MG132 on the bioactivity of plasma from LPS-stimulated blood to induce E-selectin expression.

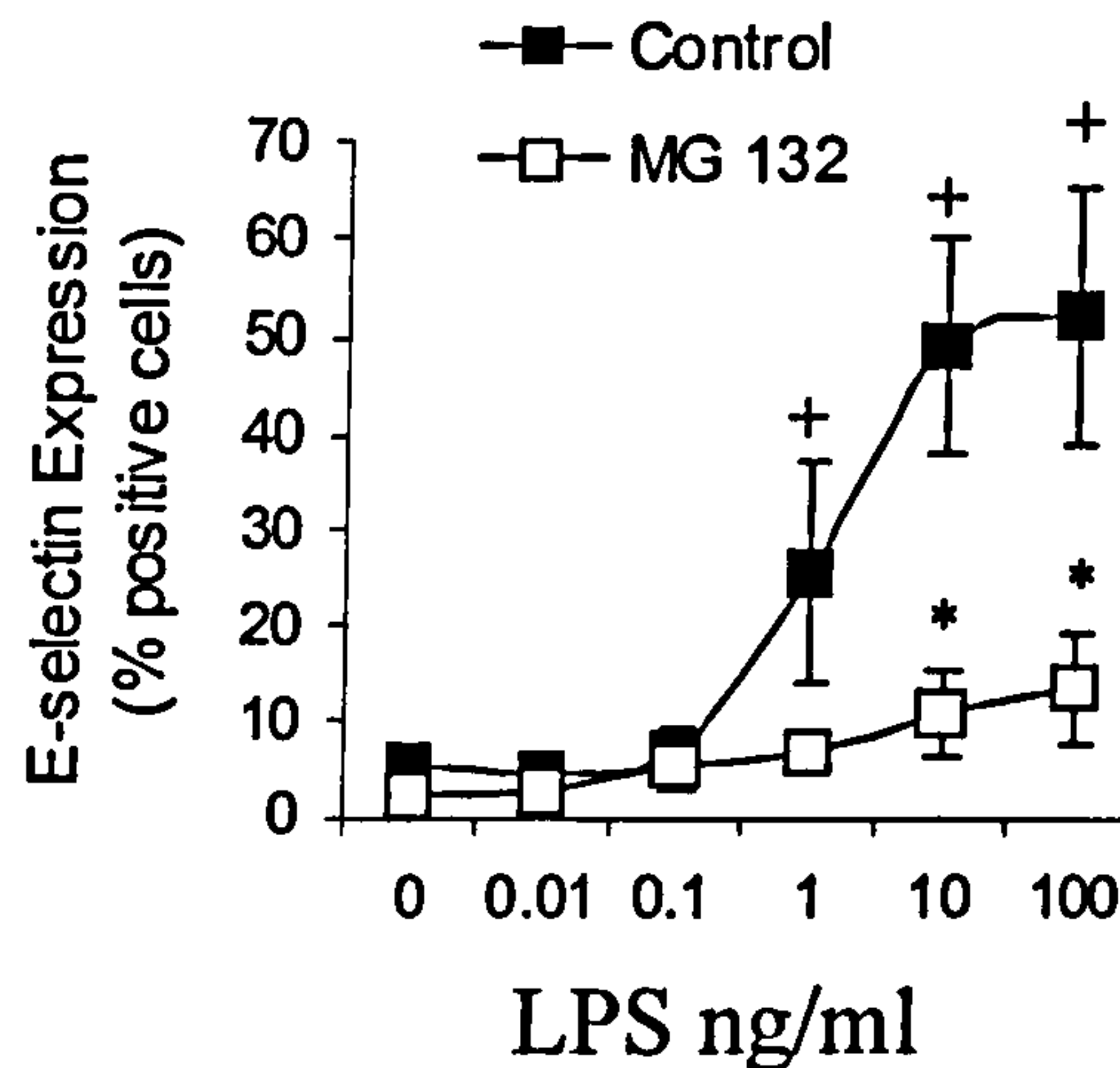


Figure 5.13. Dose dependant effect of LPS on PAEC E-selectin expression. * denotes $p < 0.05$ from untreated cells. * denotes $p < 0.05$ from corresponding control LPS response.

5.6.4 PATIENTS UNDERGOING CARDIAC SURGERY WITH AND WITHOUT CPB

Given the cytokines profile in the circulation of these patients (chapter 3, section 3.4.4) the bioactivity was investigated in only t1 and t5.

5.6.4.1 Effect of patient plasma on E-selectin expression

In this study $3.2 \pm 0.4\%$ of resting, unstimulated PAEC were E-selectin positive.

There was no significant increase in E-selectin expression above control levels following treatment with pre-operative native plasma from either the CPB (median: 3.9 (1.4 – 7.1) % positive cells, $n=13$, $p > 0.05$) or the OPCAB (median: 5.7 (1.3 – 8.5) % positive cells, $n=13$, $p > 0.05$) patients.

Native plasma taken from both groups of patients at 5 hours after the initiation of the procedure also failed to significantly alter E-selectin expression (CPB 3.5 (1.2 – 5.2) and OPCAB 4.9 (1.3 – 5.7)% of cells were E-selectin positive, $p > 0.05$) Figure 5.14.

The level of E-selectin expression induced by native plasma did not correlate with any single cytokine measurement or molar ratio in the plasma of either group of patients.

5.6.4.2 Effect of patient plasma on cytokine induced E-selectin expression

To further investigate anti-inflammatory activities in the patients' plasma following cardiac surgery with and without CPB, the effects of native plasma on pro-inflammatory cytokine-induced E-selectin expression was investigated.

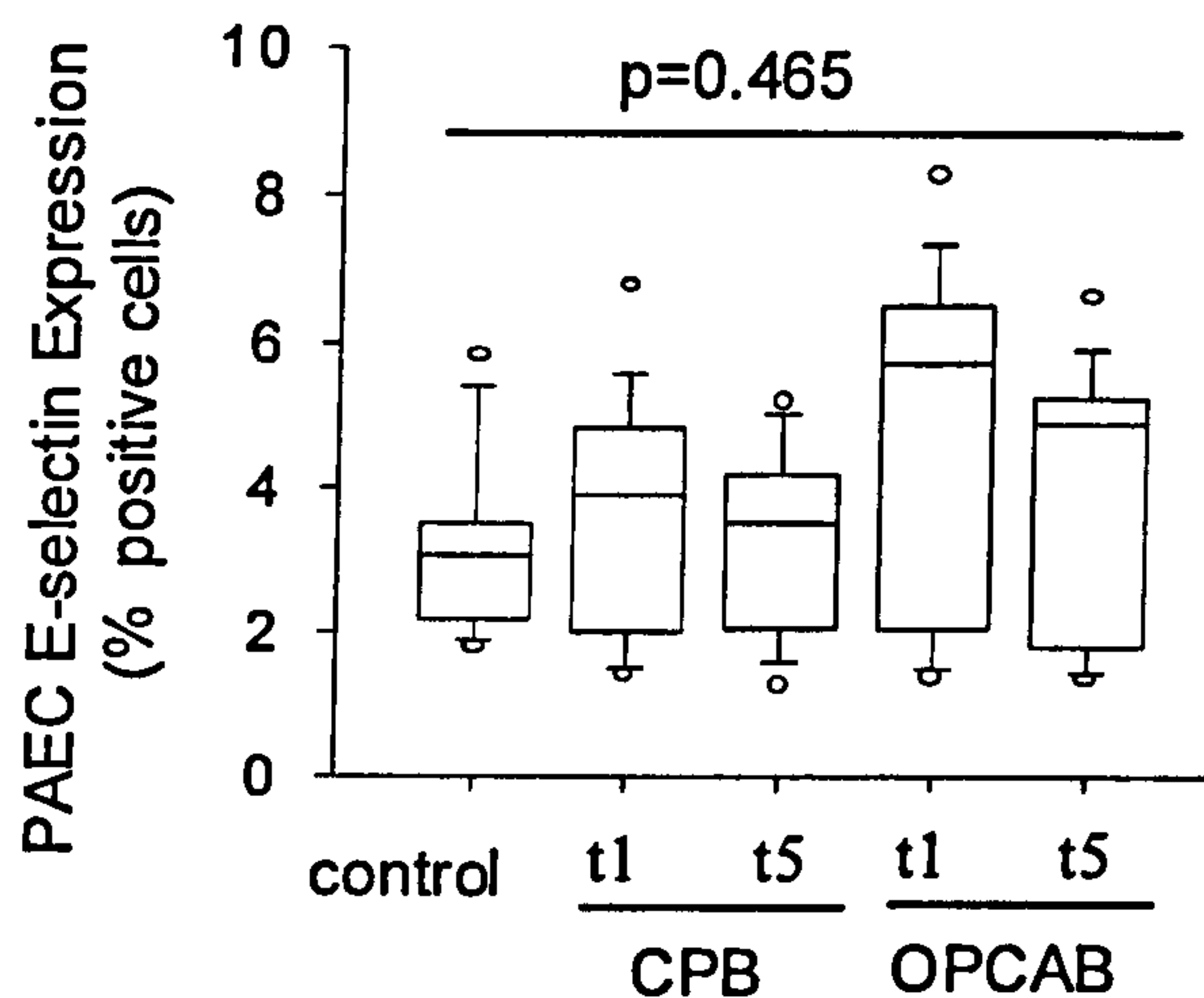


Figure 5.14. Effect of CPB on the bioactivity of plasma to induce E-selectin expression following cardiac surgery. PAEC E-selectin levels following 5 hours treatment with plasma from patients before (t1) and 5 hours after (t5) cardiac surgery with (CPB) and without (OPCAB) cardiopulmonary bypass compared to control E-selectin expression. Data expressed as the percent of E-selectin positive cells.

5.6.4.2.1 Controls

The percent of cells expressing E-selectin significantly increased following 5 hours treatment with IL-1 β (0.02ng/ml) and TNF α (0.1ng/ml) to 56.6 ± 10.6 % (n=6, p<0.05) and 51.2 ± 9.4 % (n=6, p<0.05), respectively. The presence of 50% AB serum had no effect on IL-1 β (54.6 ± 2.6 % positive cells, n=3, p=0.900) or TNF α (46.3 ± 2.3 % positive cells, n=3, p=0.741)-induced E-selectin expression.

5.6.4.2.2 Effect of plasma on IL-1 β -induced E-selectin expression

Preoperative plasma (t1) from both CPB and OPCAB patients caused PAEC E-selectin expression in response to IL-1 β (0.02ng/ml) to significantly increase to 135.8 (66.3 – 197.9) and 148.1 (98.7 – 258.4)% of the control IL-1 β response, respectively. In contrast, plasma from both the CPB and the OPCAB groups of patients obtained 5 hours after the initiation of the procedure (t5) significantly inhibited the response of the endothelial cells to IL-1 β . Post CPB plasma caused the E-selectin response to IL-1 β (0.02ng/ml) to be 47.9 (18.4 – 161.9) % of the control IL-1 β response (n=13, p<0.05) and t5 plasma from OPCAB patients caused the E-selectin response to IL-1 β to be just 44.0 (12.8 – 90.5)% of the control IL-1 β response. (n=13, p<0.05 Figure 5.15). There was no difference between the effects of CPB and OPCAB plasma in this respect.

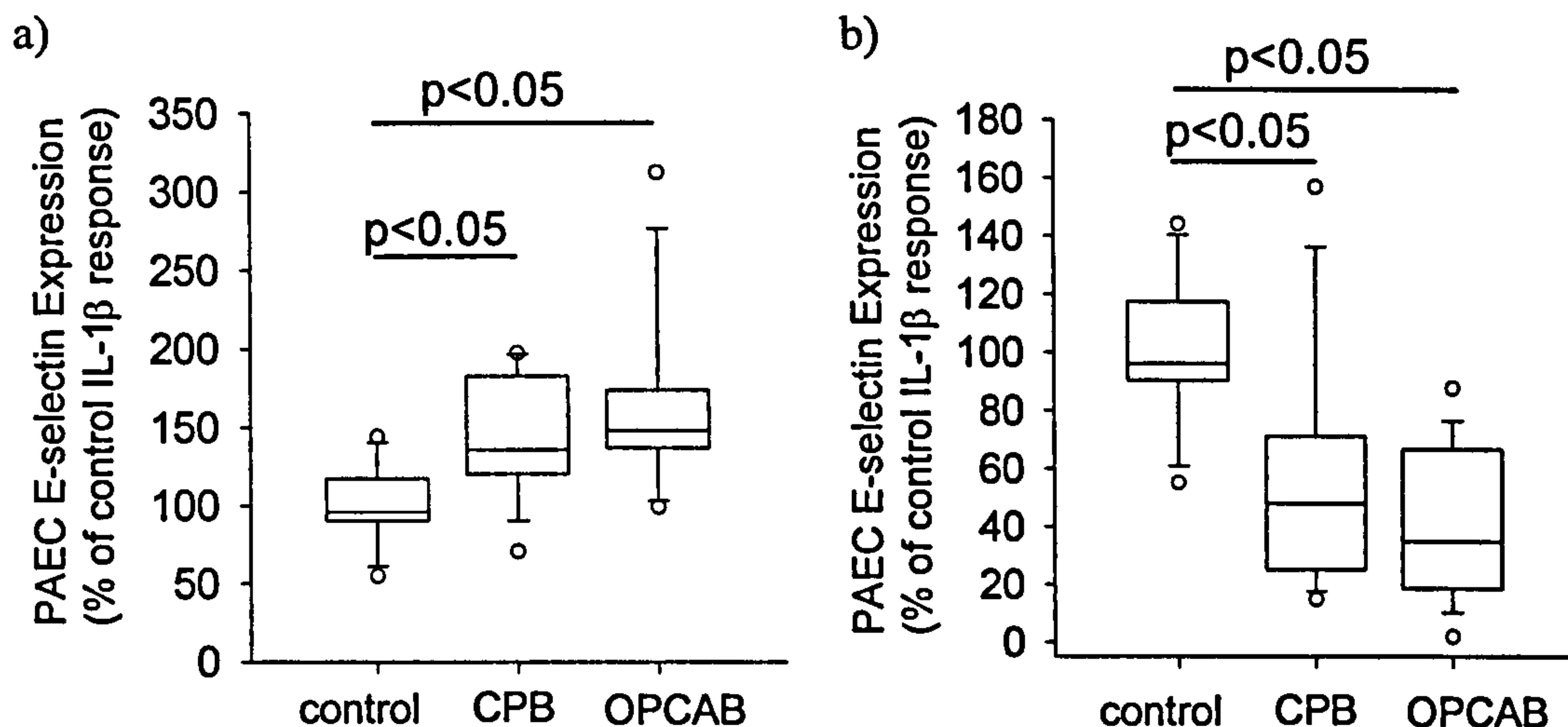


Figure 5.15. Effect of pre (a) and post (b) operative plasma from CPB and OPCAB patients on E-selectin expression induced by IL-1 β (0.02 ng/ml). Data expressed as a percent of the control response to IL-1 β 0.02ng/ml.

In the pre-operative samples there was no correlation between the effects of plasma on IL-1 β -induced E-selectin expression and any single cytokine concentration or ratio measured.

In the post-operative samples from OPCAB patients there was a significant relationship between the ability of plasma to inhibit IL-1 β -induced E-selectin expression and increased plasma concentrations of IL-1RA ($r=0.699$, $p=0.01$) and the ratio of IL-1RA/IL-1 β ($r=0.713$, $p=0.008$). In post CPB plasma the ability to inhibit IL-1 β -induced E-selectin appeared to be related to the concentration of IL-1RA however this did not reach statistical significance ($r=0.481$, $p=0.089$, Figure 5.16).

5.6.4.2.3 Effect of plasma on TNF α -induced E-selectin expression

The endothelial cell response to TNF α 0.1ng/ml in terms of E-selectin expression was not altered by pre-operative plasma from either CPB or OPCAB group of patients (98.4 (69.3 – 156.7)% and 108.8 (88.7 – 171.6) % of the control TNF α response, respectively). Similar to the effect on IL-1 β , plasma from patients 5 hours after the initiation of CPB significantly inhibited TNF α -induced E-selectin expression (74.7 (35.7 – 104.3)% of control TNF α response, $p<0.05$). However, plasma taken 5 hours after application of Octopus 3® did not significantly alter the response to TNF α (90.3 (63.1 – 123.3) %, $p>0.05$, Figure 5.17).

Inhibition of TNF α -induced E-selectin expression by post CPB plasma was associated with increased sTNFR1 concentration ($r=0.740$, $p=0.003$, Figure 5.18).

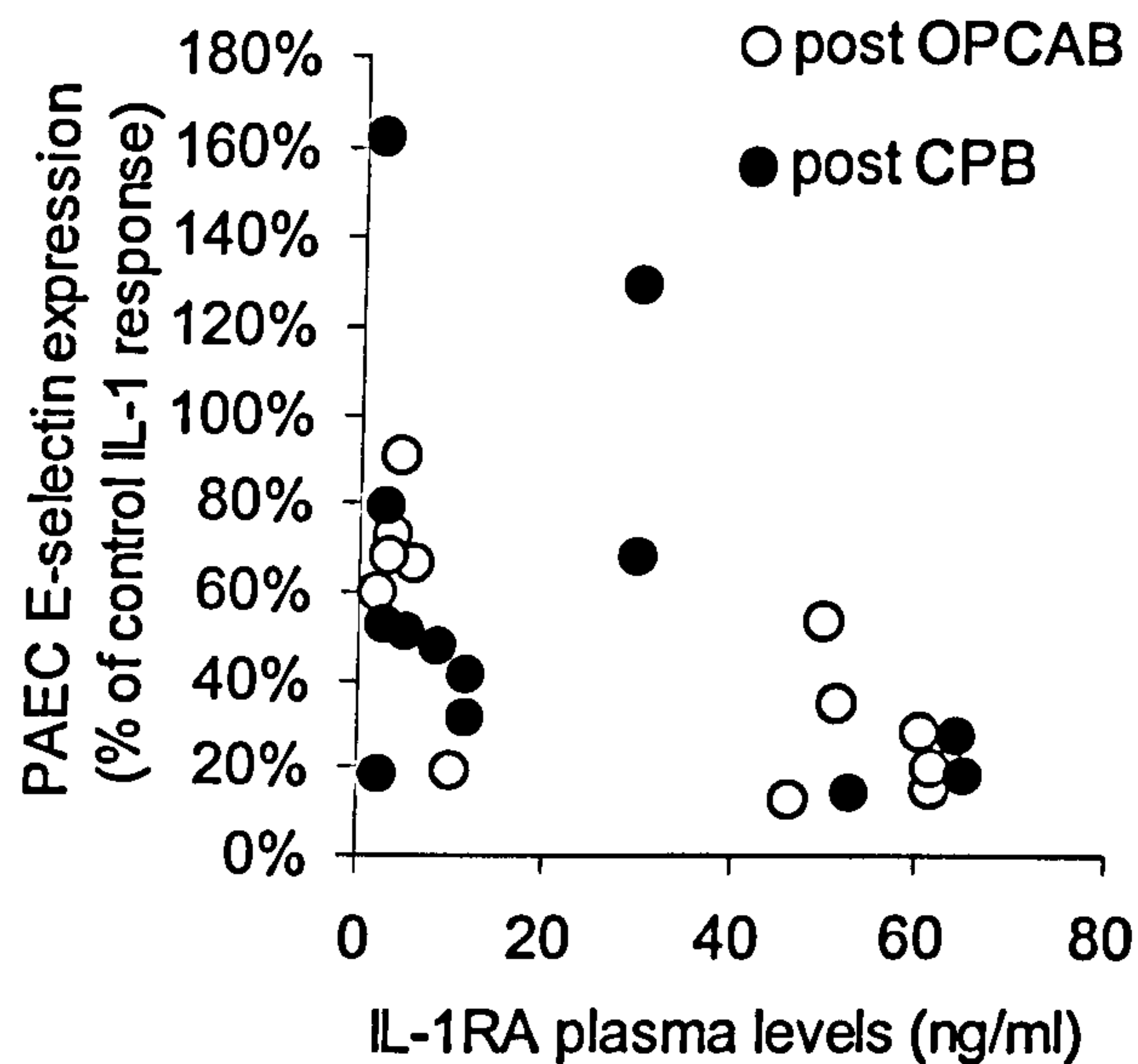


Figure 5.16 Dot plot demonstrating the relationship between plasma levels of IL-1RA and the ability of post operative plasma (CPB (●) and OPCAB (○) to reduce the effects of IL-1 β (0.02ng/ml) on E-selectin expression.

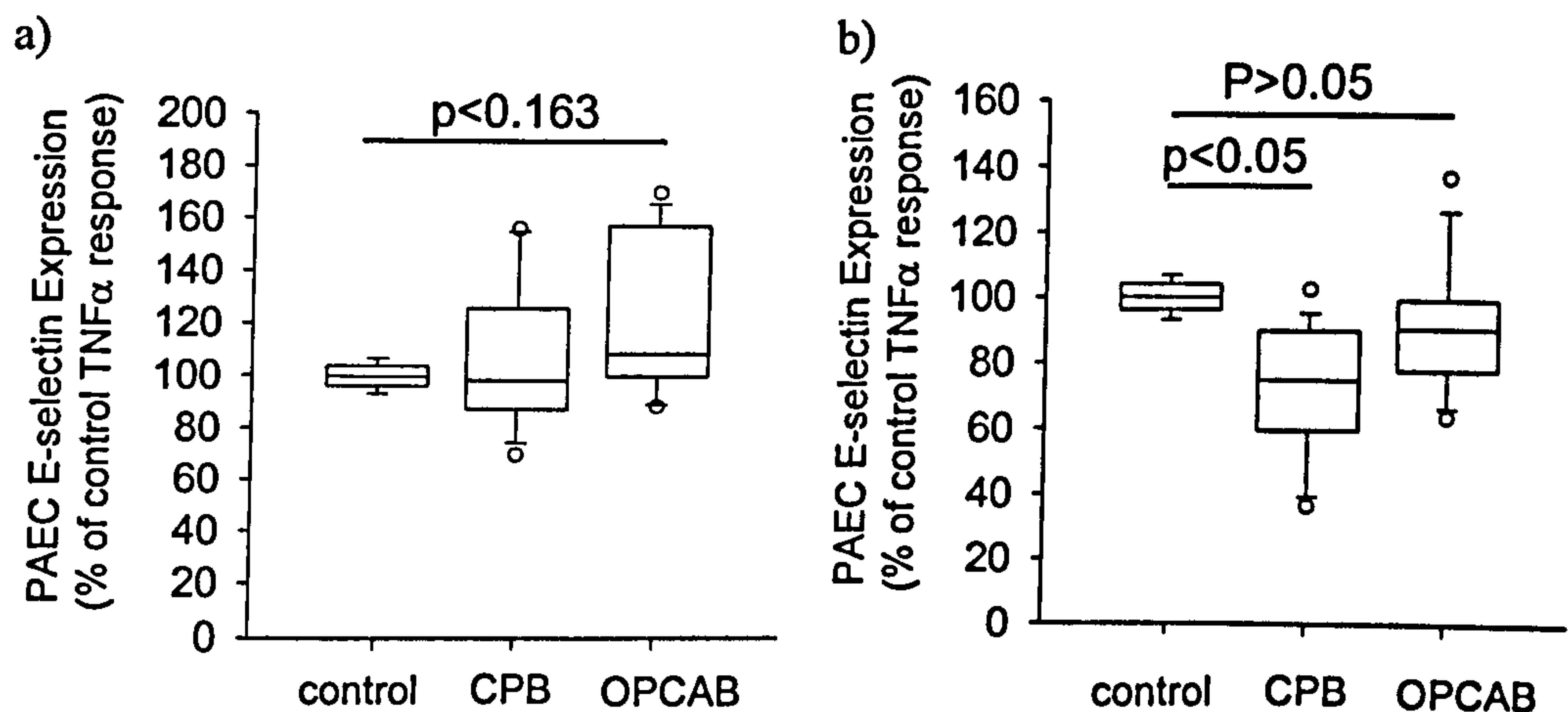


Figure 5.17. Effect of pre (a) and post (b) operative plasma from CPB and OPCAB patients on E-selectin expression induced by TNF α (0.1 ng/ml). Data expressed as a percent of the control response to TNF α 0.1ng/ml.

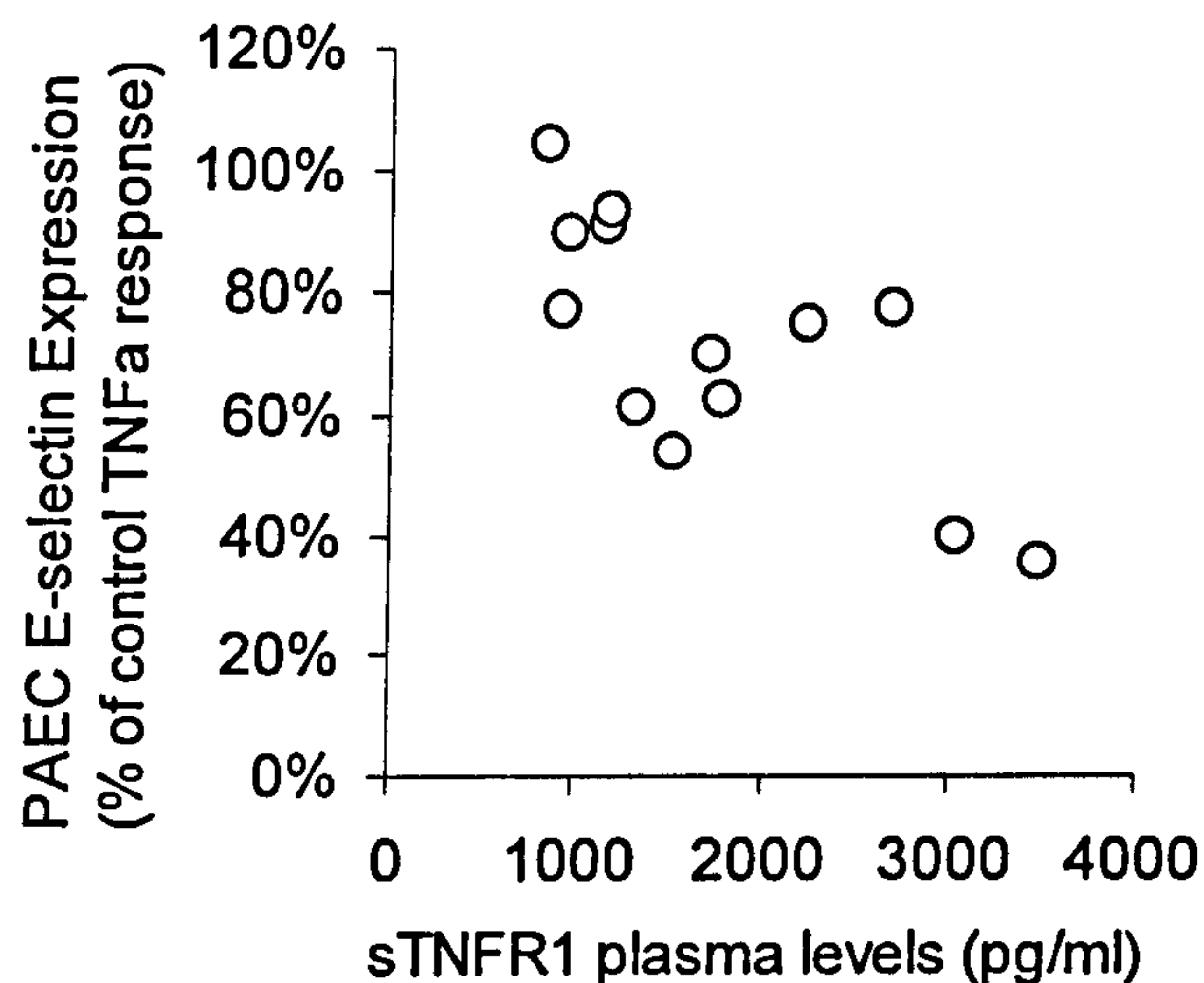


Figure 5.18. Dot plot demonstrating the relationship between plasma levels of sTNFR1 and the ability of post CPB plasma to reduce the effects TNF α (0.1ng/ml) on E-selectin expression.

5.7 DISCUSSION

5.7.1 HEART FAILURE PATIENTS

The data presented in this chapter demonstrates that soluble factors in the plasma of both Tx and LVAD patients, in contrast to control AB serum have the capacity to initiate inflammatory signal transduction pathways (NF κ B) in human pulmonary artery endothelial cells as demonstrated by the significant reduction of cellular levels of I κ B α . Despite this apparent activation of the NF κ B signal transduction pathway in endothelial cells in response to treatment with plasma from both groups of patients there was only a small increase in E-selectin expression, which only reached statistical significance in the cells treated with plasma from the Tx patients.

5.7.1.1 Plasma bioactivity to induce I κ B α depletion

The identity of the soluble factor or factors present in the patients' plasma that is responsible for the bioactivity that induced I κ B α depletion is unclear since there was no apparent correlation between any of the factors quantified and the induction of I κ B α depletion. Pro-inflammatory cytokines, IL-1 β and TNF α are both potent inducers of I κ B α depletion in these cells as demonstrated in chapter 4. TNF α was significantly elevated in the LVAD plasma compared to the other patients studied. The levels of TNF α measured in the LVAD patients' plasma had a median value of 8.5 pg/ml with

levels increasing to over 30pg/ml in some patients. Recombinant TNF α added to these cells at 10pg/ml can induce an almost 50% depletion in I κ B α in this bioassay system suggesting that the plasma concentration of TNF α , at least in some patients, may contribute to this observed inflammatory bioactivity. Furthermore, *in vitro* studies have suggested the existence of synergistic interactions between TNF α and other factors that are capable of enhancing the level of NF κ B activation induced by TNF α alone. For example, in EA.hy926 cells TNF α -induced NF κ B activation was enhanced by the presence of IFN γ . The mechanism responsible for this action appears to be, at least in part via enhanced degradation of I κ B α since a combination of TNF α and IFN γ at concentrations that alone did not activate NF κ B resulted in a significant I κ B α depletion and nuclear accumulation of NF κ B (Cheshire and Baldwin, Jr., 1997). This data indicates that, even at low concentrations TNF α may be an important contributor to the plasma bioactivity observed in this present study. TNF α neutralising experiments were performed to further investigate the importance of TNF α in this system. However this approach failed to alter the bioactivity of either the Tx or LVAD patients' plasma indicating that TNF α is unlikely to play any significant role in the inflammatory activity of the plasma to induce I κ B α depletion. These results may be explained by the relatively high levels of sTNFR in the plasma of these patients.

This negative finding regarding the influence of plasma TNF α in heart failure patients on NF κ B activation is supported by a recent study by Frantz et al (Frantz et al., 2003). In that study increased NF κ B activation in the myocytes, endothelial cells and infiltrating leucocytes in the failing myocardium also did not correlate with TNF α levels.

IL-1 β plasma levels were undetectable in the majority of these patients by the assay used in this study. However, low levels of IL-1 β may still be present and able to contribute to the bioactivity observed. The IL-1R signal transduction system is incredibly efficient requiring as few as 10 ligand-occupied receptors to induce a strong response (Dinarello, 1996; Sims and Dower, 1994). Furthermore, the results in chapter 4 demonstrate that approximately 50% I κ B α depletion can be achieved in these PAEC by IL-1 β concentrations as low as 2pg/ml over a 30 minute time course. However there were significant levels of IL-1RA present in the plasma samples that would be expected to efficiently compete with IL-1 β for binding to the cellular receptors. It is estimated

that 100-fold excess of IL-1RA over IL-1 β is required to effectively block the biological activity of IL-1 β on target cells (Arend et al., 1990). With the demonstration of 150-fold molar excess of IL-1RA to IL-1 β it seems unlikely that IL-1 β would be contributing to the observed bioactivity of the plasma. Nevertheless, specific studies to rule out the contribution of IL-1 β would need to be carried out in order to confirm this.

Heart failure is associated with the dysregulation of a number of systems that could induce the degradation of I κ B α in this system such as Angiotensin II and endothelin 1. However, this study was designed to focus on factors related to the pro-inflammatory cytokines IL-1 β and TNF α and the balance between these factors and their endogenous inhibitors. Therefore the potential involvement of other factors in contributing to the observed bioactivity of the plasma has not been investigated.

5.7.1.2 Plasma bioactivity to induce E-selectin expression

Given the level of I κ B α depletion following treatment of PAEC with plasma from Tx and LVAD patients and the essential requirement for NF κ B activation in conferring the efficient induction of the E-selectin gene, it was surprising that the expression of this adhesion molecule, in response to treatment with patients' plasma, was not increased to a greater extent. Plasma from the LVAD patients failed to induce a significant increase in E-selectin expression whilst that from Tx patients induced a very small, but significant increase above control levels. These data might suggest that either the observed NF κ B activation alone is not sufficient to confer E-selectin gene induction or the presence of inhibitory factors interfering with E-selectin expression

Due to the multi-step nature of the NF κ B activation pathway and the requirement for the co-operative action of other inducible factors for the successful transcription of genes often referred to as 'NF κ B inducible' or 'NF κ B dependant' there are numerous potential places within the sequence of events from I κ B α depletion to E-selectin protein expression where inhibition may occur. In this study increased plasma levels of IL-10 correlated with reduced E-selectin expression induced by treatment with plasma suggesting that IL-10 may be responsible for the dissociation between I κ B α depletion and E-selectin expression. IL-10 has been shown to inhibit NF κ B activation in a number of systems both *in vivo* and *in vitro*. In human monocytes, intestinal epithelial cells and bronchial epithelial cells inhibition of IKK activity and inhibition of NF κ B DNA binding activity have been suggested to be the mechanisms responsible for this action

(Schottelius et al., 1999; Tabary et al., 2003). However, data regarding the effects of IL-10 on inflammatory mechanisms in endothelial cells are limited and somewhat conflicting. Lisinski et al demonstrated that IL-10 could inhibit IL-8 and MCP-1 expression in HUVEC stimulated with LPS and *Borrelia burgdorferi* but not TNF α or IL-1 β . In addition IL-10 had no effect on E-selectin or ICAM-1 expression induced by any of these stimuli (Lisinski and Furie, 2002). Other studies however have found that IL-10 can down-regulate the expression of ICAM-1 and VCAM-1 on IL-1 β activated HUVECs (Krakauer, 1995). In contrast, Vora et al demonstrated that in human microvascular endothelial cells and HUVEC IL-10 could induce E-selectin expression, the levels of which were comparable to those observed in response to IL-1 β and TNF α (Vora et al., 1996). Most studies however fail to demonstrate any direct effect of IL-10 on human endothelial cell (HUVEC) adhesion molecule, chemokines and cytokine expression (Noble et al., 2000; Parks et al., 1998; Sironi et al., 1993).

In this present study the possibility that the presence of IL-10 in the plasma was influencing this bioassay system was further investigated. IL-10 had no effect on E-selectin expression induced by either IL-1 β or TNF α suggesting that IL-10 is not able to inhibit cytokine-induced E-selectin expression. However this does not discount the possibility that IL-10 could inhibit E-selectin expression induced by plasma factors other than these cytokines. Indeed from anti-TNF α neutralising experiments it appears that there is no significant TNF α activity present in the plasma of these patients to be able to stimulate E-selectin expression. The role of IL-10 in the bioassay was further investigated with the use of IL-10 neutralising antibodies. The E-selectin response of the endothelial cells to plasma was not altered by the presence of IL-10 neutralising antibodies providing further support for the conclusion that IL-10 activity is not influencing this bioassay.

Other soluble factors potentially present in plasma have been shown to interfere with E-selectin expression. IL-4 has been shown to inhibit TNF α -induced E-selectin in HUVEC through the binding of the transcription factor STAT6 to the STAT6 site of the E-selectin promoter. In doing so the NF κ B binding site becomes blocked preventing NF κ B-DNA interaction and preventing transactivation of the gene (Bennett et al., 1997). IL-4 has also been shown to inhibit TNF α -induced gene transcription through competition between STAT6 and NF κ B for the co-activator CBP (Ohmori and Hamilton, 2000). However, the involvement of IL-4 in the dissociation between the

bioactivity to induce I κ B α depletion and that to induce E-selectin expression is unlikely for two reasons. Firstly in the study by Bennett et al the endothelial cells required at least a 20-minute pre-incubation with IL-4 before the addition of TNF α for effective inhibition of E-selectin expression. Addition of IL-4 and TNF α to the cells simultaneously produced the same levels of E-selectin expression induced by TNF α alone. In the E-selectin bioassay in this current study, all the components present in the plasma are introduced to the cultured cells simultaneously and thus any inhibitory effect of IL-4 is unlikely. Secondly, the concentration of IL-4 used by Bennett et al as well as by others (Abu-Amer, 2001; Ohmori and Hamilton, 2000; Wei et al., 2002) to inhibit E-selectin expression or NF κ B activation was in the range of 1-10ng/ml, which exhibited about a 50% inhibition. The levels of IL-4 in the plasma of the heart failure patients (Tx and LVAD) were less than 30 pg/ml (below the level of detection in the assay). This data further suggests that IL-4 is not the factor responsible for the observed dissociation between the bioactivity in the plasma to induce I κ B α depletion and E-selectin expression.

In addition to potentially altering circulating cytokine profile, the inotropic agents, in particular the PDE(III)I, used to treat the LVAD patients could also directly influence the plasma bioactivity to induce NF κ B activation and E-selectin expression.

These agents increase intracellular levels of cAMP by inhibiting its breakdown. Early studies demonstrated that increasing intracellular cAMP with pharmacological agents could inhibit TNF α and IL-1 β -induced expression of a number NF κ B responsive genes in HUVEC including E-selectin (Fortenberry et al., 1997; Ghera et al., 1994; Ollivier et al., 1996; Pober et al., 1993). Importantly, increased intracellular cAMP had no effect on NF κ B nuclear translocation but inhibited its transcriptional activity through CREB successfully out competing NF κ B for binding to limited amounts of CBP (Parry and Mackman, 1997).

Taken together this evidence provides a possible explanation for the dissociation between the bioactivity of plasma to induce I κ B α depletion and that to induce E-selectin expression in the LVAD patients, all of whom were receiving such inotropic therapy. However, as noted when discussing the influence of these agents on the circulating cytokine profile of these patients, further analysis in this regard is severely hindered by the use multiple combinations of such drugs on a relatively small number of patients.

5.7.1.3 Conclusion

Because of the increased expression of the pro-inflammatory cytokines observed in patients with end stage heart failure, dysregulated inflammatory mechanisms are now generally regarded as being contributors to disease progression and adverse effects. However, the data presented in this chapter suggests that there is a dissociation between circulating TNF α levels and net inflammatory responses in endothelial cells induced by the native plasma of these patients. To the extent that this *in vitro* data may be extrapolated to the *in vivo* situation increased TNF α and NF κ B activation in the setting of heart failure may not be adversely contributing to the pathology of heart failure by pro-inflammatory mechanisms. It has been suggested that the increased NF κ B activity observed in the myocytes and endothelial cells of the failing myocardium is an adaptive response that plays a crucial role in regulating genes involved in the protective response against cell stress and apoptosis (Frantz et al., 2003; Grabellus et al., 2002). Under such circumstances the trigger for NF κ B activation may be local tissue hypoxia and /or released proteins from injured or stressed cells. These findings, at least in part, may also explain the failure of the recent anti-TNF α trials in heart failure.

5.7.2 CARDIAC SURGERY

5.7.2.1 Inflammatory bioactivity of native plasma

Native plasma from patients undergoing cardiac surgery with CPB induced variable levels of I κ B α depletion in the target PAEC and no difference was observed between pre and postoperative samples in respect of this bioactivity.

E-selectin expression on the target PAEC was induced to variable levels following treatment with either pre or post-operative plasma samples and, although not statistically differing from the expression in control cells, levels of this adhesion molecule following incubation with native plasma were reduced by inhibiting NF κ B activation by pre-treatment of the PAEC with MG132. These data suggest that there are variable levels of soluble factors in the circulation of these patients that are able to induce a small degree of endothelial cell activation (NF κ B activation and low level NF κ B dependant gene induction). In addition, this inflammatory bioactivity of the plasma appeared to be unaffected by cardiac surgery utilising CPB. Although a small number of these samples demonstrated increased TNF α and/or IL-1 β levels this did not relate to the ability to induce I κ B α depletion or E-selectin expression. Similarly,

increased sTNFR and IL-1RA were observed in the plasma of these patients in response to cardiac surgery with CPB yet there was no alteration in plasma bioactivity in this system. Further statistical analysis revealed that there was no correlation between the level of I κ B α depletion or E-selectin induction and any single cytokine or cytokine ratio analysed suggesting that maybe a combination of factors or factors not quantified are responsible for the observed result. Indeed the significant molar excess of sTNFR to TNF α and IL-1RA to IL-1 β in all pre-operative samples does suggest that the activity of these two pro-inflammatory cytokines is efficiently neutralised. Moreover, plasma levels of sTNFR and IL-1RA and their molar excess over TNF α and IL-1 β respectively, increased further in the post operative period in the absence of altered bioactivity to induce NF κ B activation, lending support to the role of unidentified factors in modulating the activity of NF κ B and E-selectin expression.

5.7.2.2 Inflammatory bioactivity of plasma from LPS stimulated whole blood

To compare the effects of CPB to a controlled inflammatory insult, whole blood was stimulated *in vitro* with endotoxin and the resulting bioactivity assessed. In contrast to the effects of cardiac surgery with CPB, such stimulation of whole blood with LPS resulted in a significant increase in bioactivity to induce efficient NF κ B activity (both I κ B α depletion and E-selectin expression) in the target cells.

Plasma from LPS (1ng/ml)-stimulated whole blood obtained before CPB significantly activated the NF κ B pathway in PAEC causing I κ B α depletion even when diluted 1000-fold. Post CPB samples stimulated with LPS induced variable responses on PAEC I κ B α levels that were not significantly different from control samples suggesting that LPS responses of whole blood are attenuated by cardiac surgery with CPB. This is further highlighted by the reduced bioactivity to induce E-selectin expression in the post CPB plasma following LPS stimulation compared to the pre-CPB samples.

The significant correlation between the bioactivity to induce I κ B α depletion and E-selectin expression and the plasma levels of IL-1 β and TNF α suggest that these are the factors responsible. In addition the reduced bioactivity in samples in which there was an increased ratio of sTNFR/TNF α and IL-1RA/IL-1 β further substantiate this conclusion.

This data serves as an important control demonstrating that the blood cells from all of these patients are capable of producing biologically active pro- and anti-inflammatory

mediators in response to an inflammatory stimuli and as such cardiac surgery with CPB does not appear to be a significant pro-inflammatory stimuli in most of these patients.

5.7.3 CARDIOPULMONARY BYPASS VERSUS OPCAB

The use of CPB during cardiac operation is often thought to be responsible for initiating the inflammatory response often seen in such patients. As observed already, cardiac surgery with CPB did not alter the bioactivity of plasma with respect to ability to induce E-selectin expression. Interestingly, in the few patients in which increased levels of TNF α and/or IL-1 β were observed there appeared to be an effective counteracting response capable of preventing E-selectin expression. In addition avoidance of CPB in the OPCAB patients did not alter the bioactivity of the native plasma in this respect.

The predominant response to both CPB and OPCAB surgery appeared to be an anti-inflammatory one (chapter 3) with increased circulating levels of anti-inflammatory cytokine (IL-10 and IL-6), sTNFR and IL-1RA with no consistent overall change in the expression of pro-inflammatory cytokines observed. There was no statistical difference in the extent of this response between the two surgical procedures. The nature of this anti-inflammatory activity was further evaluated by determining the effect of pre- and post-operative plasma from these two groups of patient on E-selectin expression induced by TNF α and IL-1 β .

The IL-1 β -induced increase in E-selectin expression was significantly increased by pre operative plasma from both groups of patients. Pre-operative native plasma has already been shown to induce variable levels of I κ B α depletion and E-selectin expression in this bioassay system suggesting that this effect on E-selectin expression may simply be an additive one. However pre-operative native plasma had no effect on TNF α -induced E-selectin expression implying the effect is specific for IL-1 β and the presence of soluble factors capable of a synergistic interaction with the IL-1 β activity. This observation also reveals that the signals generated by IL-1 β and TNF α to induce E-selectin expression are, at least in part, different despite both potentially activating NF κ B and E-selectin.

Plasma samples obtained post operatively from both groups of patients significantly inhibited IL-1 β -induced E-selectin expression. Statistical analysis of this data revealed that plasma levels of IL-1RA and the ratio of IL-1RA/IL-1 β strongly correlated with the IL-1 β inhibitory activity in the plasma suggesting that this may be the predominant anti-IL-1 β activity in the samples. Indeed under the conditions of this bioassay there was

100 - 3,000 times molar excess of IL-1RA to recombinant IL-1 β . In addition, the similarity in the plasma levels of IL-1RA in the post-CPB and post-OPCAB samples and the similar magnitude of inhibitory effect lends support to this assumption.

TNF α -induced E-selectin expression was also significantly inhibited by post CPB plasma. However, post OPCAB plasma had no effect in this bioassay indicating that the use of CPB during cardiac surgery induced a significantly greater anti-TNF α activity than off pump coronary surgery. The magnitude of the inhibitory effect of post CPB plasma on TNF α -induced E-selectin expression correlated with plasma levels of sTNFR1 suggesting that this may be the anti-TNF α activity in the plasma. However, plasma levels of sTNFR1 and indeed sTNFR2 were similar in the two groups of patients (CPB or OPCAB) casting doubt on the role of soluble TNF α receptors in the inhibitory effect of post CPB plasma and suggests that perhaps other factors are contributing to this effect.

5.7.3.1 Conclusion

The systemic inflammatory response that is associated with cardiac surgery, in particular that utilising CPB does not appear to be evident in the patients in this current study. In contrast, there is the presence of a significant activity in the circulation of these patients that is capable of inhibiting pro-inflammatory events in endothelial cells that appears to be attributable, at least in part to the use of CPB.

5.8 CONCLUSION

In conclusion this data illustrates the immense complexity of the cytokine network and interacting factors in the plasma of patients with heart failure and of patients following cardiac surgery. These factors are collectively able to influence the inflammatory activity of the transcription factor NF κ B in vascular endothelial cells in ways that cannot be predicted from measuring concentrations of single cytokines. Moreover, this data highlights the importance of considering multiple steps in the activation pathway of NF κ B responsive genes. This combination strategy using bioassays targeting sequential steps in pro-inflammatory gene induction appears to provide important additional information on global inflammatory events beyond single cytokine measurements. In doing so this study reveals that the increased circulating levels of pro-inflammatory cytokines associated with increasing severity of heart failure and in response to cardiac surgery with cardiopulmonary bypass may be of little importance with respect to

endothelial cell activation. In this respect the predominant activity in the circulation of these patients is an anti-inflammatory one.

Chapter Six: THE ROLE OF OXIDATIVE STRESS IN NF κ B ACTIVATION IN HUMAN VASCULAR AND RESPIRATORY CELLS

6.1 INTRODUCTION

There is compelling evidence that one of the major contributing factors to the pathogenesis of a number of inflammatory vascular events, including heart failure, atherosclerosis, ischaemia/reperfusion injury and sepsis, is increased oxidative stress. Increased oxidative stress occurs when there is an imbalance between reactive oxygen species (ROS) production and antioxidant defences, such that either ROS production is increased and/or defence mechanisms are impaired. Under pathological conditions high concentrations of ROS can cause cell injury and death by the oxidative modification of proteins and carbohydrates, lipid peroxidation and DNA strand breaks. However, recently it has become apparent that ROS and oxidant stress should be viewed as more than just a process of cell damage and are now recognised as important second messenger molecules that are important in the control of the inflammatory process. Indeed, inflammation and oxidative stress are perhaps inseparable phenomena.

6.1.1 OXIDATIVE STRESS AND INFLAMMATION

There is a large body of evidence demonstrating that the production of ROS occurs at the site of inflammation and contributes to leucocyte recruitment and activation and to the tissue damage observed under such circumstances.

The activation of neutrophils by adhesion to the activated endothelium or the extracellular matrix primes the neutrophils for a massive respiratory burst and the release of large amounts of superoxide, an event that forms an integral part of non-specific host defence (Dapino et al., 1993; Hill et al., 1994; von Asmuth and Buurman, 1995). In addition, activation of vascular cells (endothelial cell and smooth muscle cells) by a variety of inflammatory stimuli can contribute to the oxidant rich environment through the production of superoxide via NADPH oxidase activation (Lassegue and Clempus, 2003).

Increased oxidative stress in various experimental models has been shown to promote leucocyte rolling, adhesion and migration through the vascular endothelium (Hotter et al., 1997; Johnston et al., 1996; Poch et al., 1999). Furthermore, direct modulation of the ratio of oxidised to reduced glutathione (GSSG/GSH) in endothelial cells can produce a biphasic neutrophil-endothelial cell adhesion response mediated by both transcription dependant and independent surface expression of adhesion molecules (Kokura et al., 1999).

6.1.2 OXIDATIVE STRESS AND HEART FAILURE

Recently it has become apparent that increased oxidative stress is associated with heart failure. In fact increased oxidative stress has been suggested to be one of the major contributing factors in the transition from compensated ventricular hypertrophy to decompensated heart failure. Evidence in support of this hypothesis has come from both experimental and clinical studies.

6.1.2.1 Animal studies

In isolated cardiac myocytes, a moderate increase in ROS can induce a heart failure phenotype characterised by hypertrophy and apoptosis (Siwik et al., 1999). In addition, in both rat and guinea pig models of heart failure reduced myocardial anti-oxidant activity (superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx)) has been demonstrated with an accompanying increase in GSSG/GSH and oxidative stress (Dhalla et al., 1996; Dhalla and Singal, 1994; Hill and Singal, 1996). Transition through mild to moderate to severe heart failure in these models saw a progressive worsening of this state of poor anti-oxidant defences and increased oxidative stress (Hill and Singal, 1997).

In addition to reduced anti-oxidant defences in heart failure, increased production of ROS have been demonstrated in the myocardium that correlate with LV contractile dysfunction (Ekelund et al., 1999; Ide et al., 2000).

6.1.2.2 Clinical studies

Studies in patients with heart failure have extended the observations from animal models and also support the role of oxidative stress in heart failure.

Belch et al measured plasma lipid peroxides (malondialdehyde) and thiols in patients with heart failure and compared them to healthy controls. That study was one of the first to demonstrate that increased lipid peroxidation and reduced thiol content correlated with LV dysfunction (Belch et al., 1991). Indirect measures of increased oxidative stress in the pericardial fluid (isoprostanes, thiobarbituric reactive substances (TBARS)) have also demonstrated a relationship between oxidant/antioxidant imbalance in the heart and severity of ventricular dysfunction and heart failure (Ellis et al., 2000; Mallat et al., 1998). In addition, increased evidence of oxidative stress in peripheral endothelial cells of patients with heart failure has been demonstrated (Colombo et al., 2002) as has an

increased superoxide-generating capacity of neutrophils which correlated with NYHA functional class (Ellis et al., 2000).

6.1.2.3 Anti-oxidant capacity of current heart failure therapies

Current therapies used in the treatment of heart failure have been suggested to have anti-oxidant activities, which may contribute to the efficacy of such therapies. Carvedilol, a non-specific β -antagonist with α -blocking activity, also inhibits lipid peroxidation possibly by scavenging free radicals (Yue et al., 1992a) and/or by inhibiting neutrophil superoxide generation (Yue et al., 1992b). In a recent clinical study carvedilol treatment reduced myocardial HNE-modified protein levels, a marker of lipid peroxidation, by 40% along with amelioration of cardiac function (Nakamura et al., 2002).

In addition, captopril, an ACE inhibitor frequently used in the treatment of heart failure, can scavenge superoxide and hydrogen peroxide (H_2O_2), improve myocardial SOD, GPx and catalase activities as well as effectively inhibiting the generation of superoxide from activated neutrophils (Chopra et al., 1992; Khaper and Singal, 1997). Losartan an angiotensin type 1 receptor blocker, in addition to reducing cardiac remodelling and improving survival in a rat model of heart failure, also has the ability to reduce oxidative stress and increase myocardial endogenous antioxidants (GPx and catalase, Khaper and Singal, 2001).

6.1.3 OXIDATIVE STRESS AND CARDIAC SURGERY

There is also strong evidence to suggest that one of the major contributing factors to the ischaemia/reperfusion injury and the inflammatory response associated with CPB is changes in the reduction/oxidation (redox) state of the cells and their environment and that ROS and oxidative stress play a significant role in the pathogenesis of such post-operative complications.

Evidence for the involvement of redox driven processes in the development of ischaemia/reperfusion injury has come through the experimental manipulation of anti-oxidant mechanisms. Transgenic mice overexpressing glutathione peroxidase (intracellular and extracellular) are protected from ischaemia/reperfusion injury (Ishibashi et al., 1999; Yoshida et al., 1997) whilst GPx knockout mice show a greater susceptibility. Other studies have shown similar beneficial effect of SOD demonstrating that, when over expressed in coronary endothelial cell, smooth muscle cells or

myocytes, human Cu/Zn-SOD can protect against myocardial ischaemia/reperfusion injury (Chen et al., 2000; Wang et al., 1998b).

In addition to the contribution of ischaemia and reperfusion to the increase in oxidative stress during cardiac surgery a recent study using on-pump beating heart CABG demonstrated that CPB itself may also play a role in the generation of reactive oxygen species (Matata et al., 2000). That prospective randomised study demonstrated that, in the early hours after surgery, OPCAB patients displayed significantly lower levels of lipid hydroperoxides, protein carbonyls and nitrotyrosine than the CPB patients.

Further evidence for an increased oxidative stress induced by cardiac surgery utilising CPB comes from Frass and colleagues who demonstrated that intracellular thiols were depleted in peripheral leucocytes and alveolar macrophages two hours after CPB (Frass et al., 2001). Clermont et al demonstrated that there was an increase in myocardial radical activation within the first three minutes of myocardial reperfusion following cardiac surgery. In addition there was also a pronounced systemic oxidative stress during the course of open-heart surgery that was likely the result of neutrophil activation and the high oxygen tension used during CPB that has the potential to cause systemic damage (Clermont et al., 2002).

Clinical studies aimed at reducing oxidative stress and the subsequent tissue damage elicited by cardiac surgery have yielded rather varying results. In a study by Dingchao et al high dose ascorbic acid (250 mg/kg) protected the myocardium of patients undergoing cardiopulmonary bypass. They observed that postoperative cardiac index was higher in ascorbate treated patients than in their controls resulting in a shorter intensive care and hospital stay (Dingchao et al., 1994). However other studies using either ascorbic acid or α -tocopherol have failed to show such definitive clinical benefit of these anti-oxidant therapies over control patients despite reducing biochemical markers of reperfusion injury (Demirag et al., 2001; Oktar et al., 2001; Westhuyzen et al., 1997). These differences may be reflective of varied therapeutic regimens used in the different studies and/or may require the detection of more subtle, functional end points of injury in populations of low risk patients.

6.1.4 OXIDATIVE STRESS/ ROS AND NF κ B

ROS are key mediators of inflammation and have profound effects on gene transcription. ROS can stimulate protein kinase cascades coupled to inflammatory gene expression; indeed much of the influence of redox imbalance on inflammatory disease is

through the manipulation of transcription factors and the subsequent effect on inflammatory gene expression. NFκB is one such transcription factor whose activation state and transactivation ability has been suggested to be highly dependent on the redox state of the cells.

ROS have been suggested to be a universal second messenger of NFκB activation (Schreck et al., 1992). This hypothesis was born from three main lines of evidence;

- 1) NFκB was activated in cultured cells in response to H₂O₂ (Meyer et al., 1993; Schreck et al., 1991).
- 2) NFκB activation induced by a number of diverse stimuli could be inhibited by anti-oxidant compounds or over expression of anti-oxidant enzymes (Schreck et al., 1991) (Bowie et al., 1997; Brigelius-Flohe et al., 1997; Manna et al., 1998; Meyer et al., 1993; Staal et al., 1995).
- 3) In some cell lines, agents that induce NFκB activation were shown to induce ROS (Schmidt et al., 1995; Schreck, 1991).

* * *

The evidence described above provides a clear link between increased oxidative stress and both heart failure and complications associated with cardiac surgery. The consequences of such changes in redox state can be attributed to a direct injurious effect of ROS on cells and tissue and to the modulation of transcriptional events. Thus, such increased oxidative stress may potentially contribute to the systemic inflammation in these patients through the modulation of transcription factors such as NFκB. Indeed this has been suggested by Colombo and colleagues who demonstrated the co-localisation of nuclear NFκB and nitrotyrosine in peripheral endothelial cells of such patients (Colombo et al., 2002).

However, recent data suggest that ROS and oxidative stress play a very specific role in the induction of NFκB in a manner that is cell, stimuli and target gene specific and thus may potentially allow selective activation of NFκB under distinct oxidising condition.

6.2 AIMS

To investigate the role of redox events in NFκB activation in cultured human endothelial, smooth muscle and epithelial cells with relevance to inflammatory gene activation.

6.3 STUDY DESIGN

To study the influence of redox modulation on NFκB activation and adhesion molecule expression in vascular and respiratory cells two experimental regimens were employed;

- 1) Determination of the influence of pro-oxidant conditions (addition of H₂O₂) on the cultured cells.
- 2) Determination of the effects of the anti-oxidant, N-acetyl-L-cysteine (NAC) on cytokine-induced events in the cultured cells.

6.3.1 CELL TOXICITY

Firstly, using an MTT cell viability assay, any toxic effects of both pro- and anti-oxidant treatment on the cells was determined in order to establish the limits of the protocols to be employed.

MTT assay relies on the cleavage of the tetrazolium ring of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT, yellow solution) to yield purple formazan crystals, which are insoluble in aqueous media. The crystals can be dissolved in acidic isopropanol to generate a purple solution that can be measured spectrophotometrically. An increase or decrease in the amount of formazan formed is proportional to the number of cells and thus indicative of the degree of cytotoxicity.

6.3.2 INTRACELLULAR REDOX EFFECTS

Reasons for monitoring intracellular redox events were two fold;

- 1) To determine whether the conditions used to simulate pro- and anti-oxidant conditions were having the desired intracellular effects.
- 2) To establish whether conditions used to induce NFκB activation were capable of generating intracellular oxidative stress.

To this end the effects of H₂O₂, NAC and cytokines on intracellular oxidation and anti-oxidant defences were determined using two methods.

6.3.2.1 Intracellular oxidation

The method used to determine the level of intracellular oxidation was based on the measurement of an intracellular fluorescent signal generated by the oxidation of 2,7-dichlorodihydrofluorescein (DCFH) to dichlorofluorescein (DCF) in response to treatment. DCFH-diacetate (DCFH-DA) is a stable, lipid soluble compound that is

readily taken up by cells. Once in the cell the diacetate group is cleaved by intracellular enzymes trapping the resulting DCFH in the cell. When DCFH is oxidised the highly fluorescent compound DCF is generated, thus serving as a convenient index of intracellular oxidative stress (Bass et al., 1983).

6.3.2.2 Reduced glutathione

The sulphhydryl group (SH) of reduced glutathione can react with DTNB (5,5'-dithio-bis-2-nitrobenzoic acid, Ellman's reagent) to produce 5-thio-2-nitrobenzoic acid (TNB). TNB is yellow in colour and the absorbance can be measured spectrophotometrically. With the use of a GSH standard curve this method was used to provide an estimation of GSH concentrations in the cells following specific treatment. Reduction in the intracellular GSH concentration therefore suggests increased intracellular oxidants causing conversion of GSH to GSSG.

6.3.3 NFκB ACTIVATION

The ability of pro- and anti-oxidant conditions to alter NFκB activity was investigated at three levels;

- 1) Ability to interfere with the initiation of signalling events was studied by monitoring the effects of the redox agents on cellular levels of IκBα, both basal and in response to cytokine treatment.
- 2) Nuclear NFκB p65 levels were monitored to determine effects of pro and anti-oxidant condition on NFκB nuclear translocation.
- 3) Alterations in the expression of NFκB dependant adhesion molecules on the cell surface and IκBα resynthesis following cytokine-induced depletion were monitored to reflect effects on NFκB driven gene transcription and translation.

6.3.4 p38 MAPK ACTIVATION

p38 MAPK activation has been demonstrated to be sensitive to changes in the redox environment in a number of cell types (Griendling et al., 2000). Therefore to assess the responsiveness of the cells to the altered intracellular redox conditions achieved by the various treatments, p38 MAPK phosphorylation was monitored in the same samples used to measure IκBα depletion.

6.4 METHODS

6.4.1 CELL CULTURE

All cells and the culture processes used in these studies have been described previously in this thesis (Chapter 4).

6.4.2 MTT CELL VIABILITY ASSAY

Experiments were carried out on cells in 24 well plates using phenol red free medium under conditions that parallel those used to study NF κ B activation and adhesion molecule expression.

Following the experimental protocol the cells were washed in PBS and 300 μ l of MTT solution (300 μ g/ml) was added to each well. Cells were incubated at 37°C for 3 hours to allow purple formazan crystals to develop. To each well 300 μ l of solubilisation reagent (10% Triton-X, 0.1M HCl in isopropanol) was added and the plates were incubated at room temperature for 30 minutes with constant rocking. The absorbance of the solubilised formazan was determined at 570nm with a reference wavelength of 690nm using a μ QUANT™ plate reader and KCjunior™ acquisition and analysis software (Bio-Tek Instruments Inc). The results are expressed as a percent of the result obtained from cells treated with medium alone.

6.4.3 DETERMINATION OF INTRACELLULAR REDOX STATUS

6.4.3.1 Fluorescent measurement of intracellular oxidation

Cells were grown in medium without phenol red, for one week prior to use. When confluent, the cells were trypsinised, washed in PBS, resuspended in DMEM without phenol red and the cell density was adjusted to 1 million cells per ml. All subsequent steps were performed with minimum exposure to light. 100 μ l of cells was removed and incubated with 1 μ l ethanol, as vehicle, for 20 minutes at 37°C and used as unloaded control cells. 20mM DCFH-DA in ethanol was added to the remainder of the cells to give a final concentration of 200 μ M. The cells were incubated at 37°C for 20 minutes in the dark to load the cells with the dye. Cells were washed twice in phenol red free DMEM to remove the remaining extracellular dye and were resuspended to the original volume. The cell suspension was divided into two and incubated with either NAC 20mM or vehicle for 30 minutes at 37°C. After washing, the cell were resuspended in phenol red free DMEM at 5×10^5 cells/ml and 100 μ l of cells were added into the wells

of opaque, white 96 well plates containing 100µl of relevant solutions at two times the required final concentration.

Fluorescence was measured every 90 seconds over 30 minutes using a FLx800 microplate fluorescence reader (Bio-Tek Instruments Inc) using excitation wavelength of 485/20 nm and an emission wavelength of 528/20 nm. Data was acquired and analysed using KCjunior™ software.

6.4.3.2 Determination of intracellular sulphhydryl concentration

Intracellular acid soluble thiols were measured according to the method of Sedlak and Lindsay (Sedlak and Lindsay, 1968). Following experimental procedure, confluent cells in 24 well plates were washed in cold PBS and incubated with ice cold 5% trichloroacetic acid (TCA) for 30 minutes and the supernatant from duplicate wells were combined. 200µl 0.1M TRIS/EDTA solution (pH8.9) and 10µl 10mM Ellman's reagent in methanol were added to 100µl of each sample or GSH standards (0-100µM) in a 96 well plate. Absorbance was measured at 414nm using a µQUANT™ plate reader and KCjunior™ acquisition and analysis software (Bio-Tek Instruments Inc). The protein precipitate remaining in the wells after sulphhydryl extraction was washed 3 times with PBS, solubilised in 100µl of 1M NaOH and the amount of protein in each sample determined by Bradford assay. All results are expressed as nanomoles sulphhydryl per mg protein.

6.4.4 NFκB ACTIVATION

For all experiments cells were incubated for 24 hours with DMEM containing no additives prior to the start of the experiment and all experiments were performed in this medium. NFκB activation was monitored as previously described in this thesis (section 4.3.2).

6.4.5 p38 MAPK ACTIVATION

p38 MAPK activation was monitored by Western blot analysis of whole cell lysates on the same nitrocellulose membrane used to quantify total cell IκBα protein levels. p38 activation was monitored using a primary antibody specific for the dual phosphorylated activated form of this protein (clone p38-TY, 1µg/ml, Sigma-Aldrich) and a rabbit anti-mouse IgG–HRP-conjugate used at 1.3 µg/ml.

6.5 RESULTS 1: ENDOTHELIAL CELLS

6.5.1 EFFECT OF H₂O₂ ON HUMAN PAEC VIABILITY

Five hours treatment of human PAEC with H₂O₂ caused a concentration dependant toxicity. MTT assay demonstrated significantly reduced cleavage the tetrazolium ring by viable cell mitochondria dehydrogenase following treatment with 250μM (70.8 ± 7.9% of control, p=0.002, n=19), 500μM (60.8 ± 6.8% of control, p<0.001, n=15)) and 1000μM (29.3 ± 1.8% of control, p<0.001, n=15) H₂O₂ (Figure 6.1a)

6.5.2 EFFECT OF NAC ON HUMAN PAEC VIABILITY

Following 5 hours treatment of human PAEC with NAC (20mM) there was no evidence of cell death compared to control treated cells (121.6 ± 4.5% of control, p=0.070, n=9). In addition, NAC (20mM) protected the PAEC from the toxic effects of a pro-oxidant environment and prevented cell death induced by even the highest concentration of H₂O₂ (1000μM, 112.2 ± 7.8% of control versus 30.5 ± 3.0% following treatment with H₂O₂ alone, p<0.001, n=9, Figure 6.1b).

6.5.3 EFFECT OF H₂O₂ ON INTRACELLULAR OXIDATION IN HUMAN PAEC

6.5.3.1 Oxidation of DCFH

Treatment of human PAEC with H₂O₂ resulted in a time and concentration dependent increase in fluorescence as a result of oxidation of DCFH to DCF. Treatment with 500μM and 1000μM H₂O₂ resulted in a significant increase in fluorescence above that of time matched, control levels as early as three minutes after addition to the cells (893.0 ± 4.1 and 926.5 ± 7.9 RFU respectively versus 818.5 ± 13.3 RFU in control cells, p<0.05, n=4). Fluorescence continued to increase in cells treated with 500 and 1000μM H₂O₂ over the 30 minute time course studied (Figure 6.2a, Table 6.1). Treatment of cells with NAC significantly reduced the oxidation of DCFH under all conditions studied (p<0.001, n=4, Figure 6.2b).

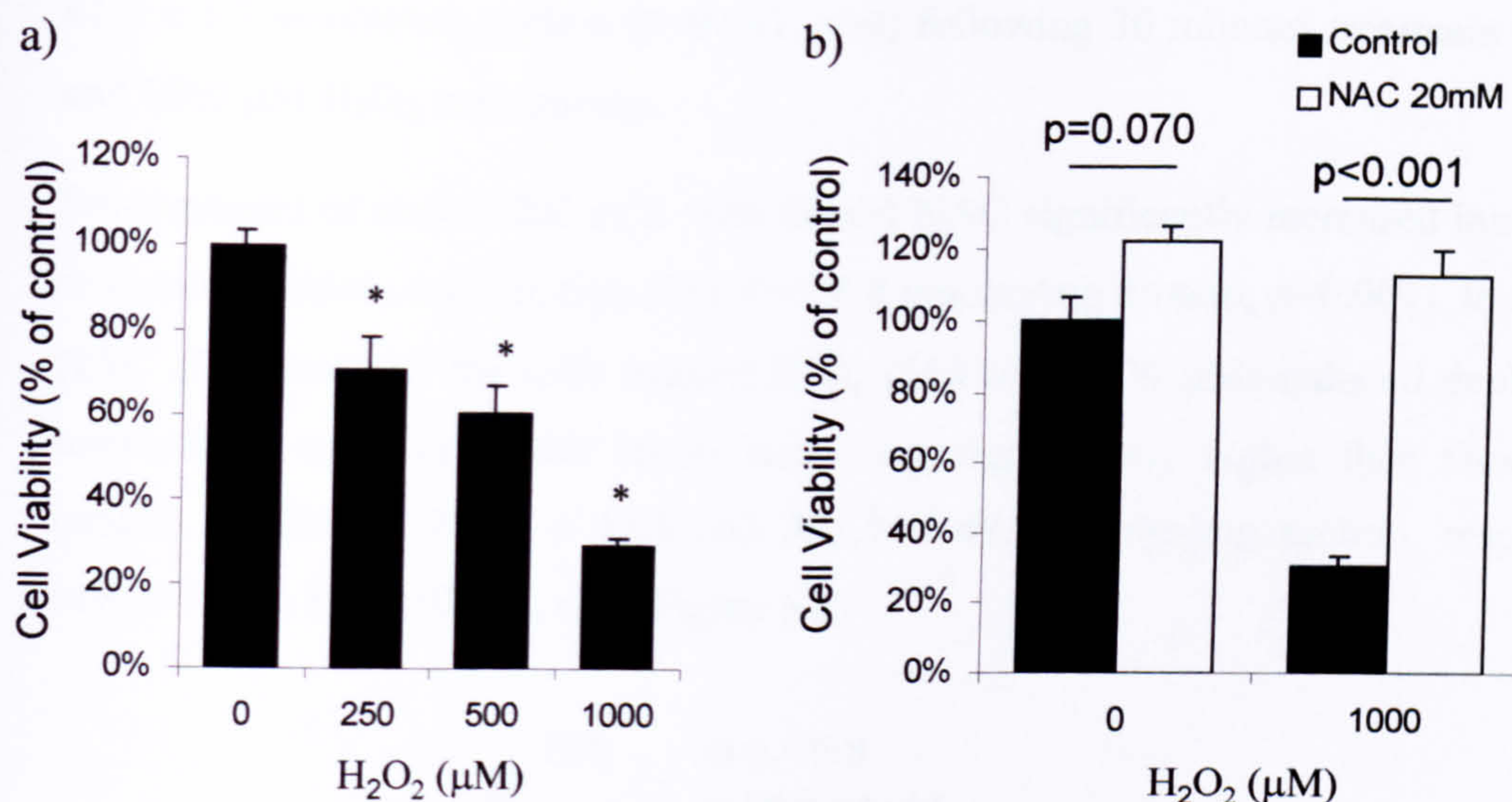


Figure 6.1. Effect of H_2O_2 and NAC on PAEC viability. a) Concentration-dependent effect of 5 hours H_2O_2 treatment. b) Effect of NAC on cell viability in control and H_2O_2 treated cells. *Denotes $p < 0.05$ from control cells.

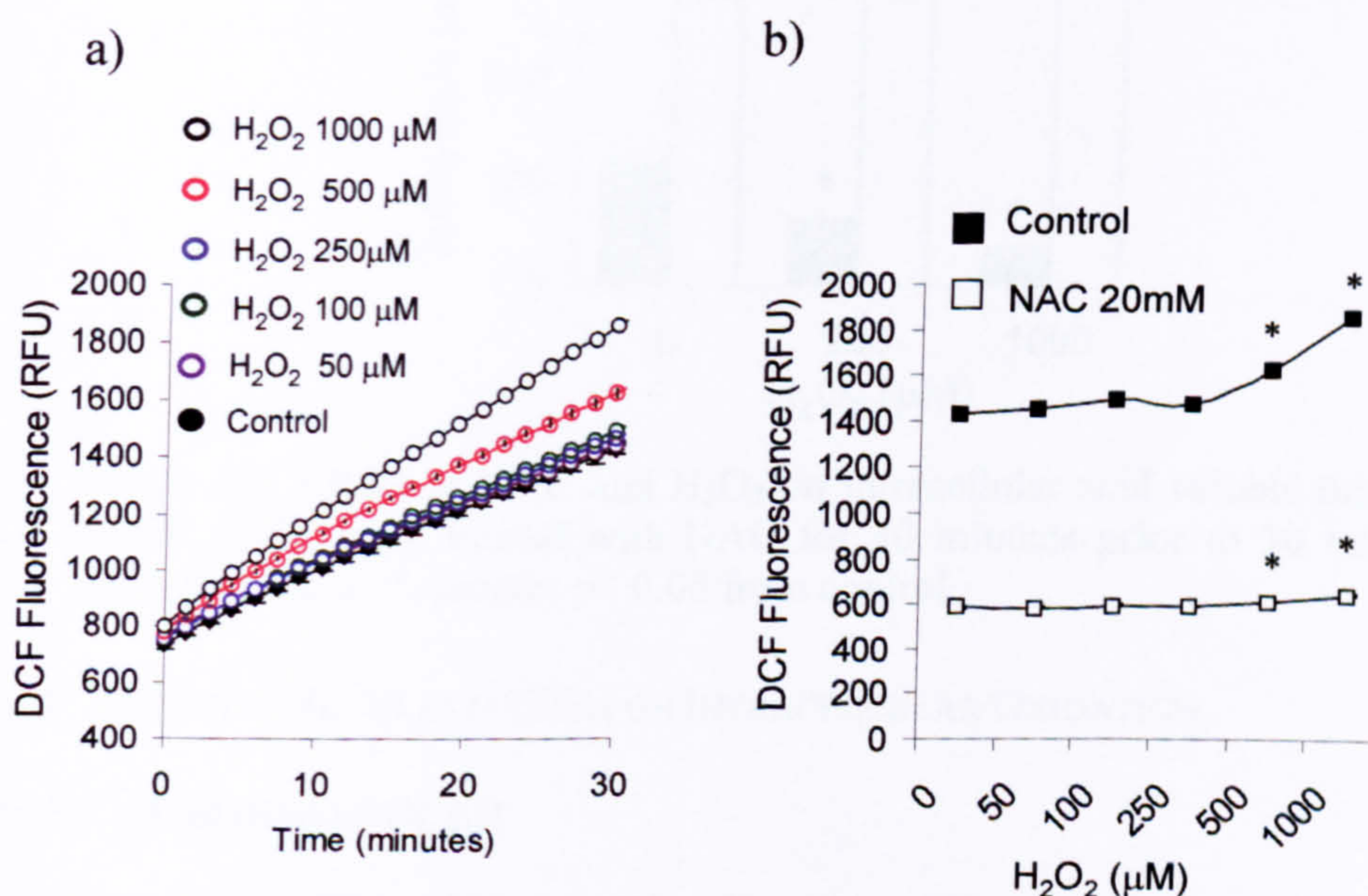


Figure 6.2. Effect of H_2O_2 and NAC on the fluorescence generated by oxidation of intracellular DCFH in PAEC. a) Time course over 30 minutes. b) Effect of NAC (20mM) on the H_2O_2 generated DCF fluorescence after 30 minutes. * denotes $p < 0.05$ from time matched control cells.

6.5.3.2 Sulphydryl concentration

In human PAEC the intracellular sulphydryl concentration was significantly reduced from 99.15 ± 8.9 nmoles/mg protein in control cells to 68.9 ± 2.4 , ($p=0.007$, $n=4$) and

41.8 ± 1.7 nmoles/mg protein (p<0.001, n=4) following 30 minutes treatment with 250 and 1000 µM H₂O₂, respectively.

Pre-treatment of endothelial cells with 20mM NAC significantly increased intracellular acid soluble thiol concentration (251.4 ± 18.8 nmoles/mg protein, p<0.001). In addition, NAC also protected the cells against H₂O₂ (250 and 1000 µM)-induced depletion of intracellular sulphhydryl with levels remaining significantly higher than those under control conditions (287.5 ± 17.8 and 301.5 ± 49.7 nmoles/mg protein, respectively versus 99.2 ± 8.9, p<0.001, n=4, Figure 6.3).

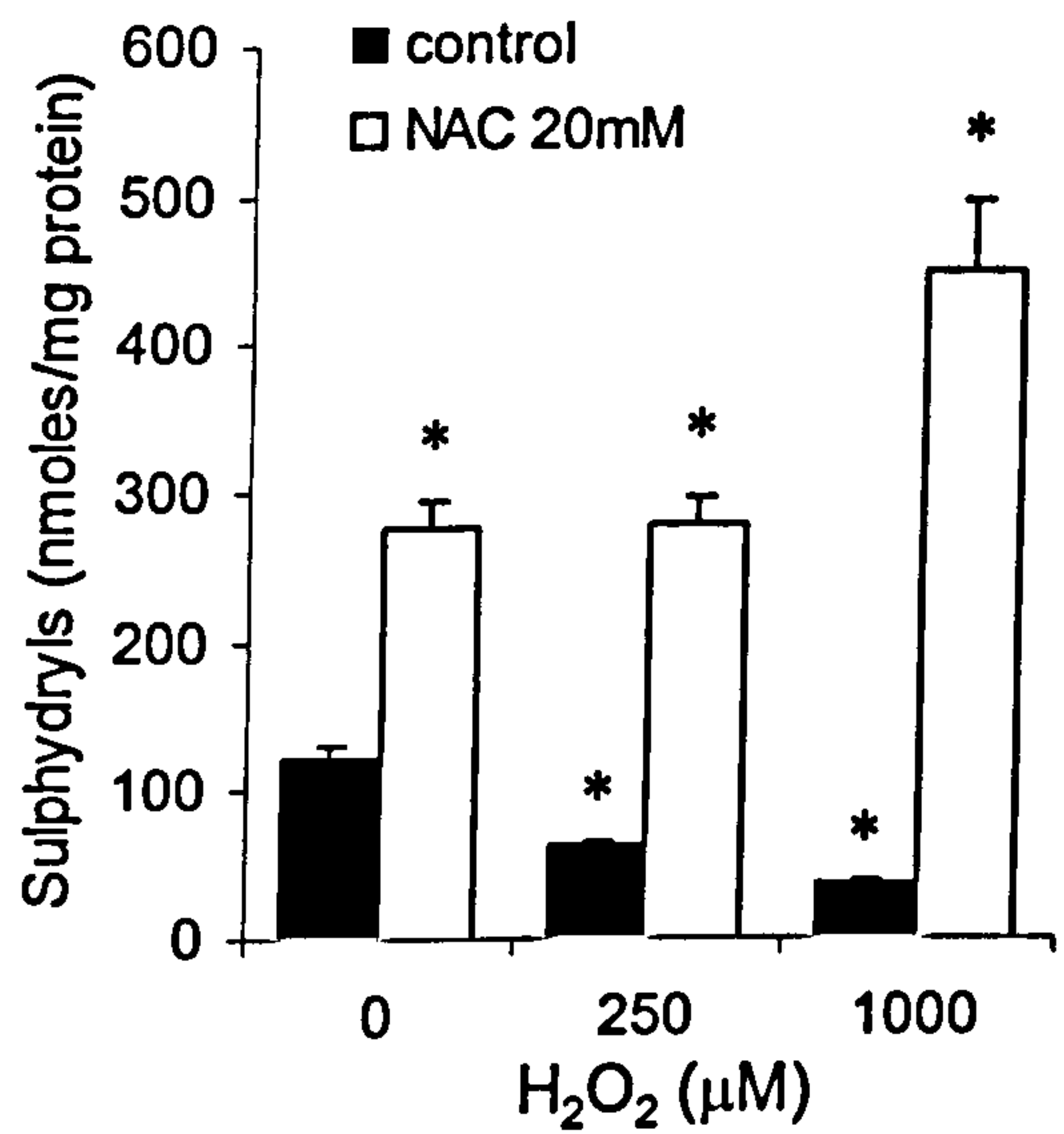


Figure 6.3. Effect of NAC and H₂O₂ on intracellular acid soluble thiols in PAEC. Cells were treated with NAC for 30 minutes prior to 30 minutes H₂O₂ treatment. * denotes p< 0.05 from control

6.5.4 EFFECT OF IL-1β AND TNFα ON INTRACELLULAR OXIDATION.

6.5.4.1 Oxidation of DCFH

In contrast to the effect of H₂O₂ neither IL-1β nor TNFα, at concentrations shown to induce NFκB and p38 MAPK activation demonstrated the ability to induce DCFH oxidation over a 30 minute time course (Figure 6.4, Table 6.1).

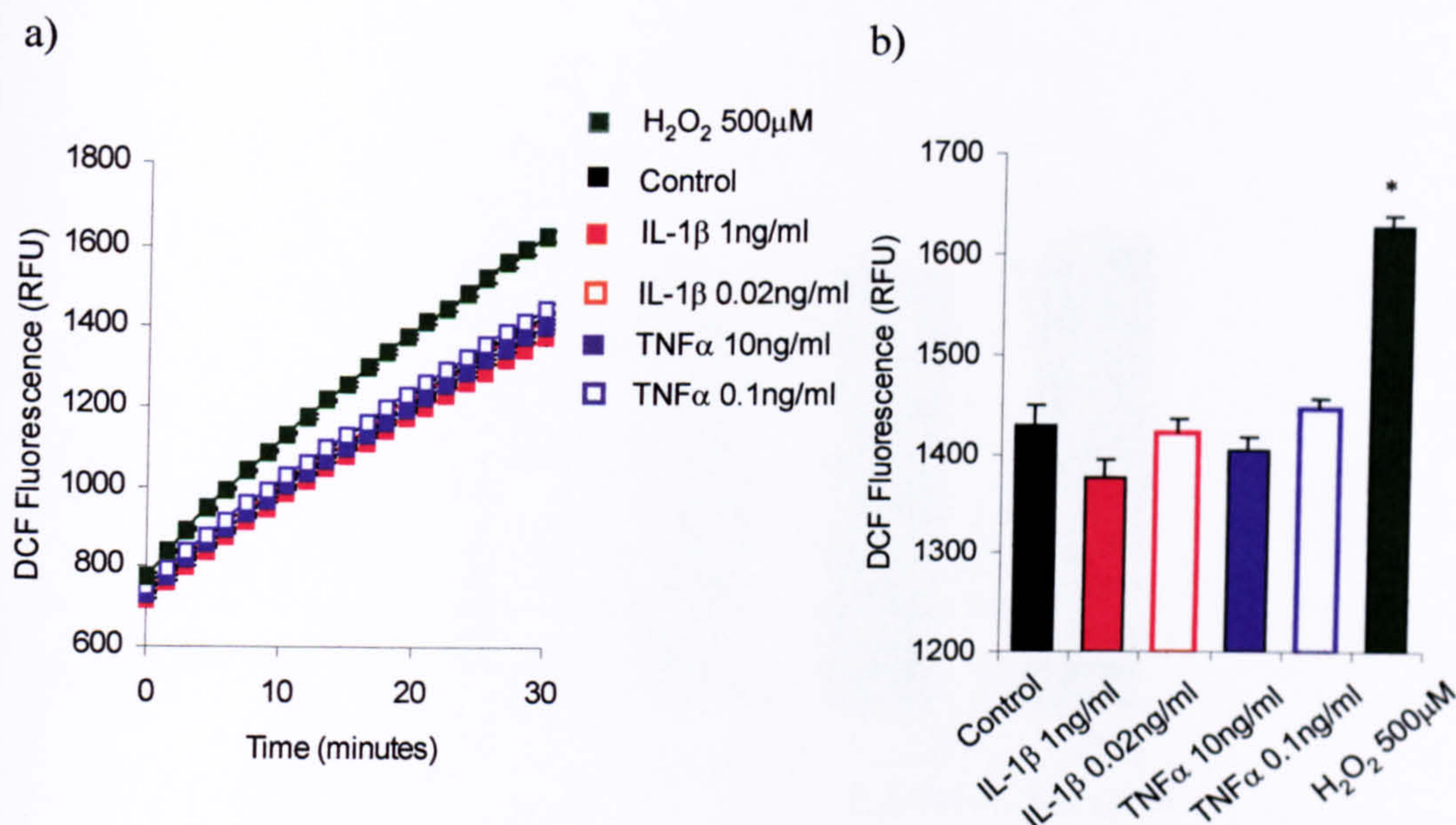


Figure 6.4. Effect of IL-1 β and TNF α on the fluorescence generated by oxidation of intracellular DCFH in PAEC. a) Time course over 30 minutes. b) Summary graph showing the fluorescence after 30 minutes of treatment. * Denotes $p < 0.05$ from time matched control cells.

TREATMENT	DCF Fluorescence (RFU) at 30 minutes		Significance from control
	Mean	SEM	
Control	1429.0	20.1	
H ₂ O ₂ (50μM)	1445.8	31.2	ns
H ₂ O ₂ (100μM)	1494.3	5.7	ns
H ₂ O ₂ (250μM)	1472.0	10.0	ns
H ₂ O ₂ (500μM)	1625.6	12.2	$p < 0.001$
H ₂ O ₂ (1000μM)	1857.0	10.2	$p < 0.001$
IL-1 β 1ng/ml	1377.3	16.6	ns
IL-1 β 0.02ng/ml	1422.3	12.6	ns
TNF α 10ng/ml	1405.3	13.2	ns
TNF α 0.1ng/ml	1447.0	10.4	ns

Table 6.1 Fluorescence signal (relative fluorescence units) generated by the oxidation of intracellular DCFH following 30 minutes treatment of PAEC.

6.5.4.2 Sulphydryl concentration

The concentration of intracellular acid soluble thiols in cultured human PAEC (68.3 ± 2.3 nmoles/mg) was not altered by treatment with either IL-1 β (1ng/ml, 70.1 ± 2.0 nmoles/mg protein) or TNF α (10ng/ml, 74.9 ± 3.2 nmoles/mg protein) over the time course demonstrated to induce NF κ B and p38 MAPK activation (30 minutes, Figure 6.5).

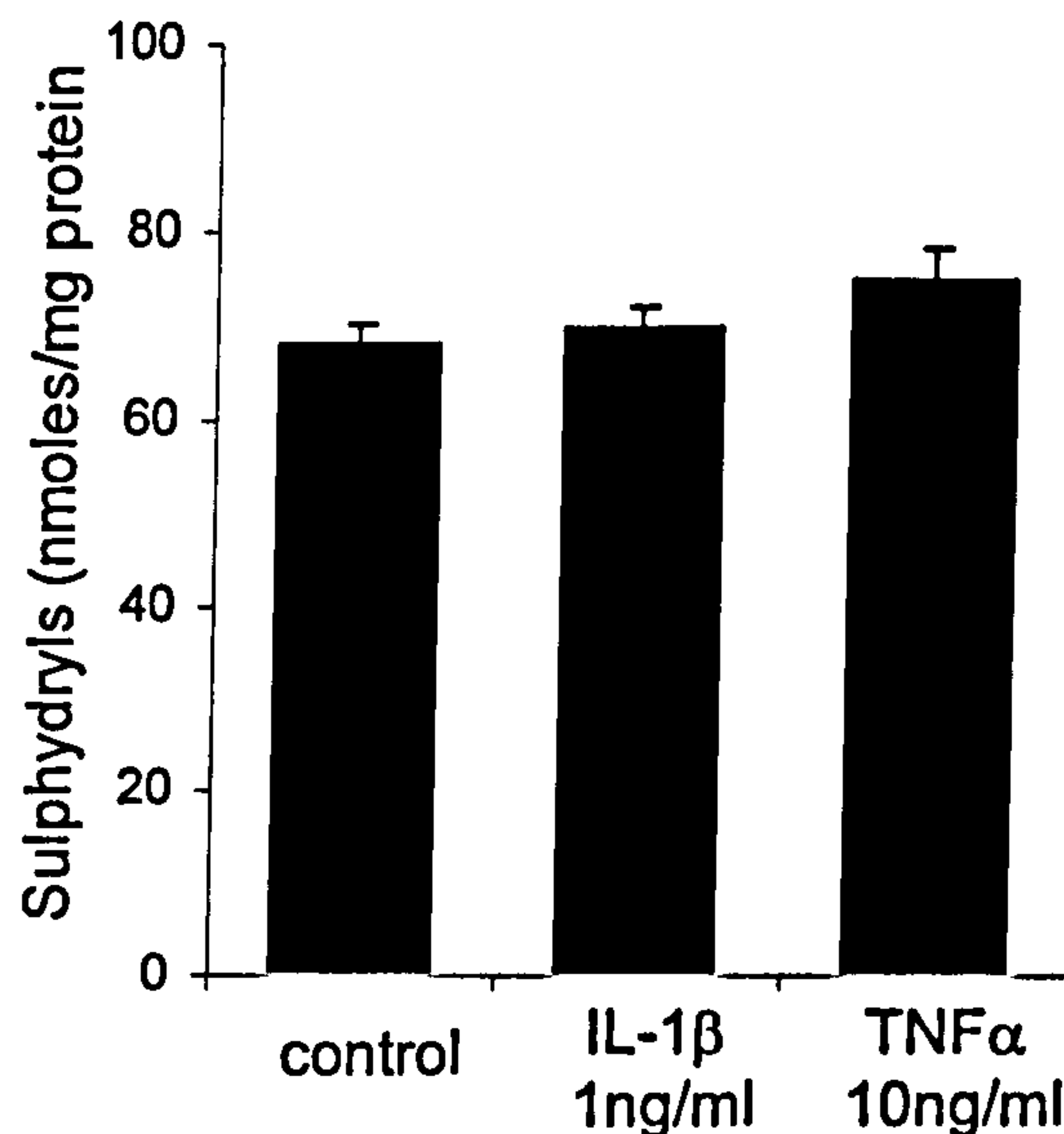


Figure 6.5. Effect of 30 minutes treatment of PAEC with IL-1 β and TNF α on intracellular acid soluble thiols.

6.5.5 INFLUENCE OF ANTI-OXIDANTS ON CYTOKINE-INDUCED NF κ B ACTIVATION

6.5.5.1 I κ B α depletion

In PAEC I κ B α depletion induced by 30 minutes treatment with IL-1 β (0.2pg/ml – 2ng/ml, pEC₅₀ 2.34 $\pm$ 0.66ng/ml) was not affected by pre-treatment with NAC (20mM, pEC₅₀ 1.60 $\pm$ 0.45), p=0.386, n=4, Figure 6.6a). Similarly, TNF α (1pg/ml – 10ng/ml)-induced I κ B α depletion after 30 minutes treatment was unaffected by the presence of NAC (20mM) (pEC₅₀ 0.83 $\pm$ 0.34 ng/ml verses 1.54 $\pm$ 0.42 ng/ml in the absence of NAC, p=0.259, p=3, Figure 6.6b).

Furthermore, this antioxidant failed to affect the resynthesis of I κ B α following IL-1 β or TNF α -induced depletion. Following 120 minutes treatment with IL-1 β (1ng/ml) I κ B α levels were 57.8 $\pm$ 24.7% of control levels in the absence of NAC and 79.7 $\pm$ 46.0% of control levels in the presence of NAC (p=0.942). Following 120 minutes treatment with TNF α (10ng/ml) I κ B α levels were 57.9 $\pm$ 11.7% of control levels in the absence of NAC and 111.5 $\pm$ 26.4 % of control levels in the presence of NAC (p=0.113, n=4, Figure 6.6c-e).

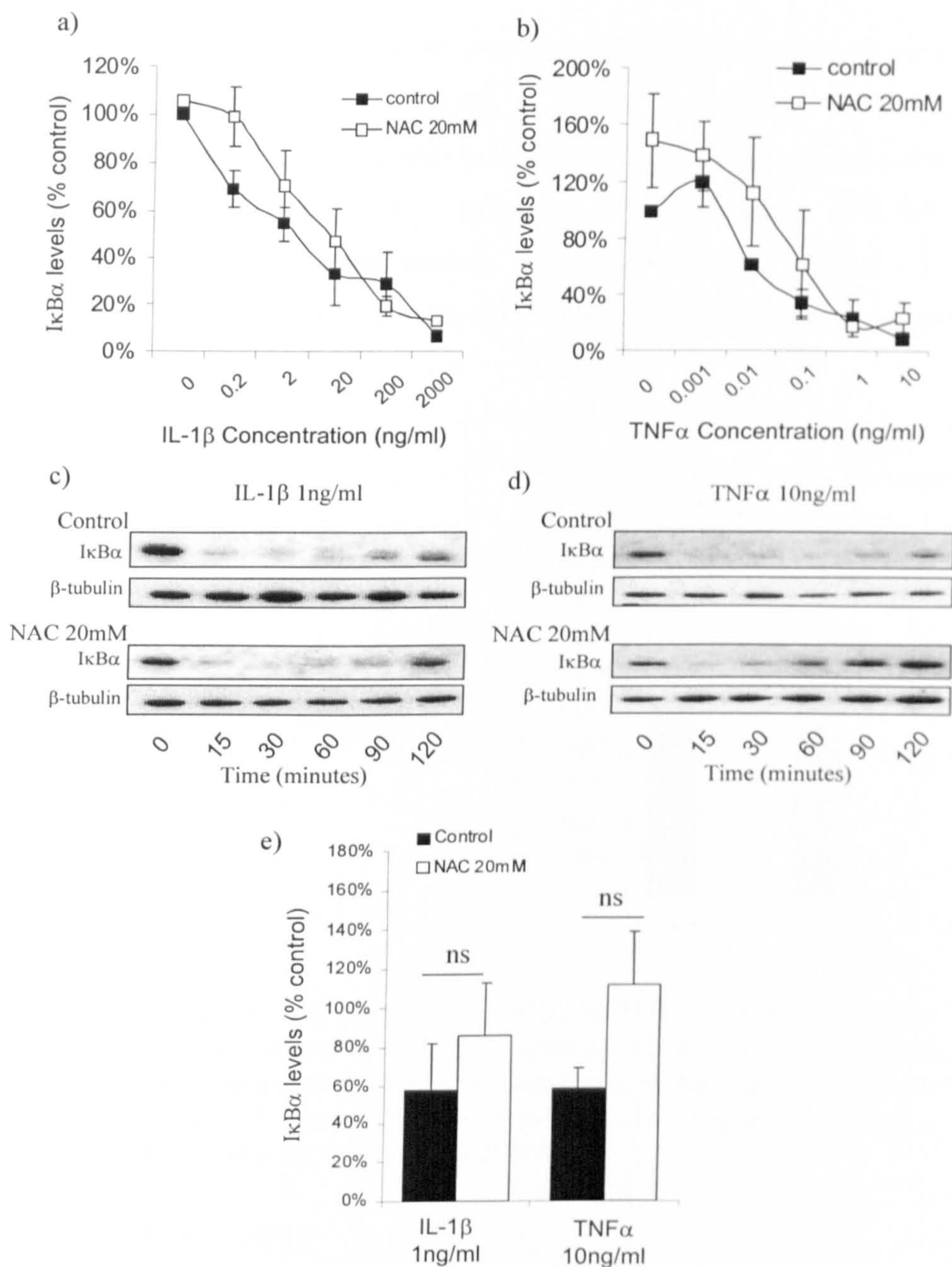


Figure 6.6. Effect of NAC (20mM) on IκBα depletion and resynthesis induced by IL-1β and TNFα in PAEC. Summary graphs of the concentration related effects of 30 minutes treatment with IL-1β (a) and TNFα (b) on the cellular levels of IκBα protein in the presence (□) and absence (■) of NAC. Representative Western blots of the time course of IκBα protein depletion and resynthesis following treatment with IL-1β 1ng/ml (c) and TNFα 10ng/ml (d) in the presence and absence of NAC. e) Summary graph demonstrating the effect of NAC on newly synthesised IκBα protein levels following 120 minutes treatment with IL-1β 1ng/ml and TNFα 10ng/ml.

6.5.5.2 NFκB p65 nuclear translocation

Increased nuclear levels of NFκB p65 protein in human PAEC following 60 minutes IL-1β (1ng/ml) treatment ($2633.4 \pm 1811.5\%$ of control levels) were not significantly altered by the presence of NAC (20mM) in the culture medium 30 minutes prior to and during cytokine treatment ($1950.8 \pm 1221.8\%$ of control levels, $p=0.785$, $n=4$). Likewise, TNFα (10ng/ml)-induced increase in nuclear NFκB p65 ($2712.4 \pm 1920.0\%$ of control) was not altered by NAC (20mM, $2090.8 \pm 1378.0\%$ of control, $p=0.801$, $n=4$, Figure 6.7).

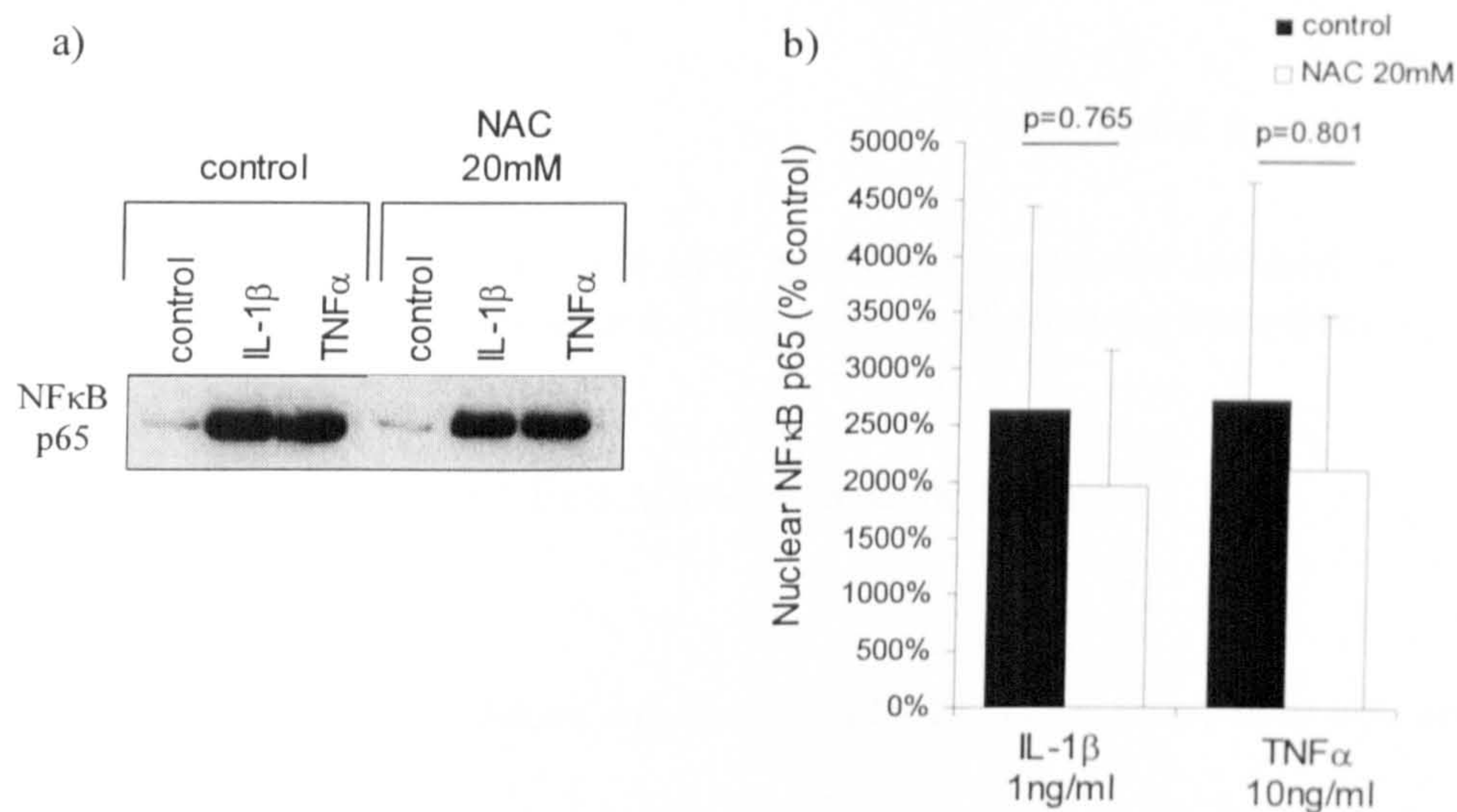


Figure 6.7. Effect of NAC (20mM) on IL-1β and TNFα-induced increase in nuclear NFκB p65 protein in PAEC. Representative Western blots (a) and summary graph showing NFκB p65 protein levels in nuclear extracts from PAEC following 60 minutes treatment with IL-1β (1ng/ml) and TNFα (10ng/ml) in the presence and absence of NAC.

6.5.5.3 E-selectin Expression

PAEC E-selectin expression induced by 5 hours treatment with IL-1β (0.2pg/ml – 2ng/ml) was unaffected by the presence of NAC (20mM, $p>0.05$, $n=3$, Figure 6.8a).

In contrast, PAEC E-selectin expression induced by sub-maximal concentrations of TNFα (0.1ng/ml, $46.7 \pm 12.0\%$ positive cells and 1ng/ml, $65.2 \pm 14.6\%$ positive cells) were significantly reduced by the presence of 20mM NAC (8.6 ± 1.9 and $20.6 \pm 2.3\%$ positive cells respectively, $p<0.05$, $n=3$, Figure 6.8b).

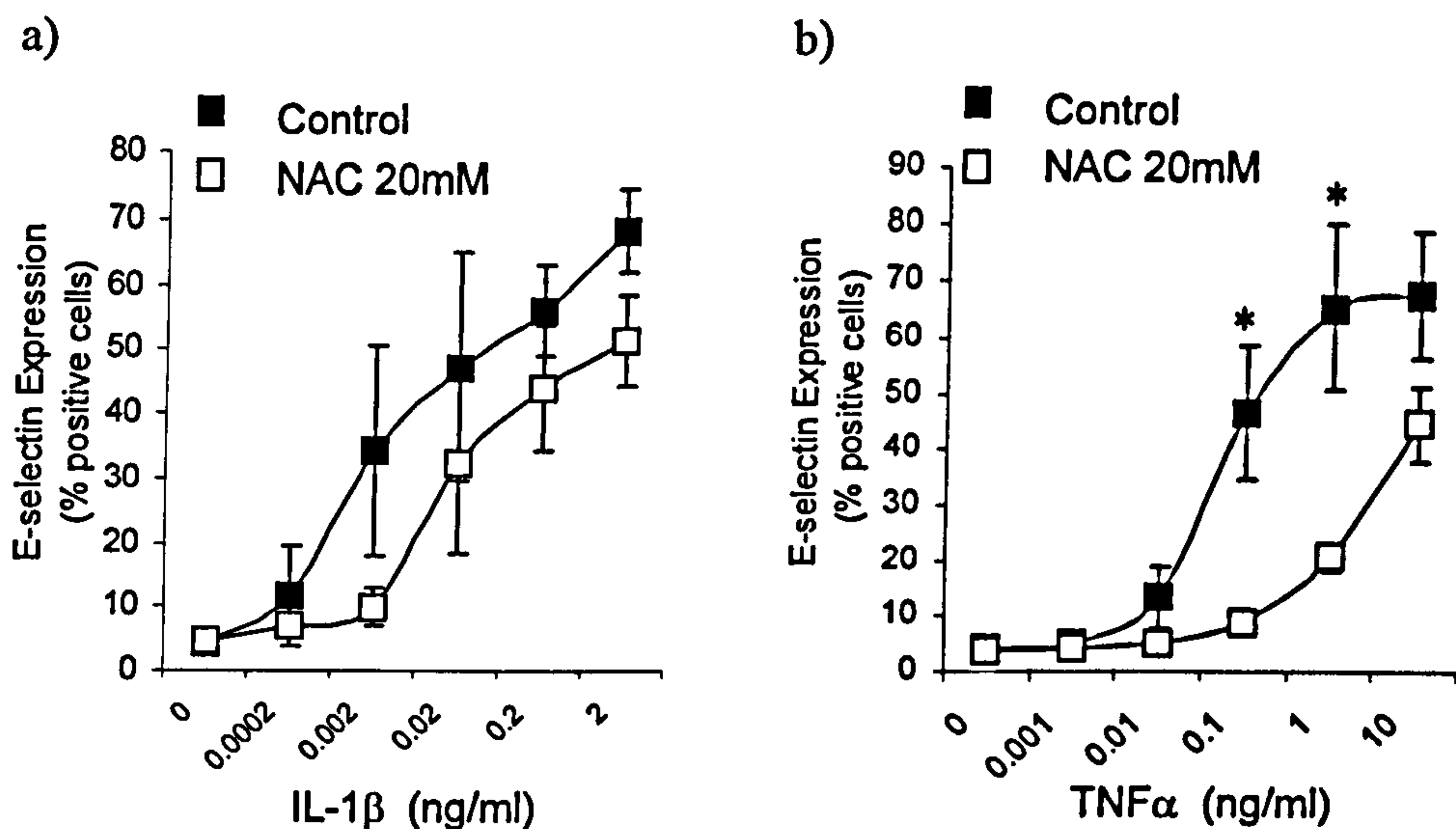


Figure 6.8. Effect of NAC on PAEC E-selectin expression induced by 5 hours IL-1 β and TNF α -treatment. *Denotes $p < 0.05$ from the corresponding control cytokine response.

6.5.6 INFLUENCE OF H₂O₂ ON NF κ B ACTIVITY IN RESTING CELLS

6.5.6.1 I κ B α depletion

H₂O₂ (50-1000 μ M) failed to induce any change in PAEC I κ B α levels over a 30 minute time course (Figure 6.9).

6.5.6.2 NF κ B p65 nuclear translocation

Nuclear NF κ B p65 levels did not change from that in resting cells following 60 minutes treatment with H₂O₂ 250 μ M ($140.8 \pm 14.0\%$ of that in control cells, $n=5$).

6.5.6.3 E-selectin expression

Due to the significant toxic effect of H₂O₂ on PAEC over a 5 hours time period (section 6.5.1) it was not possible to test the effect of pro-oxidant conditions on E-selectin expression.

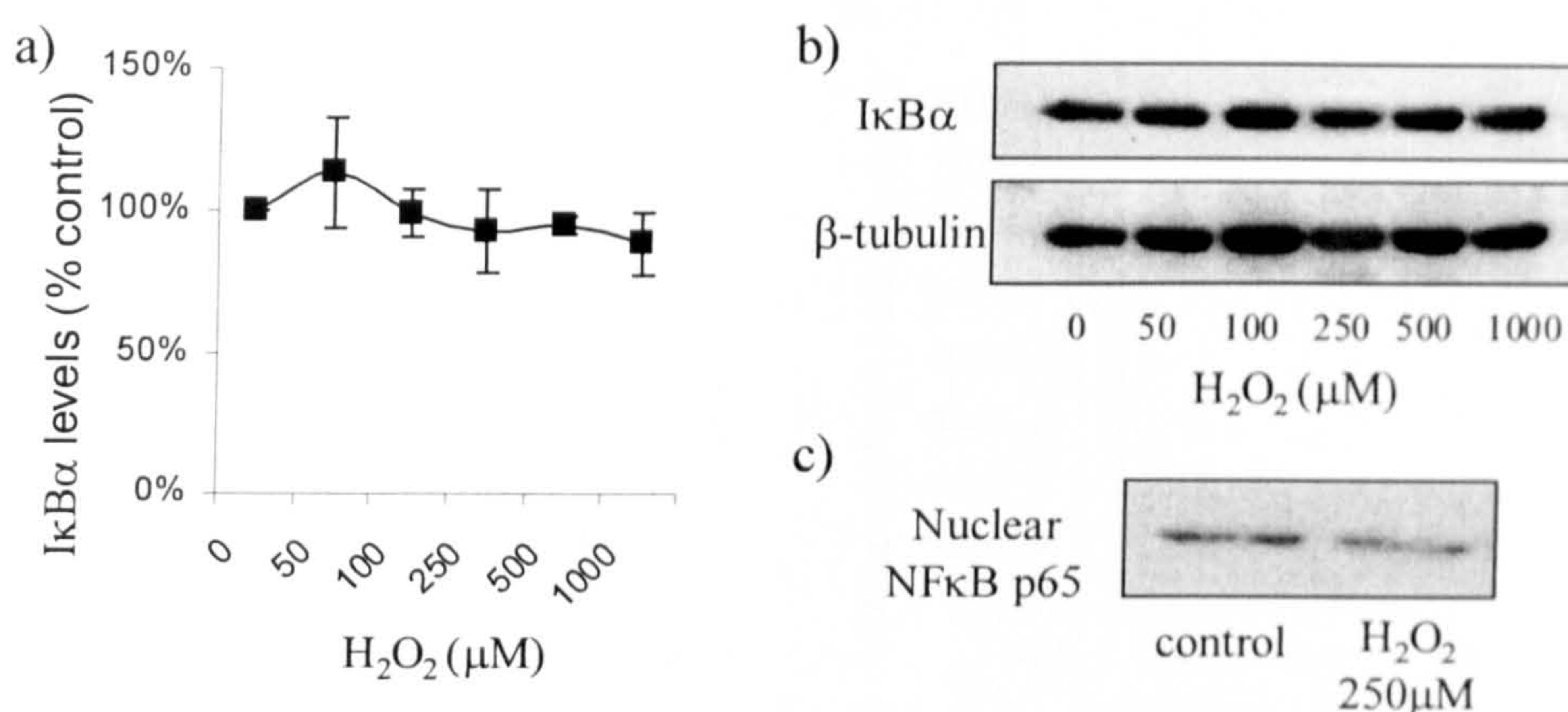


Figure 6.9. Effect of H₂O₂ on NFκB activation in PAEC. a) Summary graph of the cellular levels of IκBα protein following 30 minutes treatment with H₂O₂. b) Representative Western blot of (b) cellular levels of IκBα protein following 30 minutes treatment with H₂O₂, c) NFκB p65 in isolated nuclear proteins following 60 minutes H₂O₂ treatment.

6.5.7 INFLUENCE OF ANTI-OXIDANTS ON CYTOKINE-INDUCED P38 MAPK ACTIVATION

In PAEC, IL-1β (1ng/ml) induced a transient activation of p38 MAPK peaking at 30 minutes ($630.2 \pm 206.6\%$ of control levels) and returning to control levels by 120 minute ($111.1 \pm 27.1\%$ of control, Figure 6.10a). Pre-treatment of the cells with 20mM NAC had no effect on the level of p38 activation following 30 minutes treatment with IL-1β (1ng/ml, $443.4 \pm 159.3\%$ of control levels, $p=0.556$, $n=4$, Figure 6.10c).

TNFα (10ng/ml) also induced a transient activation of p38 MAPK in PAEC peaking at 15 minutes ($540.8 \pm 193.8\%$ of control) and returning to baseline levels by 60 minutes ($153.5 \pm 71.4\%$, Figure 6.10b). In contrast to IL-1β-induced p38 activation, that induced by 15 minutes treatment with TNFα (10ng/ml) was completely abolished by pre-treatment of the cells with 20mM NAC ($110.3 \pm 11.0\%$, $p=0.036$, $n=3$, Figure 6.10c).

6.5.8 INFLUENCE OF H₂O₂ ON P38 MAPK ACTIVATION

Exposing PAEC to a pro-oxidant environment resulted in p38 MAPK activation, as determined by increased levels of the phosphorylated form of this protein. At 30 minutes a maximum response was achieved with 250μM H₂O₂ resulting in a $319.7 \pm 71.4\%$ increase in p38 MAPK activation above control levels (Figure 6.11).

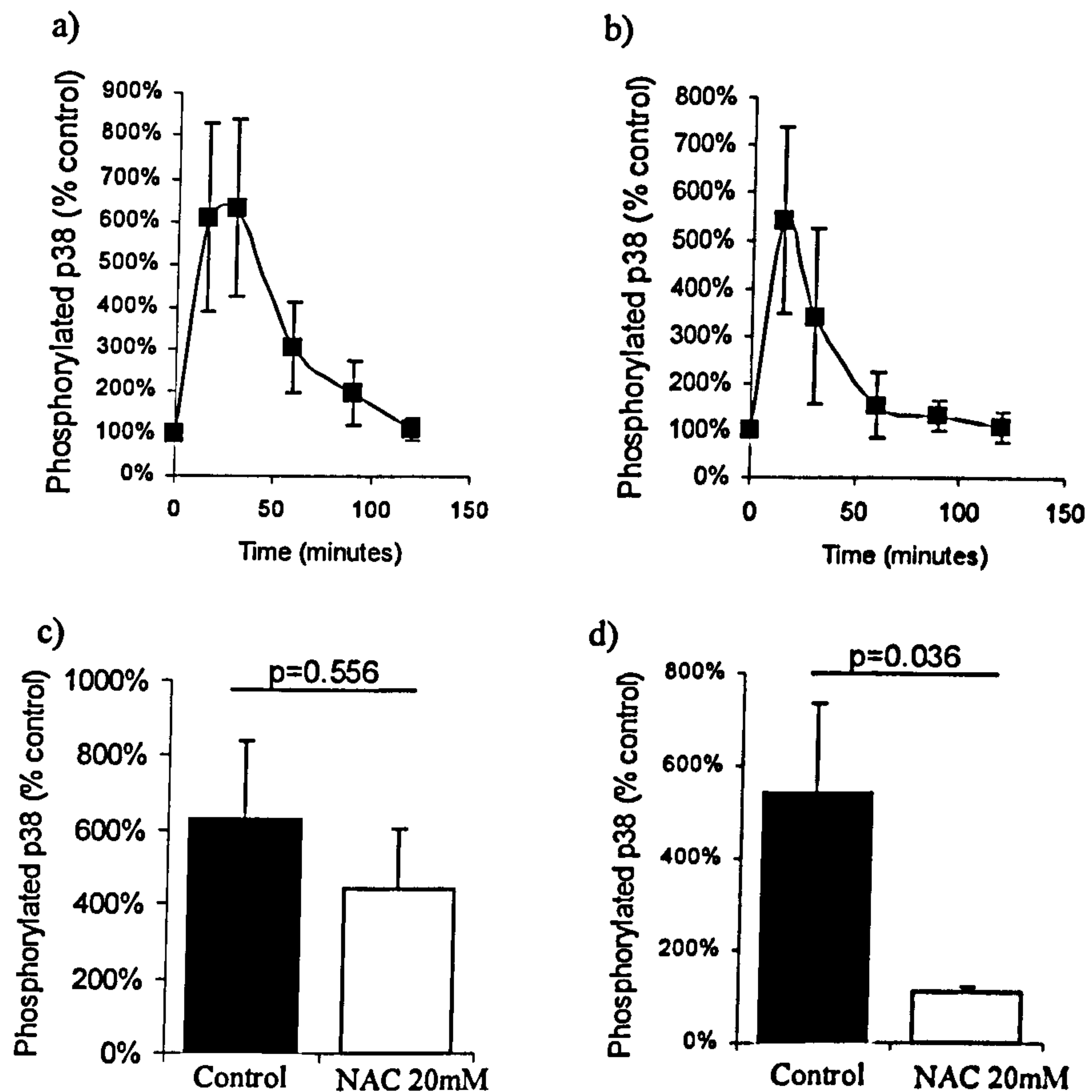


Figure 6.10. Effect of NAC on cytokine-induced p38 MAPK activation. Summary graphs of the time course of p38 phosphorylation induced by a) IL-1 β 1ng/ml and b) TNF α 10ng/ml. Effect of NAC (20mM) on p38 phosphorylation induced by c) 30 minutes IL-1 β (1ng/ml) treatment and d) 15 minutes TNF α (10ng/ml) treatment.

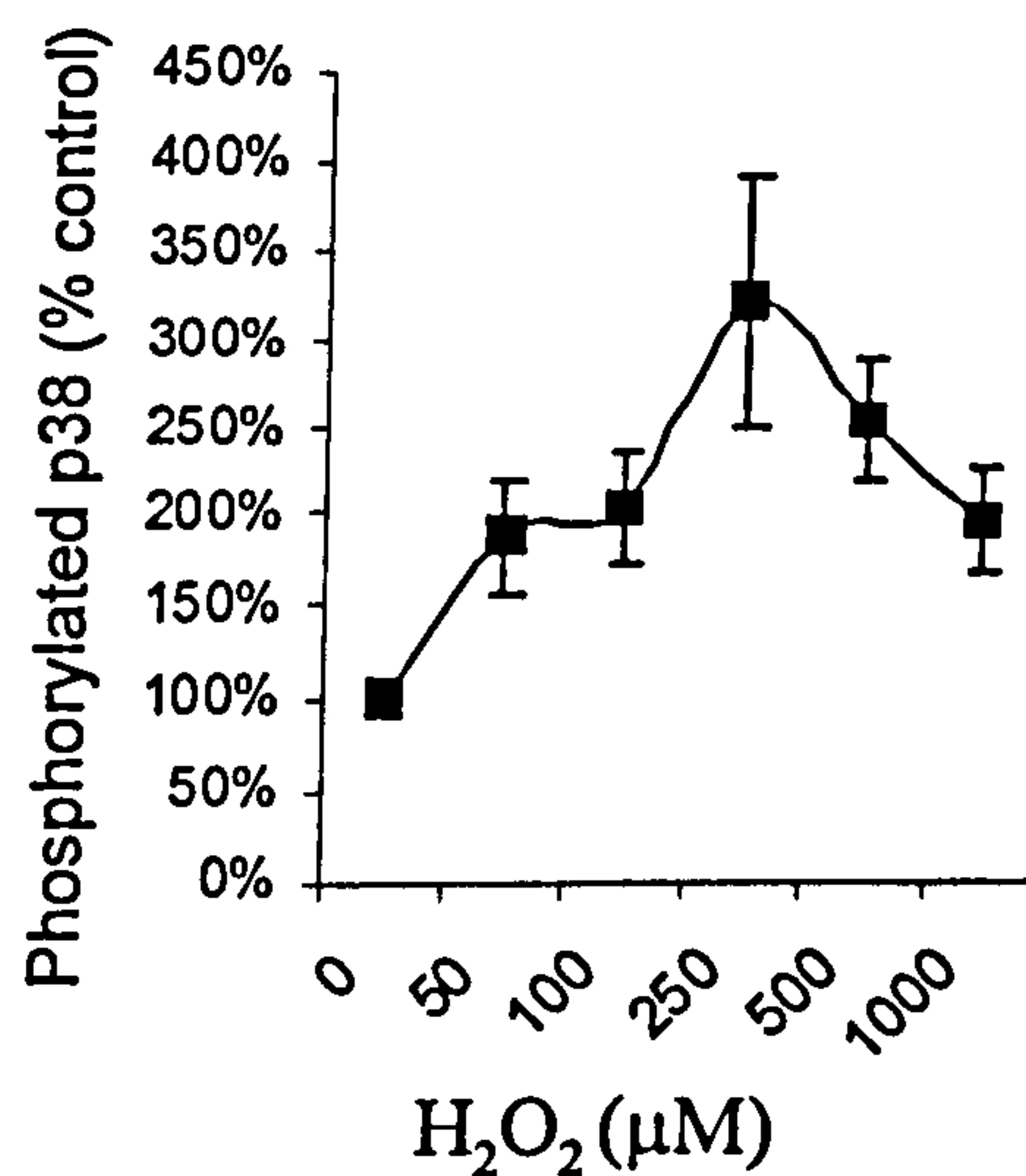


Figure 6.11. Effect of 30 minutes treatment of PAEC with H₂O₂ on p38 activation.

6.6 RESULTS 2: SMOOTH MUSCLE CELLS

6.6.1 EFFECT OF H₂O₂ ON HUMAN VASCULAR SMC VIABILITY

Treatment of human aortic SMC with H₂O₂ for 18 hours caused a concentration dependant toxicity. MTT assay demonstrated significantly reduced cleavage of the tetrazolium ring by viable cell mitochondria dehydrogenase following treatment with 250μM (84.8 ± 4.5 % of control, p=0.038 n=5), 500μM (82.9 ± 3.5% of control, p=0.015, n=5) and 1000μM (58.6 ±5.0% of control, p<0.001, n=5, Figure 6.12a) H₂O₂. Shorter exposure (3 hours) of these SMC to H₂O₂ (250, 500 and 1000μM) resulted in toxic effects observed only with 1000μM (56.5 ±3.3% of control, p<0.05, n=6, Figure 6.12b).

6.6.2 EFFECT OF NAC ON HUMAN VASCULAR SMC VIABILITY

Following 18 hours treatment with NAC (20mM), human aortic SMC viability was significantly reduced to 16.2 ± 1.4% of control levels (p<0.001, n=5, Figure 6.12). Cell viability remained unaltered from control following 3 hours treatment with NAC (20mM, 93.9 ± 1.2% of control, p>0.05, n=6, Figure 6.12). In addition, over a 3 hour time period NAC inhibited the toxic effects of 1000μM H₂O₂ (38.7 ± 1.5% of control versus 93.9 ± 3.9% in NAC pre-treated cells, p<0.05, n=6).

6.6.3 EFFECT OF H₂O₂ ON INTRACELLULAR OXIDATION

6.6.3.1 Oxidation of DCFH

Treatment of human aortic SMC with H₂O₂ resulted in a time- and concentration-dependent increase in fluorescence as a result of oxidation of DCFH to DCF. Treatment with 250, 500, 1000μM H₂O₂ resulted in a significant increase in fluorescence above that of time matched, control levels as early as six minutes after addition to the cells (723.8 ± 17.6, 881.3 ± 9.5, 991.8 ± 6.3 RFU respectively vs. 663.0 ± 9.0 RFU in control cells, p<0.05, n=4). Fluorescence continued to increase in cells treated with 250, 500 and 1000μM H₂O₂ over the 30 minute time course studied (Figure 6.13a, Table 6.2).

Pre-treatment of cells with NAC for 30 minutes prior to the addition of H₂O₂ significantly reduced the oxidation of DCFH under all conditions studied (p< 0.05, n=4, Figure 6.13b).

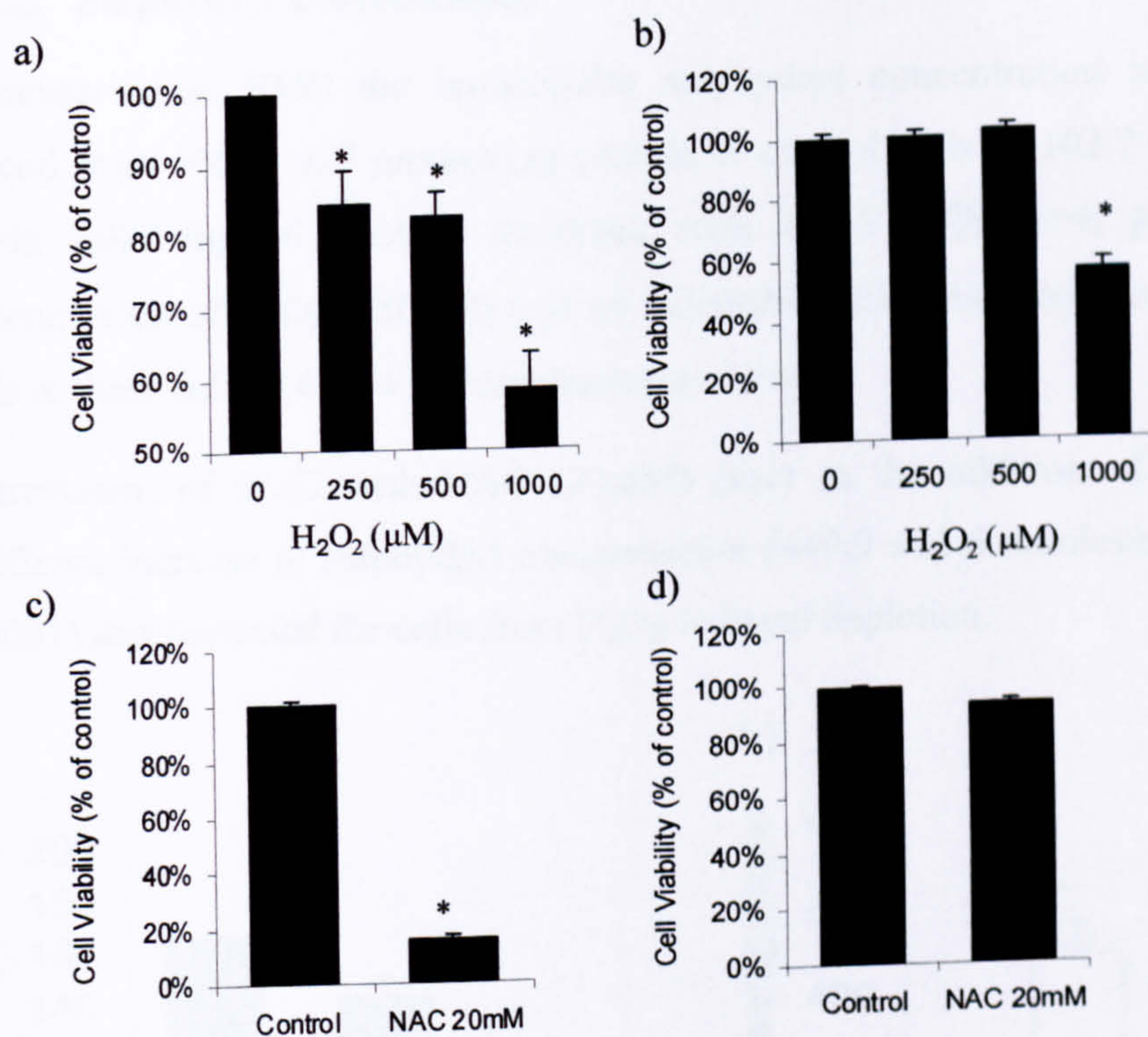


Figure 6.12. Effect of H₂O₂ and NAC on aortic SMC viability. Concentration-dependent effect of a) 18 hours and b) 3 hours H₂O₂ treatment. Effect of c) 18 hours and d) 3 hours NAC (20mM) on cell viability. *Denotes p < 0.05 from control cells.

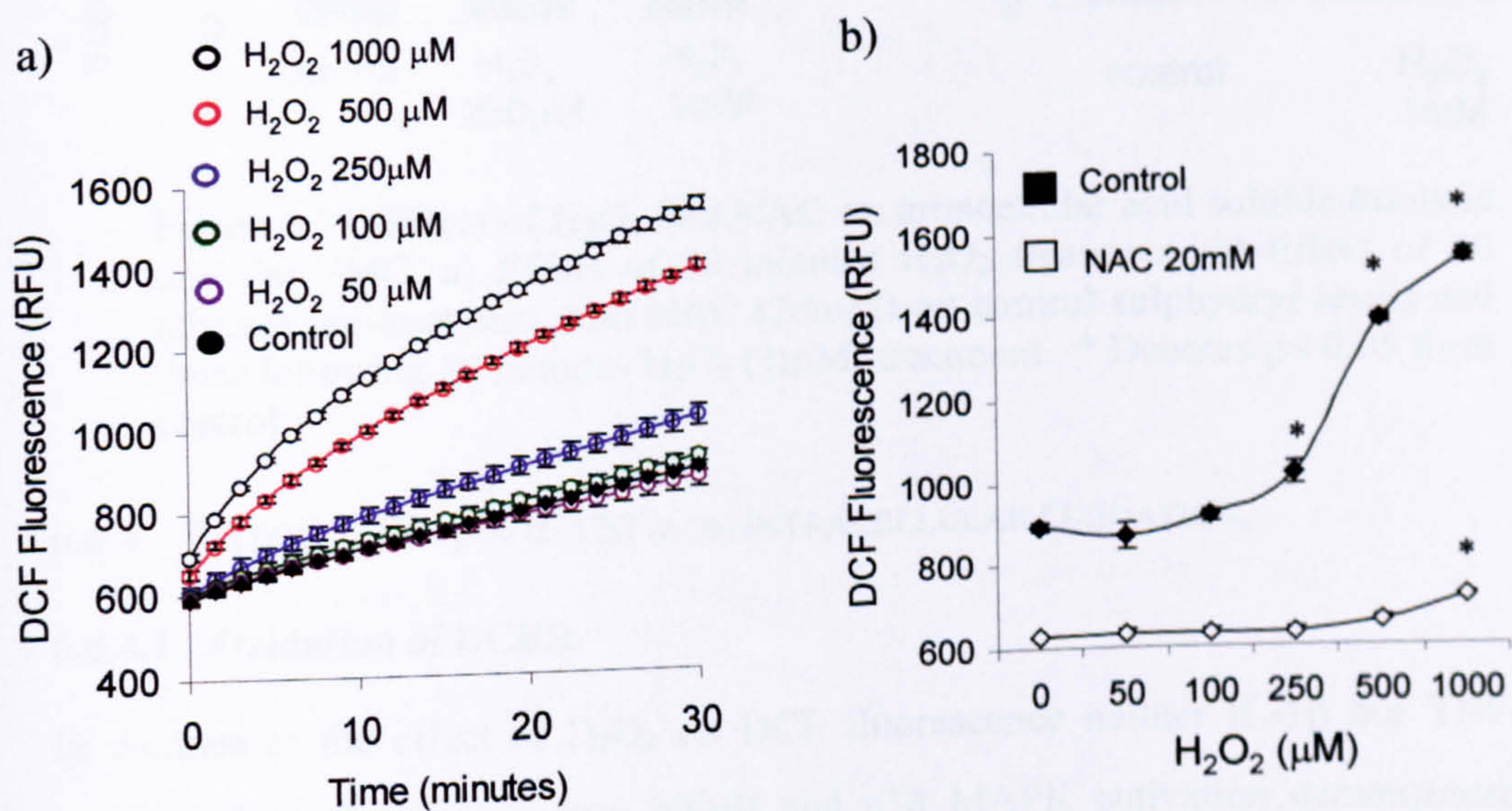


Figure 6.13. Effect of H₂O₂ and NAC on the fluorescence generated by oxidation of intracellular DCFH in SMC. a) Time course over 30 minutes. b) Effect of NAC (20mM) on the H₂O₂ generated DCF fluorescence after 30 minutes. * denotes p < 0.05 from time matched control cells.

6.6.3.2 Sulphydryl concentration

In human aortic SMC the intracellular sulphydryl concentration was significantly reduced from 165.4 ± 6.7 nmoles/mg protein in control cells to 102.7 ± 5.0 nmoles/mg protein following 30 minutes treatment with 1mM H_2O_2 ($n=4$, $p<0.001$). Lower concentrations of H_2O_2 ($250\mu M$) had no detectable effect on intracellular acid soluble thiols in these cells (141.3 ± 7.5 nmoles/mg protein).

Pre-treatment of SMC with NAC (20mM) prior to the addition of H_2O_2 caused a significant increase in sulphydryl concentration (449.9 ± 31.6 nmoles/mg protein, $n=4$, $p<0.001$) and protected the cells from H_2O_2 -induced depletion.

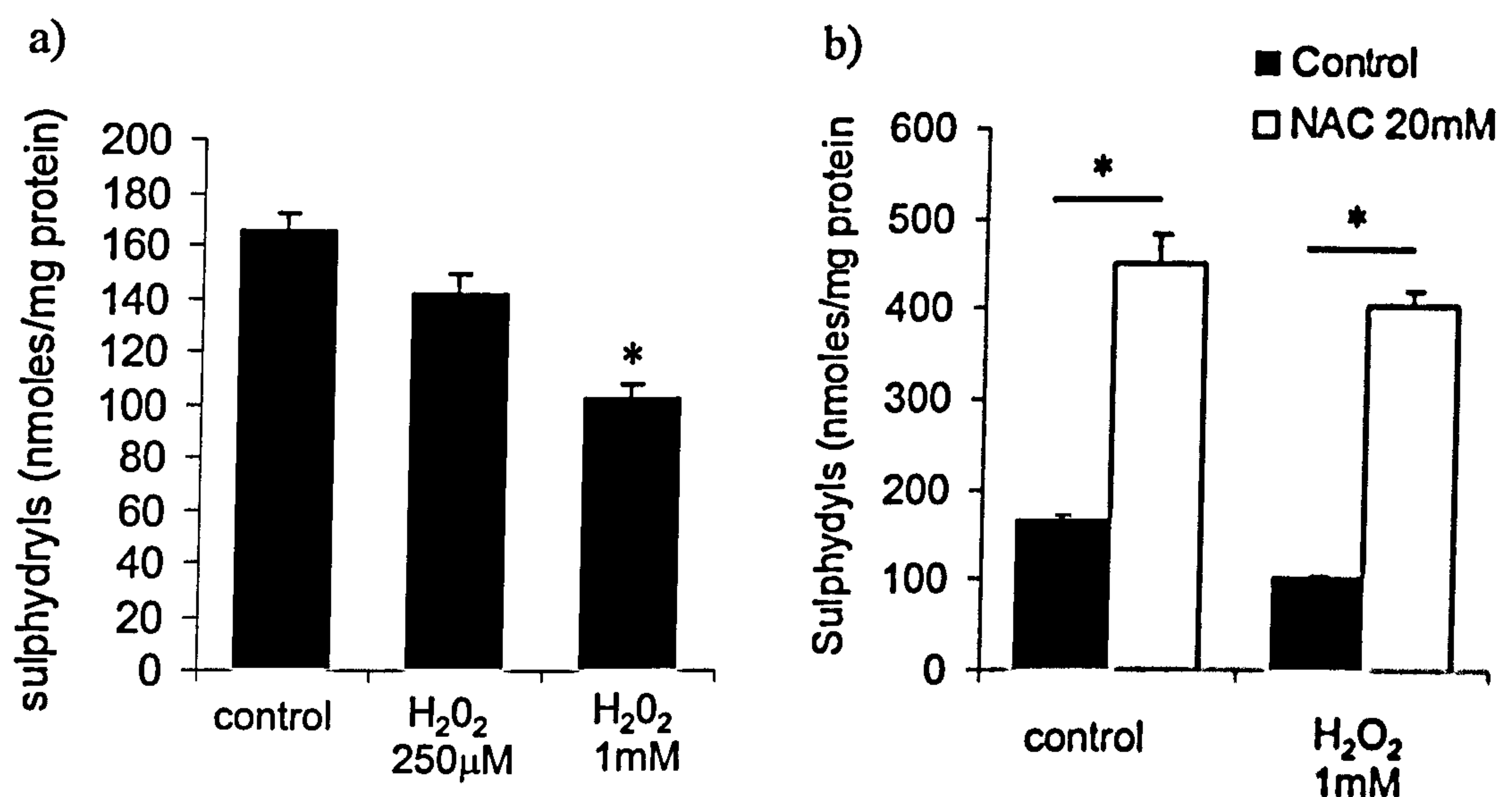


Figure 6.14. Effect of H_2O_2 and NAC on intracellular acid soluble thiols in vascular SMC. a) Effect of 30 minutes H_2O_2 treatment. b) Effect of 30 minutes pre-treatment with NAC (20mM) on control sulphydryl levels and those following 30 minutes H_2O_2 (1mM) treatment. * Denotes $p<0.05$ from control.

6.6.4 EFFECT OF IL-1 β AND TNF α ON INTRACELLULAR OXIDATION.

6.6.4.1 Oxidation of DCFH

In contrast to the effect of H_2O_2 on DCF fluorescence neither IL-1 β nor TNF α , at concentrations shown to induce NF κ B and p38 MAPK activation demonstrated the ability to induce DCFH oxidation over a 30 minute time course (Figure 6.15, Table 6.2).

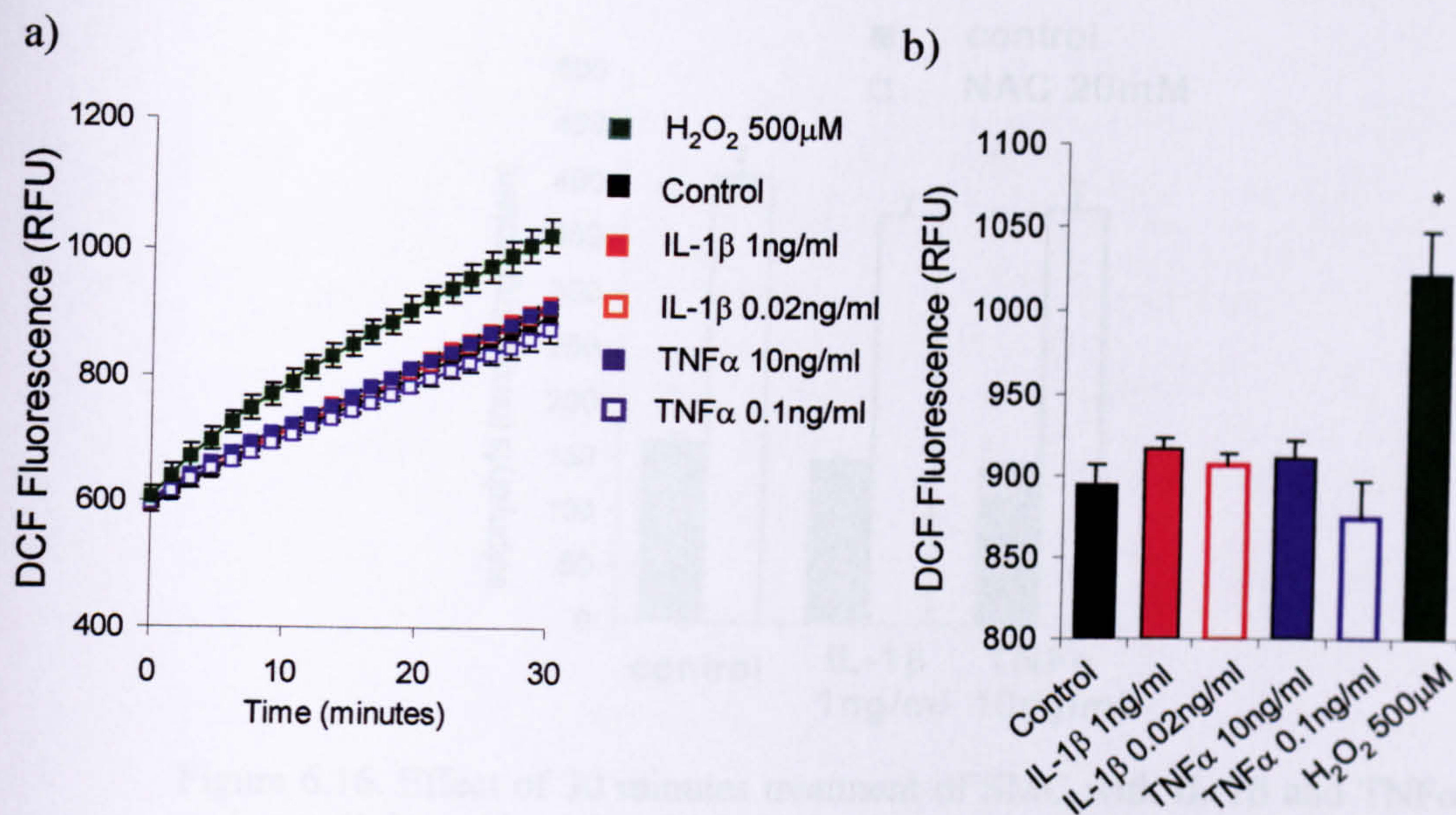


Figure 6.15. Effect of IL-1 β and TNF α on DCF fluorescence in SMC. a) Time course over 30 minutes. b) Summary graph showing the fluorescence after 30 minutes of treatment. * Denotes $p < 0.05$ from time matched control cells.

TREATMENT	DCF Fluorescence (RFU) at 30 minutes		Significance from control
	Mean	SEM	
Control	894.5	11.5	
H ₂ O ₂ (50μM)	872.5	30.6	ns
H ₂ O ₂ (100μM)	921.0	14.1	ns
H ₂ O ₂ (250μM)	1021.3	26.3	$p < 0.001$
H ₂ O ₂ (500μM)	1386.3	9.7	$p < 0.001$
H ₂ O ₂ (1000μM)	1539.25	15.5	$p < 0.001$
IL-1 β 1ng/ml	916.5	7.2	ns
IL-1 β 0.02ng/ml	906.0	7.3	ns
TNF α 10ng/ml	910.5	11.5	ns
TNF α 0.1ng/ml	874.3	20.6	ns

Table 6.2 Fluorescence signal (relative fluorescence units) generated by the oxidation of intracellular DCFH following 30 minutes treatment of SMC.

6.6.4.2 Sulphydryl concentration

The concentration of intracellular acid soluble thiols in cultured human aortic SMC was 165.4 ± 6.7 nmoles/mg protein. Both IL-1 β (1ng/ml) and TNF α (10ng/ml) failed to alter the concentration of intracellular acid soluble thiols in these cells over the time course used to induce I κ B α depletion (148.4 ± 4.3 and 143.3 ± 7.5 nmoles/mg protein, respectively, $n=4$, Figure 6.16).

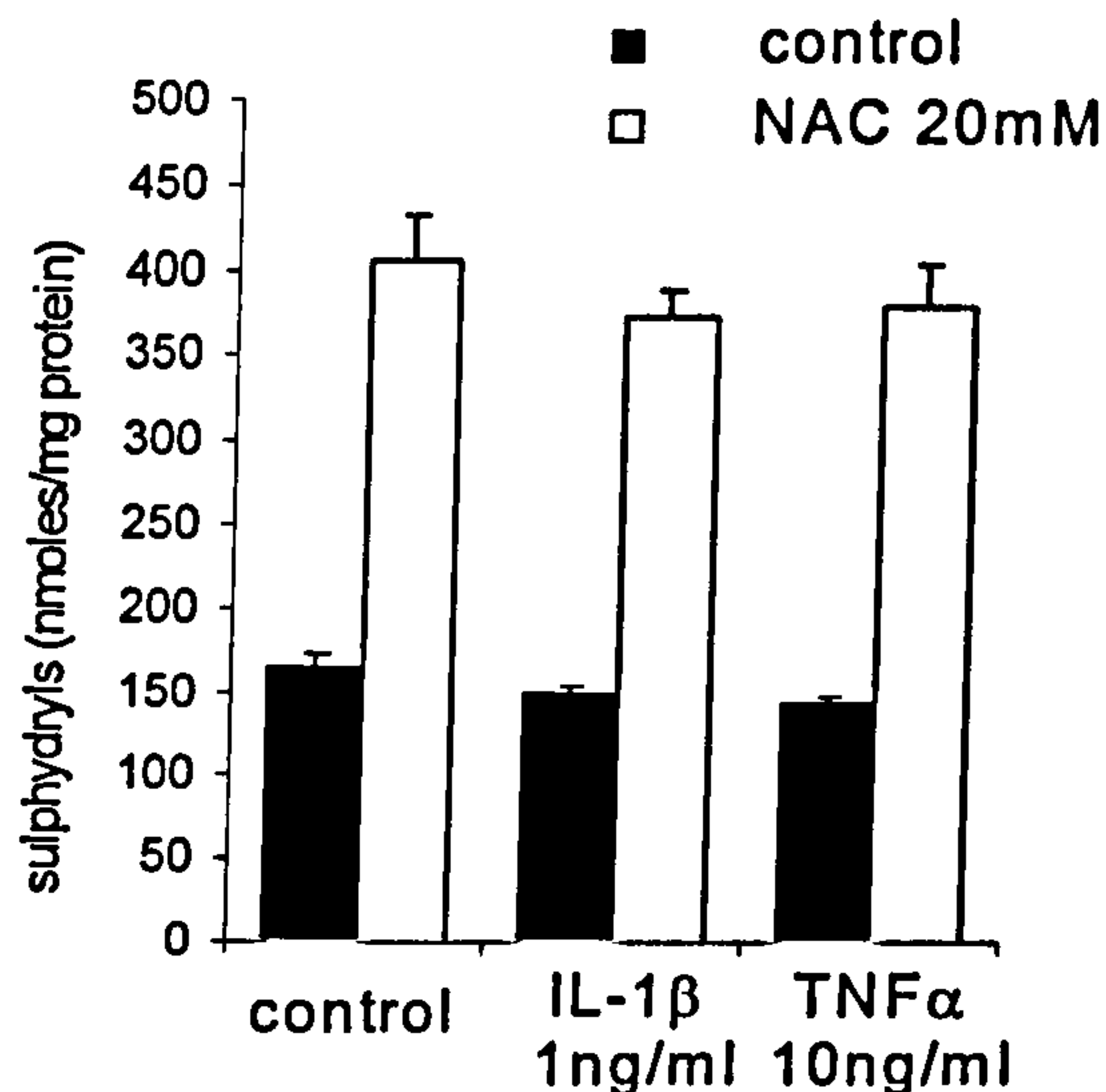


Figure 6.16. Effect of 30 minutes treatment of SMC with IL-1 β and TNF α on intracellular acid soluble thiols in the presence and absence of NAC.

6.6.5 INFLUENCE OF ANTI-OXIDANTS ON CYTOKINE-INDUCED NF κ B ACTIVATION

6.6.5.1 I κ B α depletion

Western blot analysis of total SMC lysates demonstrated that NAC had no significant effect on the kinetics of I κ B α depletion following pro-inflammatory cytokine treatment.

After 30 minutes of IL-1 β (1ng/ml) treatment I κ B α levels in NAC treated cells were 12.9 ± 3.9 % of that in resting cells compared to 19.8 ± 5.1 % in control treated cells, $n=10$ $p>0.05$, Figure 6.17a and c). Similarly, following 30 minutes TNF α (10ng/ml) treatment I κ B α levels were 18.1 ± 5.8 % of that in resting cells compared to 20.8 ± 5.8 % in cells pre-treated with NAC ($n=4$, $p>0.05$, Figure 6.17b and d).

6.6.5.2 I κ B α resynthesis

NAC had no influence on I κ B α resynthesis in human aortic SMC following depletion induced by IL-1 β or TNF α . Treatment of SMC for 90 minutes with IL-1 β (1ng/ml) in the presence of 20mM NAC resulted in I κ B α levels increasing to 58.2 ± 14.2 % of that in resting cells compared to 68.9 ± 10.4 % in cells treated with IL-1 β alone ($n=6$ $p>0.05$, Figure 6.17a and c). Similarly, following TNF α (10ng/ml)-induced depletion, I κ B α protein levels increased to 48.3 ± 9.3 % and 42.2 ± 16.7 % of that in resting cells following 120 minutes treatment in the presence and absence of NAC (20mM), respectively ($n=4$, $p>0.05$, Figure 6.17b and d).

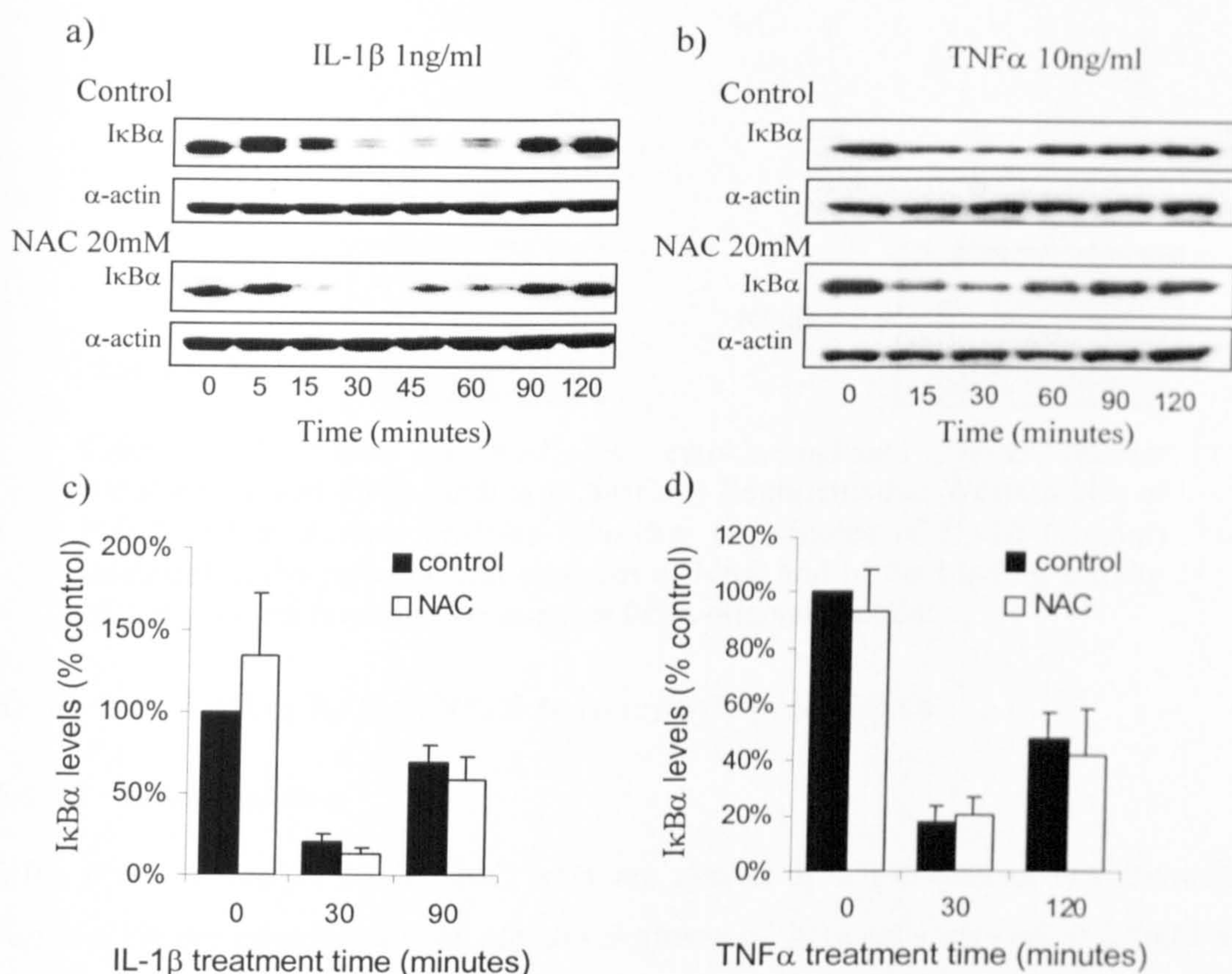


Figure 6.17. Effect of NAC on cytokine-induced I κ B α depletion and resynthesis in vascular SMC. Representative Western blots of the time course of changes in the cellular levels of I κ B α protein following treatment IL-1 β 1ng/ml (a) and TNF α 10ng/ml (b) in the presence and absence of NAC (20mM). Summary graphs showing I κ B α levels at 0, 30 and 90 minutes treatment with IL-1 β 1ng/ml (c) and TNF α 10ng/ml (d) in the presence and absence of NAC (20mM).

6.6.5.3 NF κ B p65 nuclear translocation

Nuclear levels of NF κ B p65 protein in human aortic SMC increased $850.5 \pm 231.8\%$ above control levels following 60 minutes IL-1 β (1ng/ml) treatment. The presence of NAC (20mM) in the culture medium during cytokine treatment did not alter the level of NF κ B p65 protein in the cell nuclei ($1943.4 \pm 1002.2\%$ of control levels, $n=7$, $p=0.309$, Figure 6.18a).

6.6.5.4 NF κ B /DNA binding

EMSA demonstrated that the binding of nuclear protein from IL-1 β treated human aortic SMC to an NF κ B specific DNA sequence was not altered by pre-treatment of the cells with NAC (20mM, Figure 6.18a).

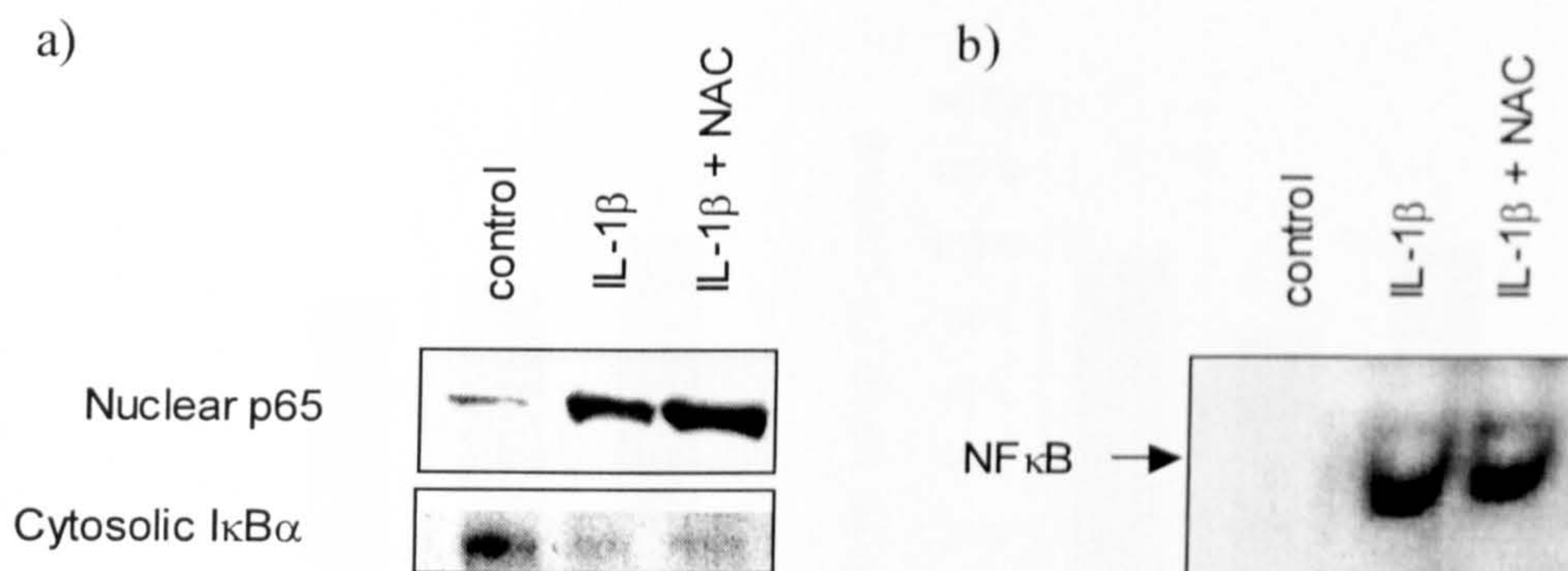


Figure 6.18. Effect of NAC on cytokine-induced NFκB nuclear translocation and DNA binding in SMC. a) Representative Western blot of NFκB p65 in nuclear proteins following 60 minutes of IL-1β (1ng/ml) treatment in the presence and absences of NAC and b) the binding activity of these nuclear proteins to consensus DNA oligonucleotide.

6.6.6 INFLUENCE OF H₂O₂ ON NFκB ACTIVITY IN RESTING CELLS

6.6.6.1 IκBα depletion

IκBα levels in human aortic SMC were not altered by a pro-oxidant environment. Figure 6.19a demonstrates that 60 minutes treatment of these cells with either 0.1mM or 1mM H₂O₂ failed to cause any notable reduction in IκBα protein levels (104.2 ± 7.3 and 120.3 ± 27.7 % of that in resting cells respectively, n=3). In addition, neither 30 nor 60 minutes treatment with 10mM H₂O₂ appeared to alter IκBα levels from that observed under resting conditions. The protein synthesis inhibitor, cycloheximide was present throughout these experiments to prevent resynthesis of IκBα and eliminate potential masking of IκBα depletion (Figure 6.19).

6.6.6.2 NFκB/IκBα association

Immunoprecipitating NFκB p65 from H₂O₂ (10mM) treated aortic SMC, and immunoblotting the resulting protein samples to determine the amount of IκBα co-precipitated revealed that the p65 subunit of NFκB remained complexed to IκBα for the duration of H₂O₂ exposure (Figure 6.19c).

6.6.6.3 NFκB p65 nuclear translocation

In addition, EMSA failed to detect any NFκB DNA binding activity in nuclear proteins isolated from H₂O₂ treated cells.

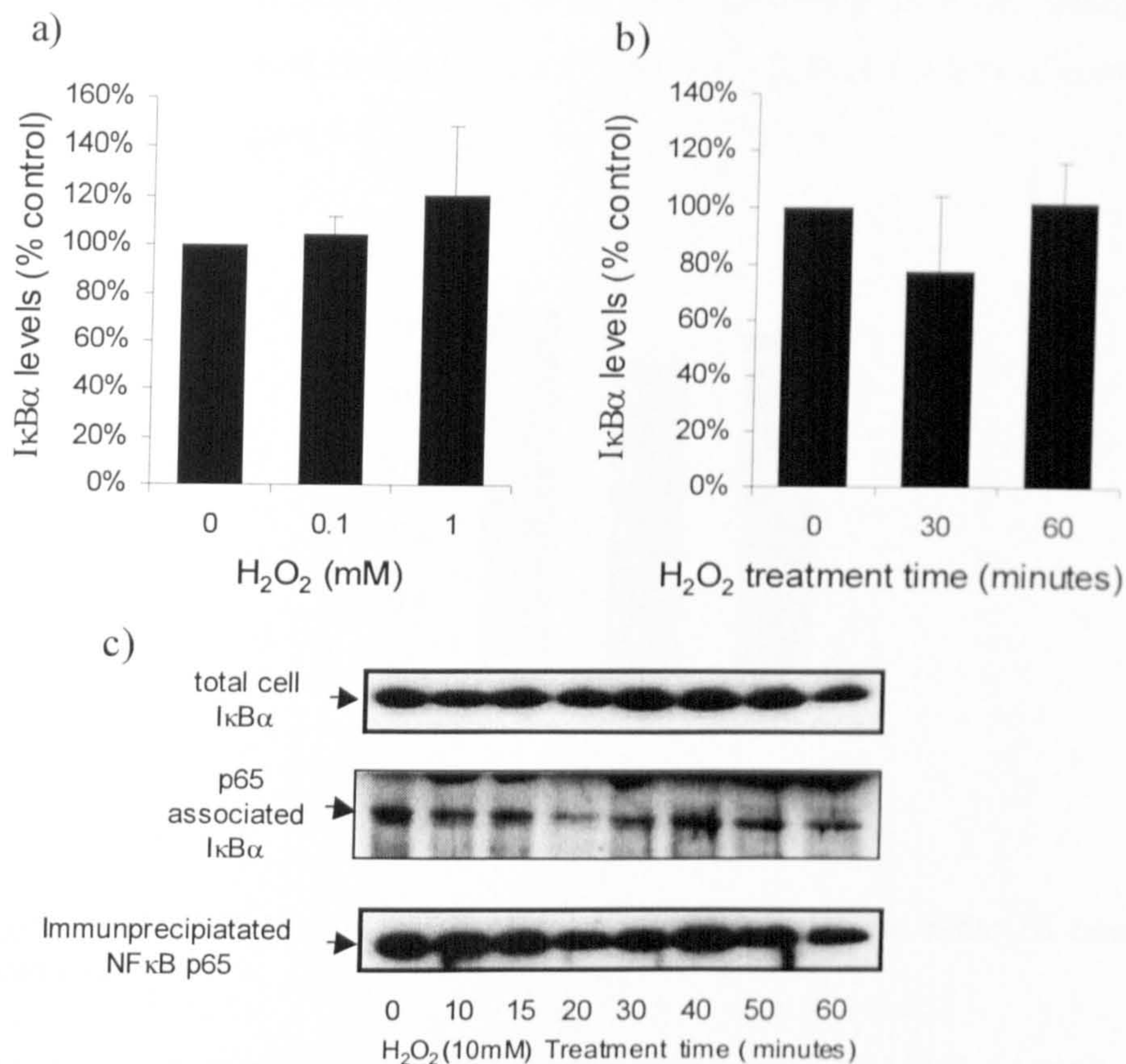


Figure 6.19. Effect of H₂O₂ on SMC IκBα . a) Summary graph of the cellular levels of IκBα protein in SMC following 60 minutes treatment with increasing concentrations of H₂O₂. b) IκBα protein levels following 30 and 60 minutes treatment of cells with 10mM H₂O₂. c) Representative Western blot of IκBα protein co-precipitated with NFκB p65 following treatment of cells with H₂O₂ over a 60 minute time course.

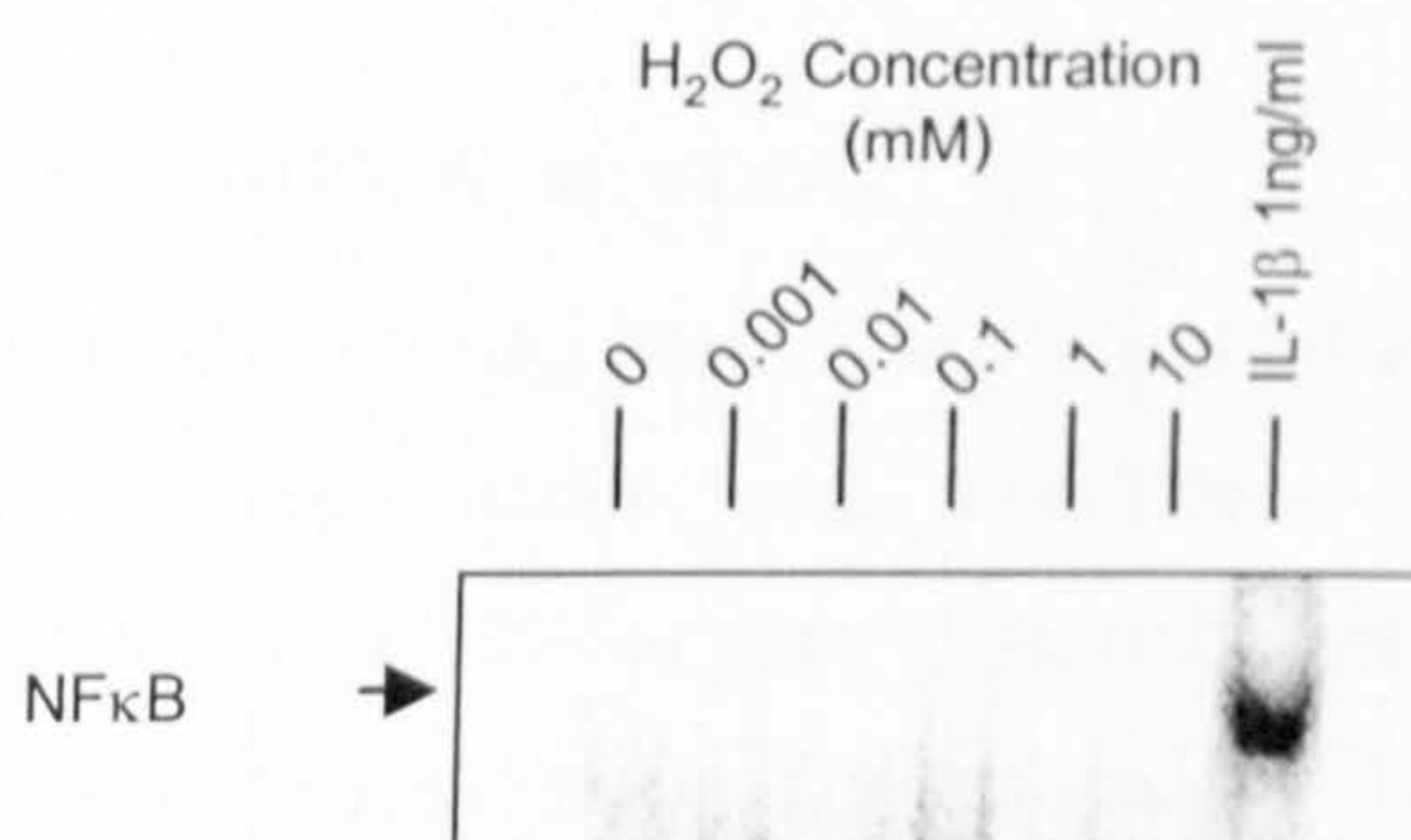


Figure 6.20. NFκB DNA binding activity of nuclear proteins isolated from SMC following 60 minutes treatment with H₂O₂.

6.6.6.4 ICAM-1 expression

Due to the toxic effects of H₂O₂ (section 6.6.1) the effect of pro-oxidant conditions on ICAM-1 expression on SMCs was limited to H₂O₂ concentrations of 250μM and lower.

ICAM-1 expression remained unchanged on SMC following 18 hours treatment with 50 μ M, 100 μ M and 250 μ M H₂O₂ (105.8 $\pm$ 7.7, 91.9 $\pm$ 4.5, 99.4 $\pm$ 4.5 % of control levels respectively, n=3-4, Figure 6.21).

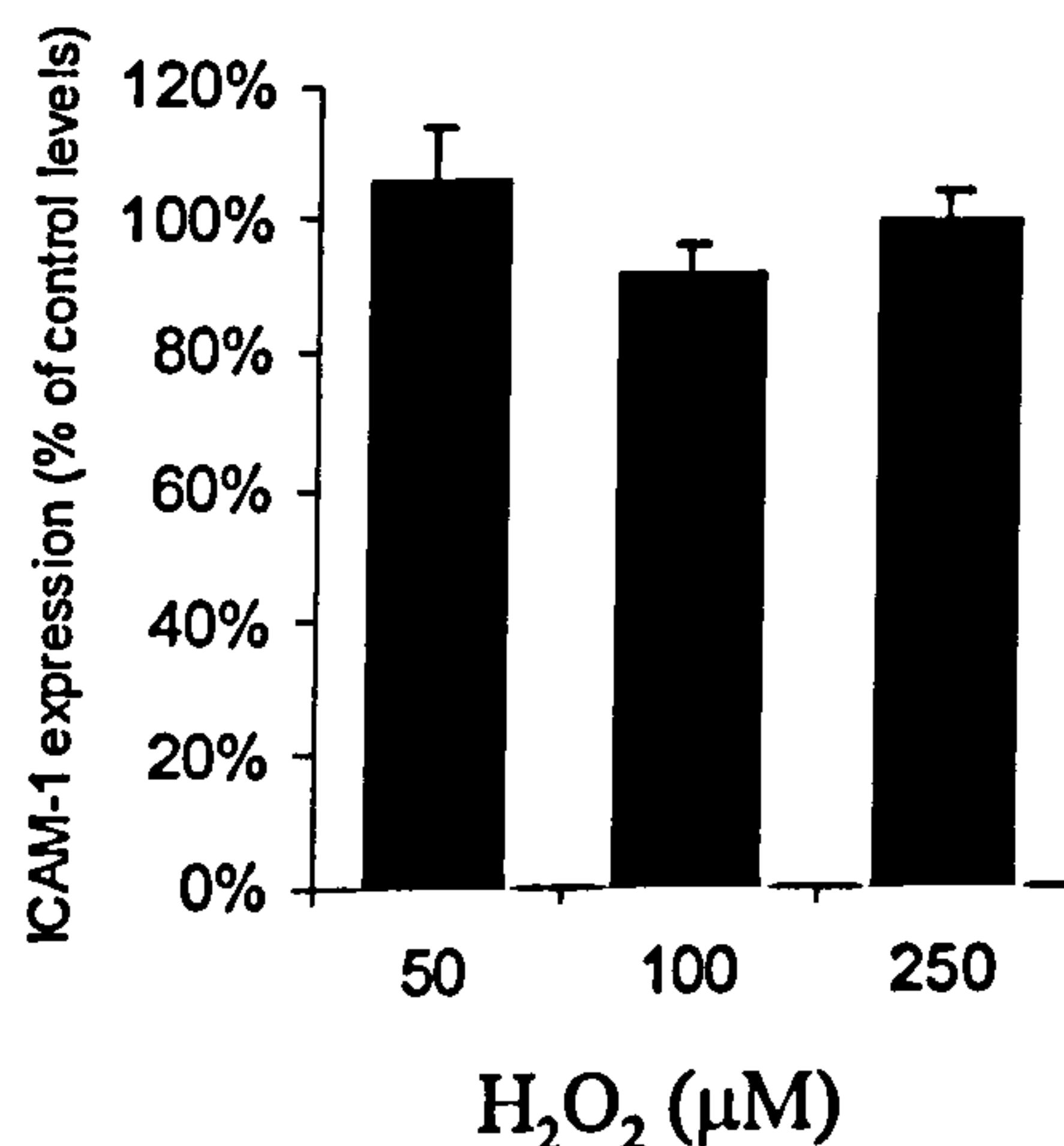


Figure 6.21. Effect of H₂O₂ on SMC ICAM-1 expression after 18 hours treatment.

6.6.7 EFFECT OF ANTI-OXIDANTS ON CYTOKINE-INDUCED p38 MAPK ACTIVATION

Western blot analysis of phosphorylated p38 demonstrated that p38 activation induced by 30 minutes treatment with either 1ng/ml IL-1 β (507.9 $\pm$ 146.4% of control levels, n=5) or 10ng/ml TNF α (469.1 $\pm$ 71.3% of control levels, n=4) were not altered by pre-treatment with 20mM NAC (631.9 $\pm$ 383.9 and 517.3 $\pm$ 115.3 % of control levels, respectively, Figure 6.22).

6.6.8 EFFECT OF H₂O₂ ON p38 MAPK ACTIVATION

Despite the lack of effect of H₂O₂ on I κ B α protein levels and NF κ B activity in human SMC, p38 MAPK was activated under such conditions. Cellular levels of phosphorylated p38 were 1406.8 $\pm$ 711.8% of that observed under resting conditions following 15 minutes treatment with 1mM H₂O₂. The anti-oxidant NAC (20mM) had no effect on basal levels of phosphorylated p38 (130.0 $\pm$ 57.9 % of control levels) however, pre-treatment of the cells with NAC completely abolished p38 phosphorylation induced by 1mM H₂O₂ (77.1 $\pm$ 39.2% of control levels, p<0.05, n=5, Figure 6.23)

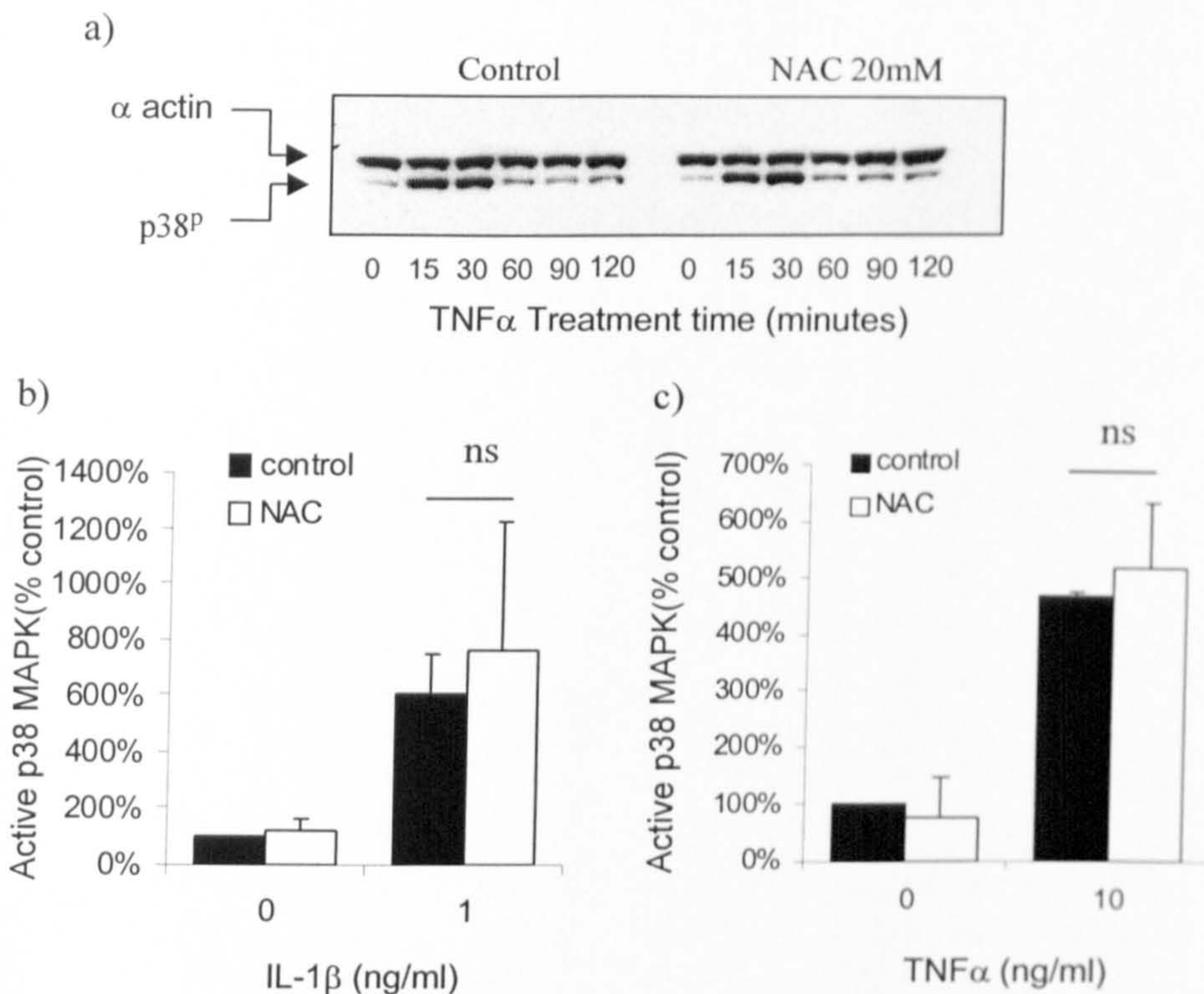


Figure 6.22. Effect of NAC on cytokine-induced p38 MAPK activation in human SMC. a) Representative Western blot demonstrating the time course of p38 phosphorylation induced by TNFα 10ng/ml in the presence and absence of NAC (20mM). Summary graph of the effects of NAC (20mM) on p38 phosphorylation induced by 30 minutes treatment with b) IL-1β (1ng/ml) or c) TNFα (10ng/ml).

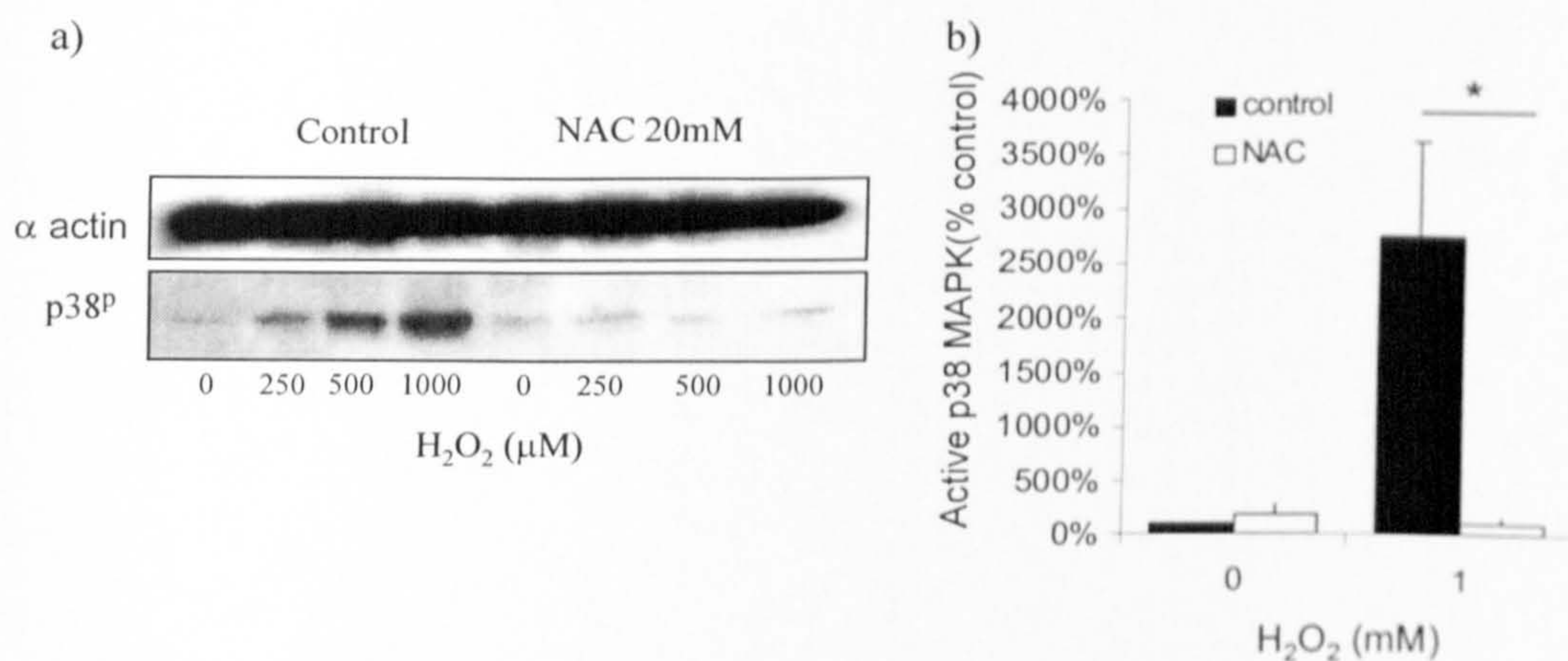


Figure 6.23. Effect of H₂O₂ on p38 MAPK activation in human vascular SMC. a) Representative Western blot of phosphorylated p38 following 15 minutes treatment with increasing concentration of H₂O₂ in the presence and absence of 20mM NAC. Summary graph of the effects of NAC on p38 MAPK phosphorylation induced by 15 minutes treatment with 1mM H₂O₂.

6.7 RESULTS 3: PULMONARY EPITHELIAL CELLS

6.7.1 EFFECT OF H₂O₂ ON A549 CELL VIABILITY

Analysis of A549 cell viability using the MTT assay revealed that high concentrations of H₂O₂ (1mM) over 18 hour period caused a small but significant amount of cell death ($78.7 \pm 2.3\%$ of control, $p < 0.05$, $n=3$). Lower concentration of H₂O₂ (50 μ M and 250 μ M) did not significantly affect cell viability over the 18 hour time period (94.7 ± 4.5 and $85.0 \pm 5.5\%$ of control respectively, $p > 0.05$, $n=3$, Figure 6.24a). Over a 3 hour time period 1mM H₂O₂ had no significant effect on cell viability ($89.2 \pm 1.2\%$ of control, $p > 0.05$, $n=4$, Figure 6.24b).

6.7.2 EFFECT OF NAC ON A549 CELL VIABILITY

Following 18 hours treatment with NAC (20mM), A549 cell viability was significantly reduced to $24.9 \pm 4.4\%$ of control ($p < 0.001$, $n=6$, Figure 6.24c). 3 hours treatment with 20mM NAC had no effect on A549 cell viability ($106.3 \pm 3.4\%$ of control, $p < 0.05$, $n=3$, Figure 6.24d).

6.7.3 EFFECT OF H₂O₂ ON INTRACELLULAR OXIDATION

6.7.3.1 Oxidation of DCFH

Treatment of A549 cells with H₂O₂ resulted in a time- and concentration-dependent increase in fluorescence as a result of oxidation of DCFH to DCF. Treatment with 500, 1000 μ M H₂O₂ resulted in a significant increase in fluorescence above that of time matched, control levels as early as three minutes after addition to the cells (309.8 ± 12.0 , 379.8 ± 13.4 RFU respectively vs. 267.0 ± 5.8 RFU in control cells, $p < 0.05$, $n=4$). Fluorescence continued to increase in cells treated with 500 and 1000 μ M H₂O₂ over the 30 minute time course studied (Figure 6.25a).

Pre-treatment of cells with NAC for 30 minutes prior to the addition of H₂O₂ significantly reduced DCF fluorescent signal generated by H₂O₂ treatment. ($p < 0.05$, $n=4$, Figure 6.25b).

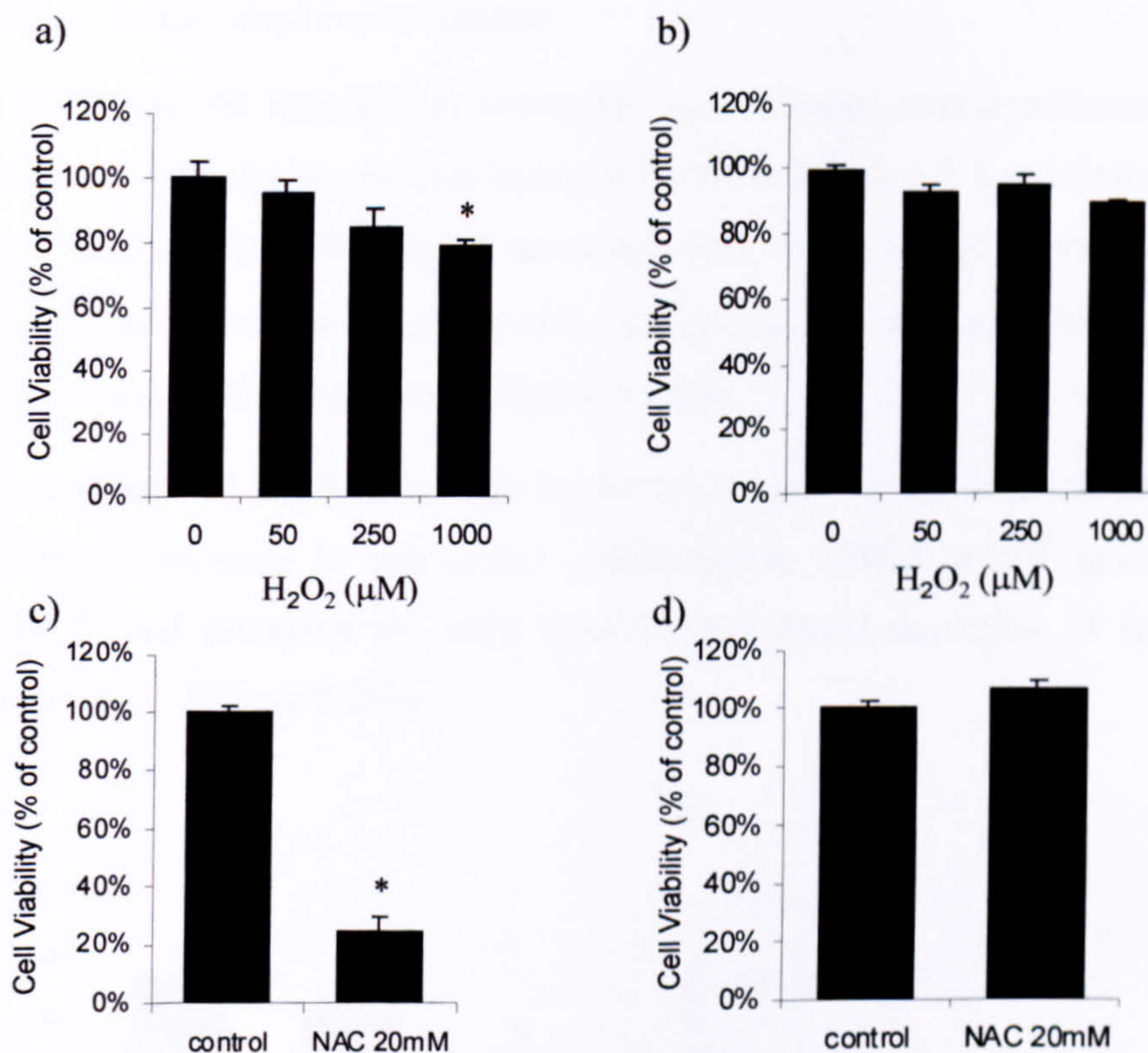


Figure 6.24. Effect of H_2O_2 and NAC on A549 cell viability. Cell viability following a) 18 hours and b) 3 hours treatment with increasing concentrations on H_2O_2 . c) 18 hours and d) 3 hours NAC treatment. *Denotes $p < 0.05$ from control cells.

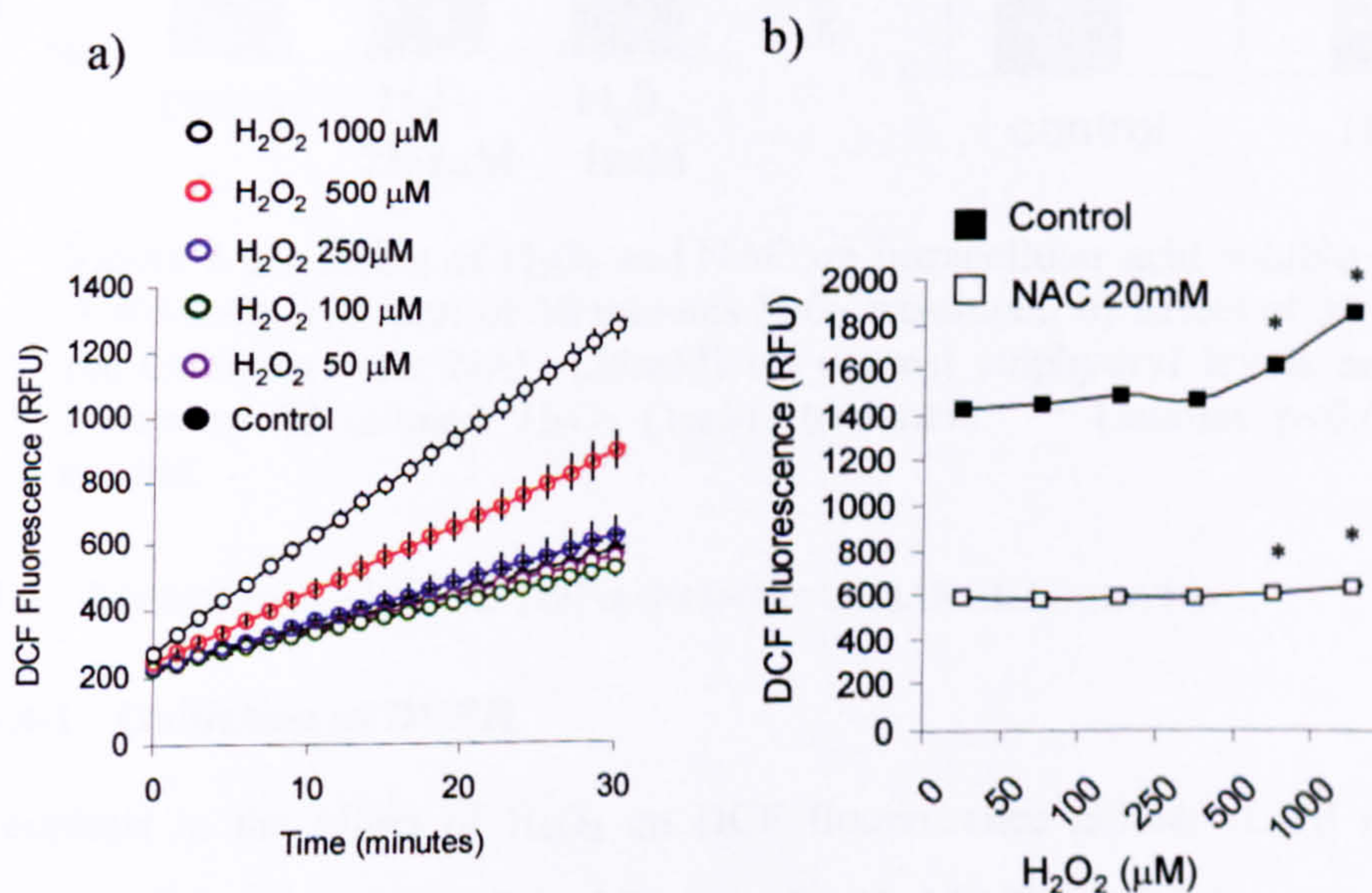


Figure 6.25. Effect of H_2O_2 and NAC on the fluorescence generated by oxidation of intracellular DCFH in A549 cells. a) Time course over 30 minutes. b) Effect of NAC (20mM) on H_2O_2 generated DCF fluorescence after 30 minutes. * denotes $p < 0.05$ from time matched control cells.

6.7.3.2 Sulphydryl concentration

In A549 cells the intracellular sulphydryl concentration was significantly reduced from 234.7 ± 8.9 nmoles/mg protein in control cells to 202.5 ± 5.3 nmoles/mg protein ($n=4$, $p<0.001$) following 30 minutes treatment with 1mM H_2O_2 . Lower concentrations of H_2O_2 (250 μ M) had no detectable effect on intracellular acid soluble thiols in these cells (224.1 ± 4.6 nmoles/mg protein, Figure 6.26a).

Pre treatment of A549 cells with 20mM NAC prior to the addition of H_2O_2 caused a significant increase in sulphydryl concentration (398.8 ± 25.0 nmoles/mg protein, $p<0.001$) and protected the cells from H_2O_2 -induced depletion of intracellular acid soluble thiols (Figure 6.26b).

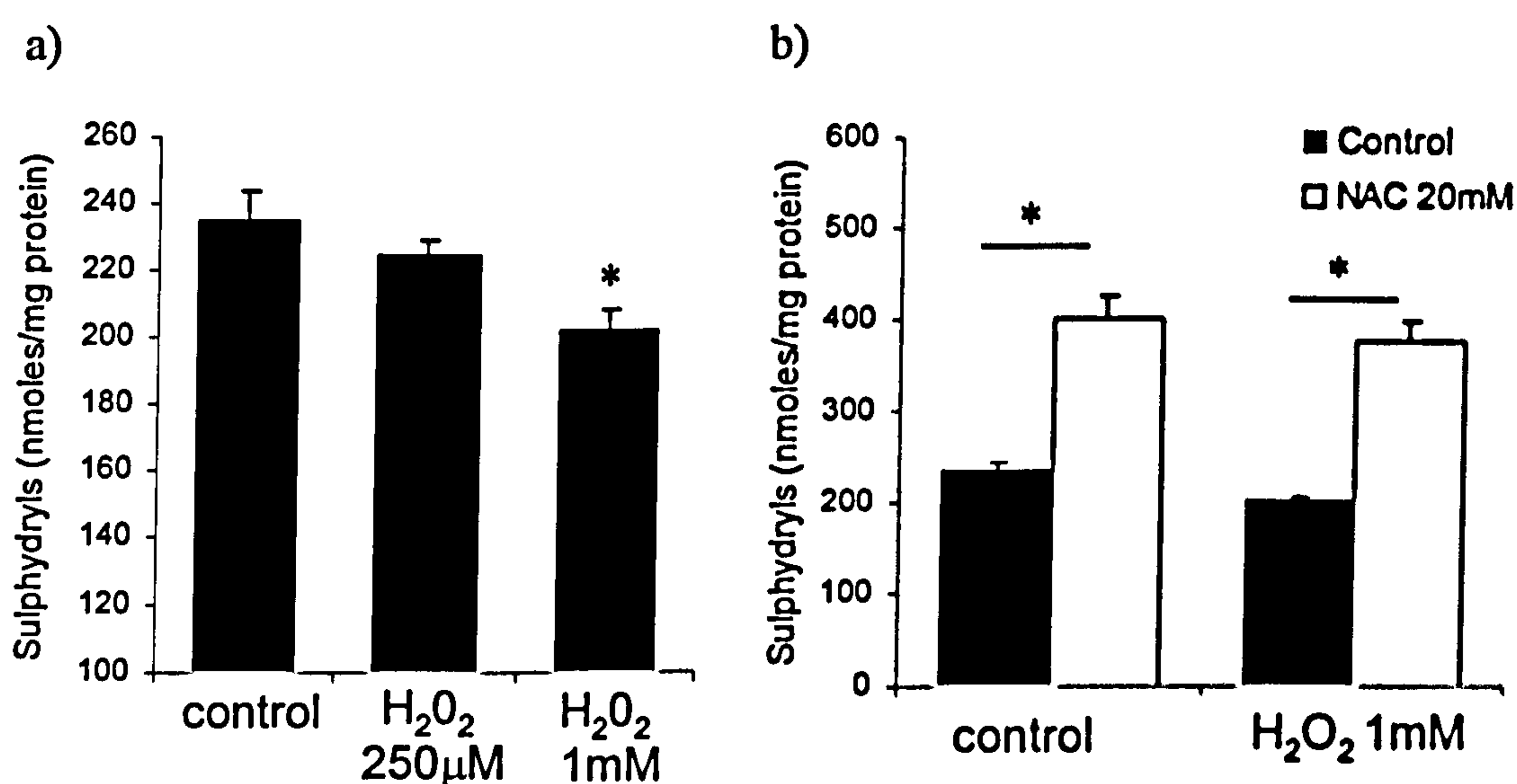


Figure 6.26. Effect of H_2O_2 and NAC on intracellular acid soluble thiols in A549 cells. a) Effect of 30 minutes H_2O_2 treatment. b) Effect of 30 minutes pre-treatment with NAC (20mM) on control sulphydryl levels and those following 30 minutes H_2O_2 (1mM) treatment. * Denotes $p<0.05$ from control.

6.7.4 EFFECT OF $IL-1\beta$ AND $TNF\alpha$ ON INTRACELLULAR OXIDATION.

6.7.4.1 Oxidation of DCFH

In contrast to the effect of H_2O_2 on DCF fluorescence neither $IL-1\beta$ nor $TNF\alpha$, at concentrations shown to induce $NF\kappa B$ and p38 MAPK activation demonstrated the ability to induce DCFH oxidation over a 30 minute time course ($n=4$, Figure 6.27, Table 6.3).

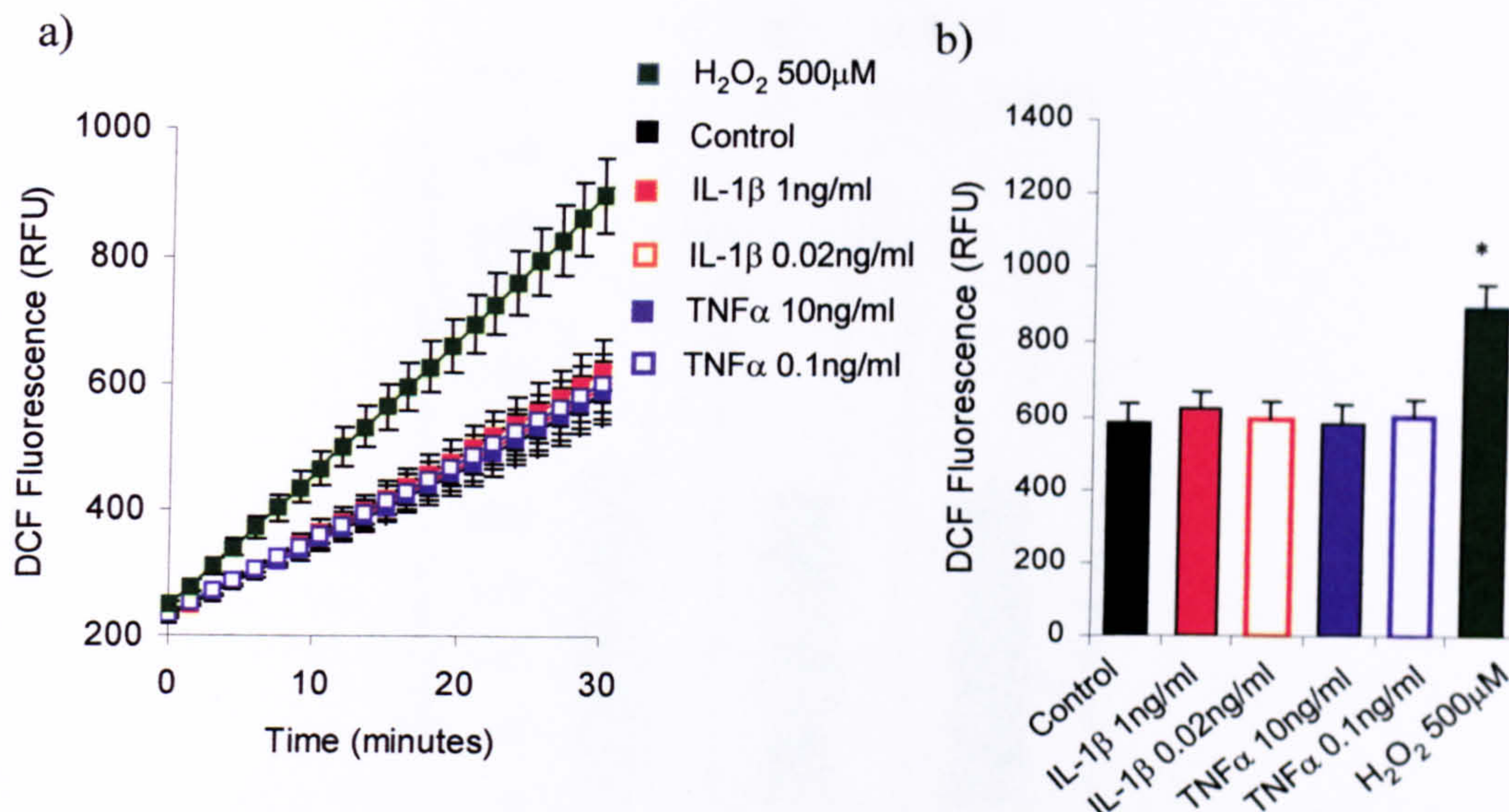


Figure 6.27. Effect of IL-1 β and TNF α on the fluorescence generated by oxidation of intracellular DCFH in A549 cells. a) Time course over 30 minutes. b) Summary graph showing the fluorescence after 30 minutes of treatment. * Denotes $p < 0.05$ from time matched control cells.

TREATMENT	DCF Fluorescence (RFU) at 30 minutes		Significance from control
	Mean	SEM	
Control	586.3	47.8	
H ₂ O ₂ (50μM)	569.0	45.4	ns
H ₂ O ₂ (100μM)	532.3	27.5	ns
H ₂ O ₂ (250μM)	634.8	56.8	ns
H ₂ O ₂ (500μM)	895.0	57.8	$p < 0.001$
H ₂ O ₂ (1000μM)	1276.0	38.6	$p < 0.001$
IL-1 β 1ng/ml	621.8	50.7	ns
IL-1 β 0.02ng/ml	596.3	48.8	ns
TNF α 10ng/ml	587.0	51.9	ns
TNF α 0.1ng/ml	605.5	45.9	ns

Table 6.3 Fluorescence signal (relative fluorescence units) generated by the oxidation of intracellular DCFH following 30 minutes treatment of A549.

6.7.4.2 Sulphydryl concentration

Both IL-1 β (1ng/ml) and TNF α (10ng/ml) failed to alter the concentration of intracellular acid soluble thiols in A549 cells over the time course used to induce I κ B α depletion (220.3 ± 6.2 and 224.0 ± 8.0 nmoles/mg protein, respectively, $n=4$) compared to control levels (234.7 ± 8.8 nmoles/mg protein, Figure 6.28).

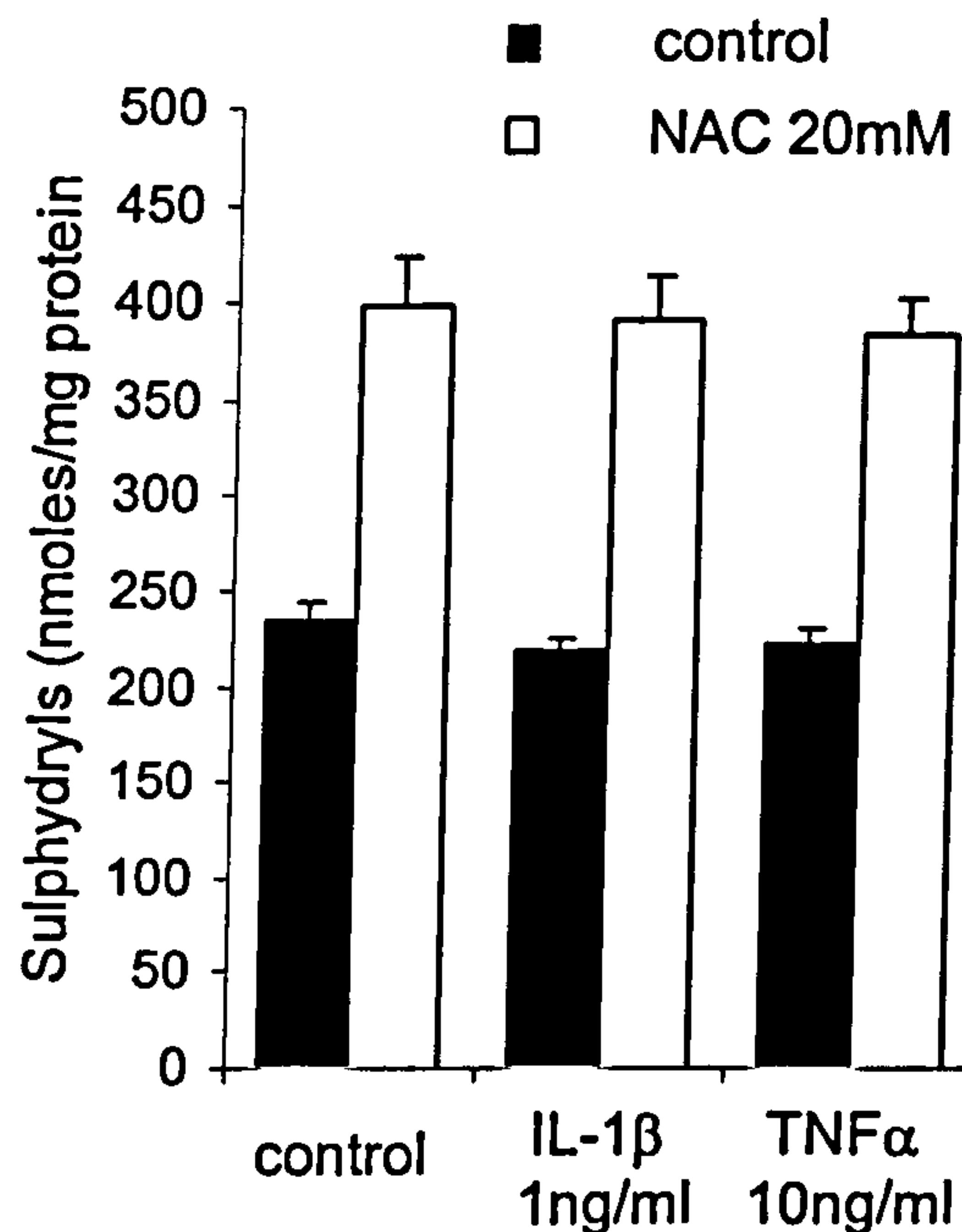


Figure 6.28. Effect of 30 minutes treatment of A549 cells with IL-1 β and TNF α on intracellular acid soluble thiols in the presence and absence of NAC.

6.7.5 INFLUENCE OF NAC ON CYTOKINE-INDUCED NF κ B ACTIVATION.

6.7.5.1 I κ B α depletion and resynthesis

In A549 cells there was no significant difference between the dose response effect of IL-1 β (0.2pg/ml – 1 ng/ml) with respects to I κ B α levels after 30 minutes treatment in either the presence or absence of NAC (20mM, pEC₅₀ 2.53 $\pm$ 0.66 ng/ml versus 1.83 $\pm$ 0.60 ng/ml, respectively p=0.472, n=3, Figure 6.29a). Similarly, TNF α (1pg/ml – 10ng/ml)-induced I κ B α depletion after 30 minutes treatment was unaffected by the presence of NAC (20mM, pEC₅₀ 1.12 $\pm$ 0.01ng/ml versus 1.11 $\pm$ 0.02 ng/ml in the absence of NAC, p=0.696, n=3, Figure 6.29b).

Furthermore this antioxidant failed to affect the resynthesis of I κ B α following IL-1 β or TNF α -induced depletion. Following 120 minutes treatment with IL-1 β (1ng/ml) I κ B α levels were 91.7 $\pm$ 24.5 % of control levels in the absence of NAC and 58.8 $\pm$ 9.6 % of control levels in the presence of NAC (p=0.246, n=5, Figure 6.29c and e). Following 120 minutes TNF α (10ng/ml) treatment I κ B α levels were 78.5 $\pm$ 22.7% of control levels in the absence of NAC and 58.3 $\pm$ 12.5 % of control levels in the presence of NAC (p=0.187, n=3, Figure 6.29d and e).

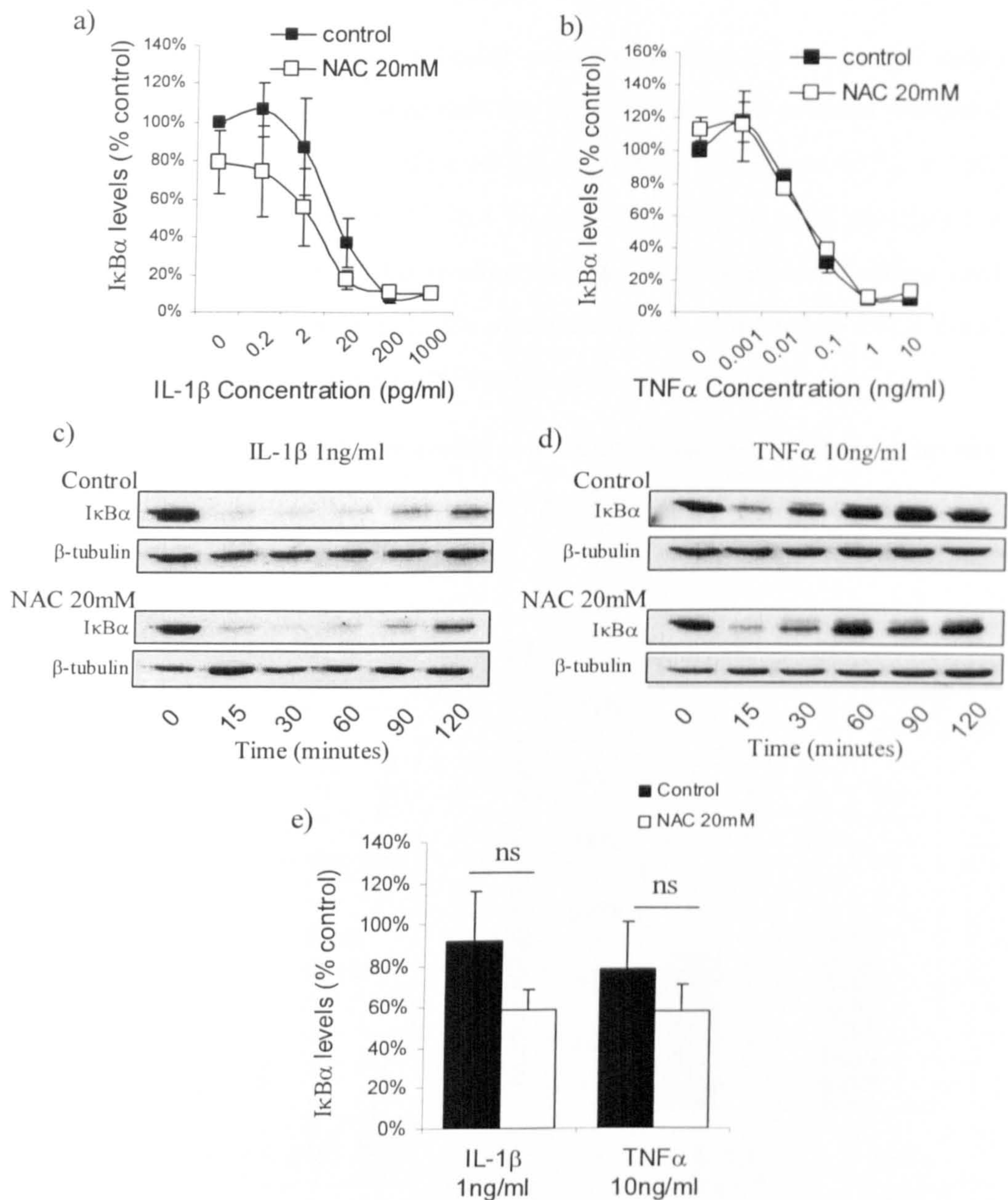


Figure 6.29. Effect of NAC (20mM) on IκBα depletion and resynthesis induced by IL-1β and TNFα in A549 cells. Summary graphs of the concentration related effects of 30 minutes treatment with IL-1β (a) and TNFα (b) on the cellular levels of IκBα protein in the presence (□) and absence (■) of NAC. Representative Western blots of the time course of IκBα protein depletion and resynthesis following treatment with IL-1β 1ng/ml (c) and TNFα 10ng/ml (d) in the presence and absence of NAC. e) Summary graph demonstrating the effect of NAC on newly synthesised IκBα protein levels following 120 minutes treatment with IL-1β 1ng/ml and TNFα 10ng/ml.

6.7.5.2 Nuclear translocation of NFκB p65

Pre-treatment of cells with NAC (20mM) prior to cytokine treatment also failed to significantly influence nuclear accumulation of NFκB p65. 60 minutes treatment of A549 cells with IL-1β caused nuclear NFκB p65 levels to increase $475.2 \pm 150.2\%$ (n=3) in control cells and $826.9 \pm 136.4 \%$ (p=0.158, n=3) in cells pre-treated with NAC. Similarly, 60 minutes TNFα treatment in the presence of NAC caused nuclear NFκB p65 to increase to $769.2 \pm 25.2\%$ of control levels compared to $547.2 \pm 83.2\%$ (p=0.063, n=3) in cells treated with TNFα alone (Figure 6.30a and b).

Limited experiments on primary epithelial cells indicate that NAC (20mM) also has no effect on IL-1β (2ng/ml) and TNFα (10ng/ml)-induced nuclear translocation of NFκB p65 in these cells (Figure 6.30c).

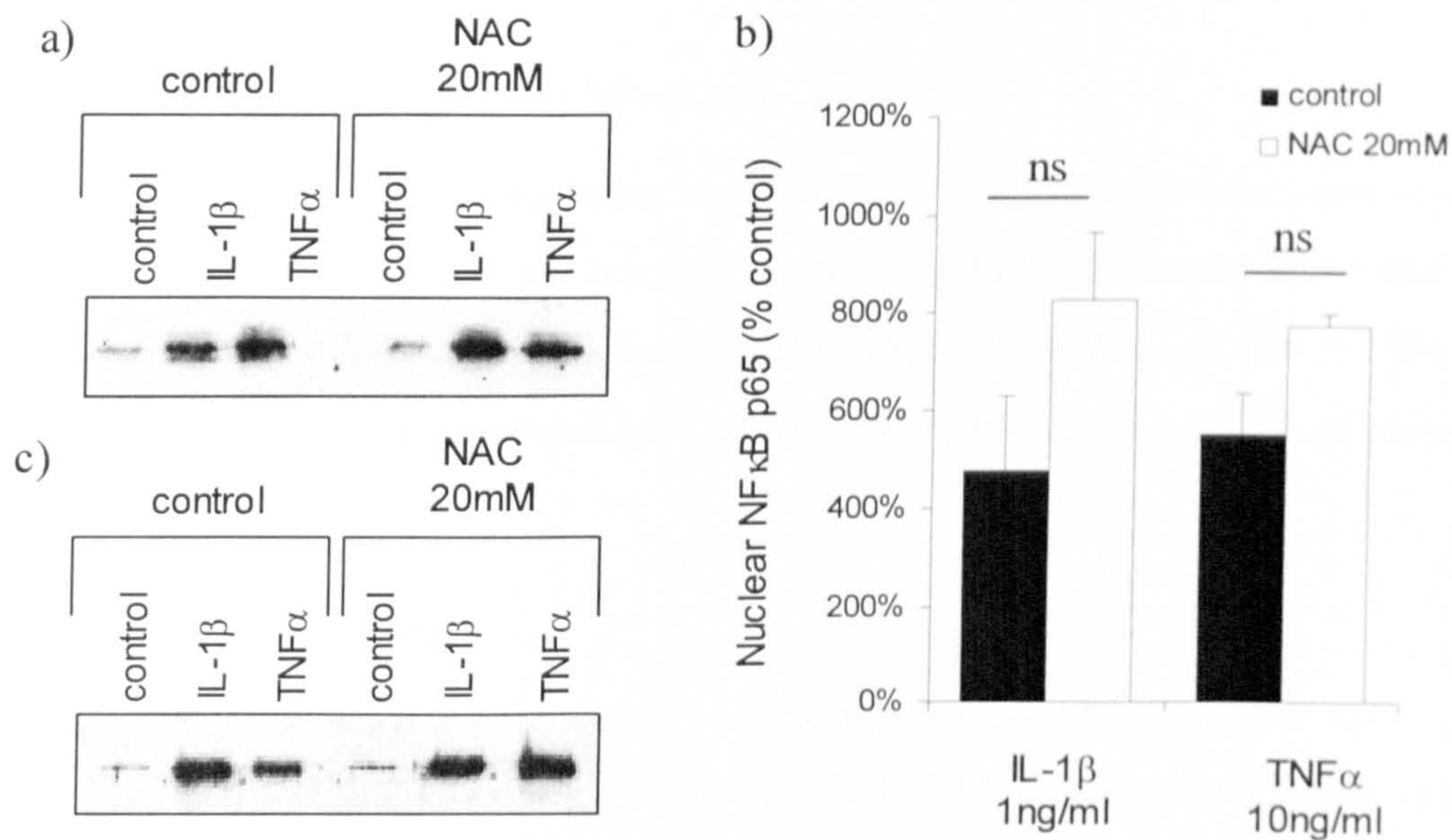


Figure 6.30. Effect of NAC (20mM) on IL-1β and TNFα-induced nuclear NFκB p65 protein levels in A549 cells. Representative Western blots (a) and Summary graph (b) showing NFκB p65 levels in nuclear protein extracts from A549 cells following 60 minutes treatment with IL-1β (1ng/ml) and TNFα (10ng/ml) in the presence and absence of NAC. c) Western blot of nuclear proteins from primary airway epithelial cells following 120 minutes treatment with IL-1β (2ng/ml) and TNFα (10ng/ml) in the presence and absence of NAC (20mM).

6.7.5.3 ICAM-1 expression

It was not possible to investigate the effect of NAC (20mM) on cytokine-induced ICAM-1 expression due to the toxic nature of NAC over the extended time course required to induce ICAM-1 on these cells (section 6.7.2).

6.7.6 EFFECT OF H₂O₂ ON NFκB IN RESTING CELLS

6.7.6.1 IκBα depletion

The effect of H₂O₂ on IκBα levels was tested in the presence of the protein synthesis inhibitor cycloheximide to prevent IκBα resynthesis that could potentially mask the depletion of this protein.

H₂O₂ (250μM) had no effect on IκBα levels in A549 cells or primary epithelial cells over the 120 minute time course studied (Figure 6.31a and b).

6.7.6.2 Nuclear translocation of NFκB p65

The inability of H₂O₂ to deplete IκBα was paralleled with downstream events in the activation pathway. There was no increase in nuclear NFκB p65 levels above that of control A549 cells following 60 minutes treatment with 250μM H₂O₂ ($146.3 \pm 7.0\%$ of control, n=4) or in primary epithelial cells after 120 minutes ($104.2 \pm 16.6\%$ of control, n=5).

6.7.6.3 ICAM-1 expression

Treatment of A549 cells for 18 hours with increasing concentrations of H₂O₂ (0-250μM) failed to induce a significant increase in ICAM-1 surface expression (13.7 ± 3.8 , 12.3 ± 3.9 , 13.6 ± 4.4 and $15.8 \pm 8.65\%$ positive cells for 0, 50, 100, 250μM H₂O₂ respectively, p=0.950, n=3, Figure 6.32a). A549 cells were treated for up to 24 hours with 250μM H₂O₂ and no change in the level of ICAM-1 expression was observed (Figure 6.32b).

In primary airway epithelial cells 24 hours treatment with H₂O₂ (250μM) had no significant effect on the surface expression of ICAM-1 ($10.4 \pm 2.4\%$ positive cells) compared to control levels ($8.5 \pm 1.7\%$, p=0.529, n=5, Figure 6.32c).

6.7.7 EFFECT OF H₂O₂ ON CYTOKINE-INDUCED I κ B α DEPLETION AND RESYNTHESIS

The profile of I κ B α -induced depletion and resynthesis induced by IL-1 β (1ng/ml) or TNF α (10ng/ml) over a 120 minute time period was not affected by the presence of H₂O₂ 250 μ M. A significant I κ B α depletion was seen within 15 minutes ($31.9 \pm 8.7\%$ and $20.2 \pm 8.7\%$ of control) in response to both IL-1 β and TNF α respectively in the presence of H₂O₂ and levels returned to that in resting cells by 120 minutes (Figure 6.33a and b).

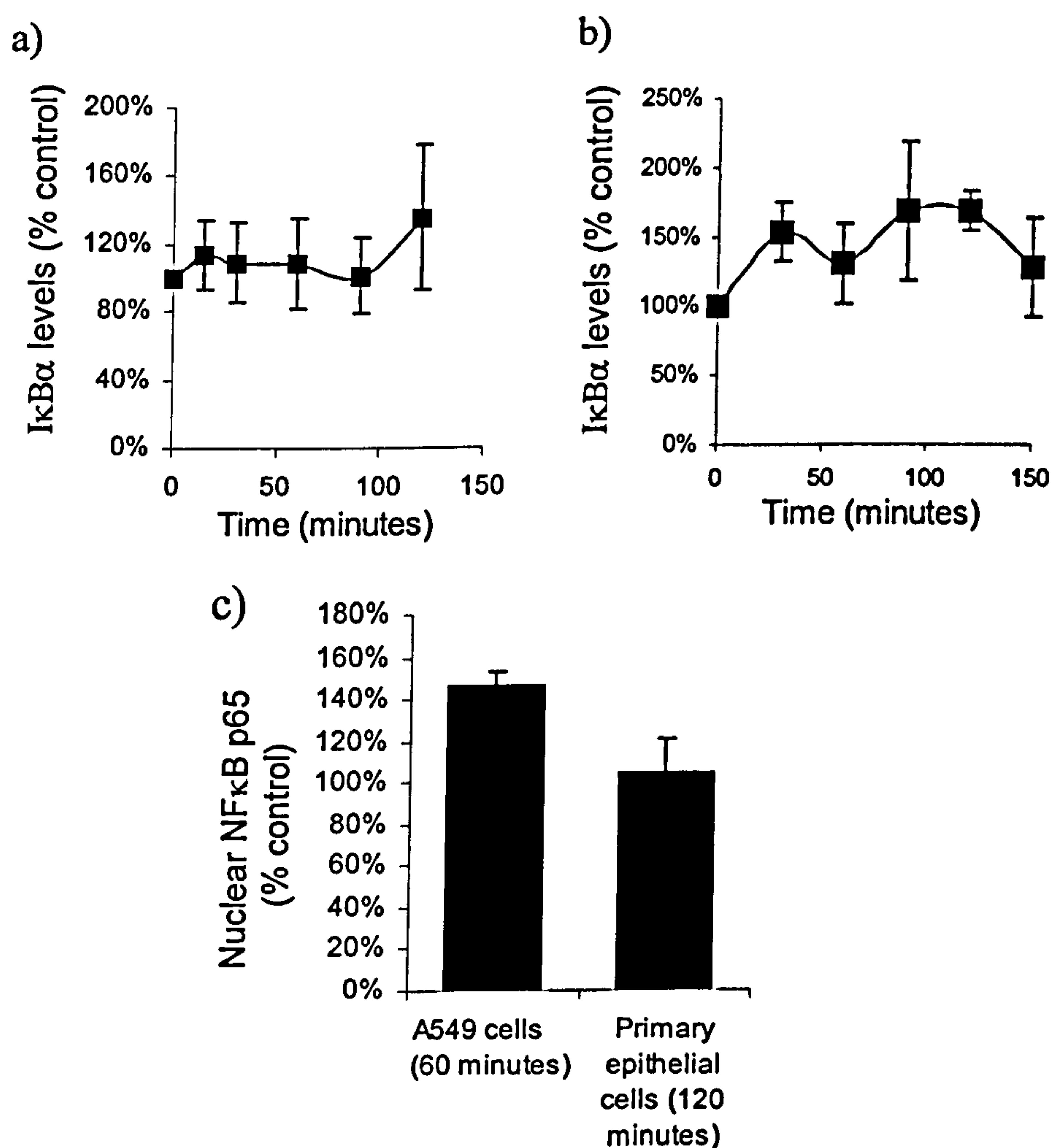


Figure 6.31. Effect of H₂O₂ on NF κ B activation in pulmonary epithelial cells. I κ B α levels following H₂O₂ treatment of A549 cells (a) and primary airway epithelial cells (b) over 120 minute time course. c) Nuclear expression of NF κ B p65 in A549 cells and primary airway epithelial cells following 60 and 120 minutes treatment with H₂O₂ (250 μ M).

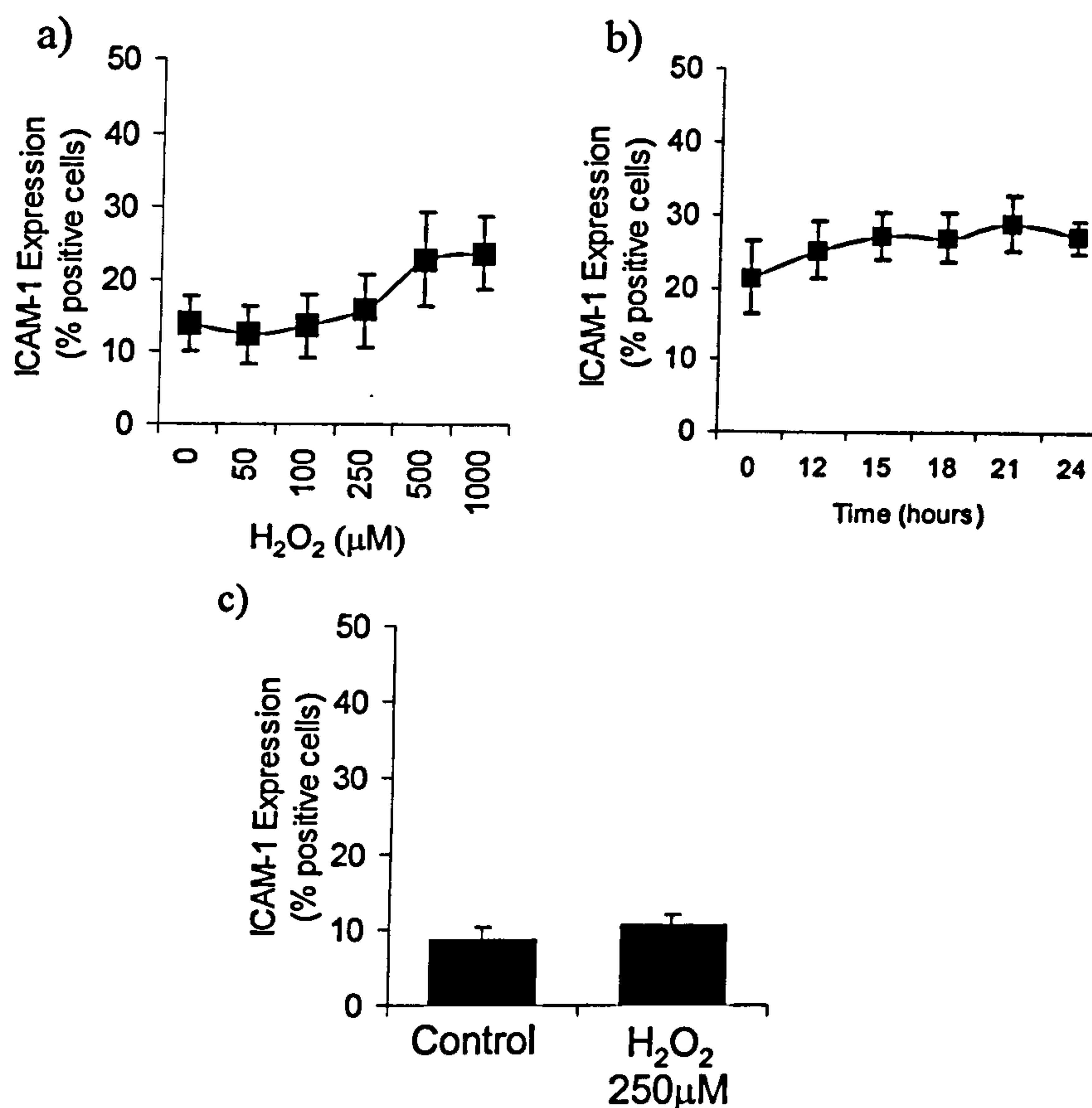


Figure 6.32. Effect of H₂O₂ on ICAM-1 expression in pulmonary epithelial cells. a) Surface expression of ICAM-1 on A549 cells following 18 hours treatment with increasing concentrations of H₂O₂. b) A549 cells ICAM-1 expression following treatment with H₂O₂ (250 μM) over a 24 hour time course. c) ICAM-1 expression on primary airway epithelial cells following 24 hours treatment with (250 μM) H₂O₂.

6.7.8 EFFECT OF H₂O₂ ON CYTOKINE-INDUCED ICAM-1 EXPRESSION

In A549 cells, neither IL-1β (0.002-1 ng/ml) nor TNFα (0.001 – 10 ng/ml)-induced ICAM-1 expression (pEC₅₀ 1.37 ± 0.08 ng/ml and 0.21 ± 0.37 ng/ml, respectively) were significantly altered by co-treatment with H₂O₂ (250 μM, pEC₅₀ 1.37 ± 3.8 ng/ml, p=0.609, n=3 and pEC₅₀ 1.08 ± 0.56 ng/ml, p=0.240, n=4, respectively, Figure 6.33c and d).

Similarly in primary epithelial cells, ICAM-1 expression induced by 24 hours treatment with IL-1β (2 ng/ml, 22.9 ± 6.3% ICAM-1 positive cells) or TNFα (10 ng/ml, 29.3 ± 9.6%) were not altered by co-treatment with H₂O₂ (250 μM, 16.7 ± 3.7%, p=0.427, n=4 and 19.0 ± 5.1%, p=0.379, n=4, respectively, Figure 6.33e).

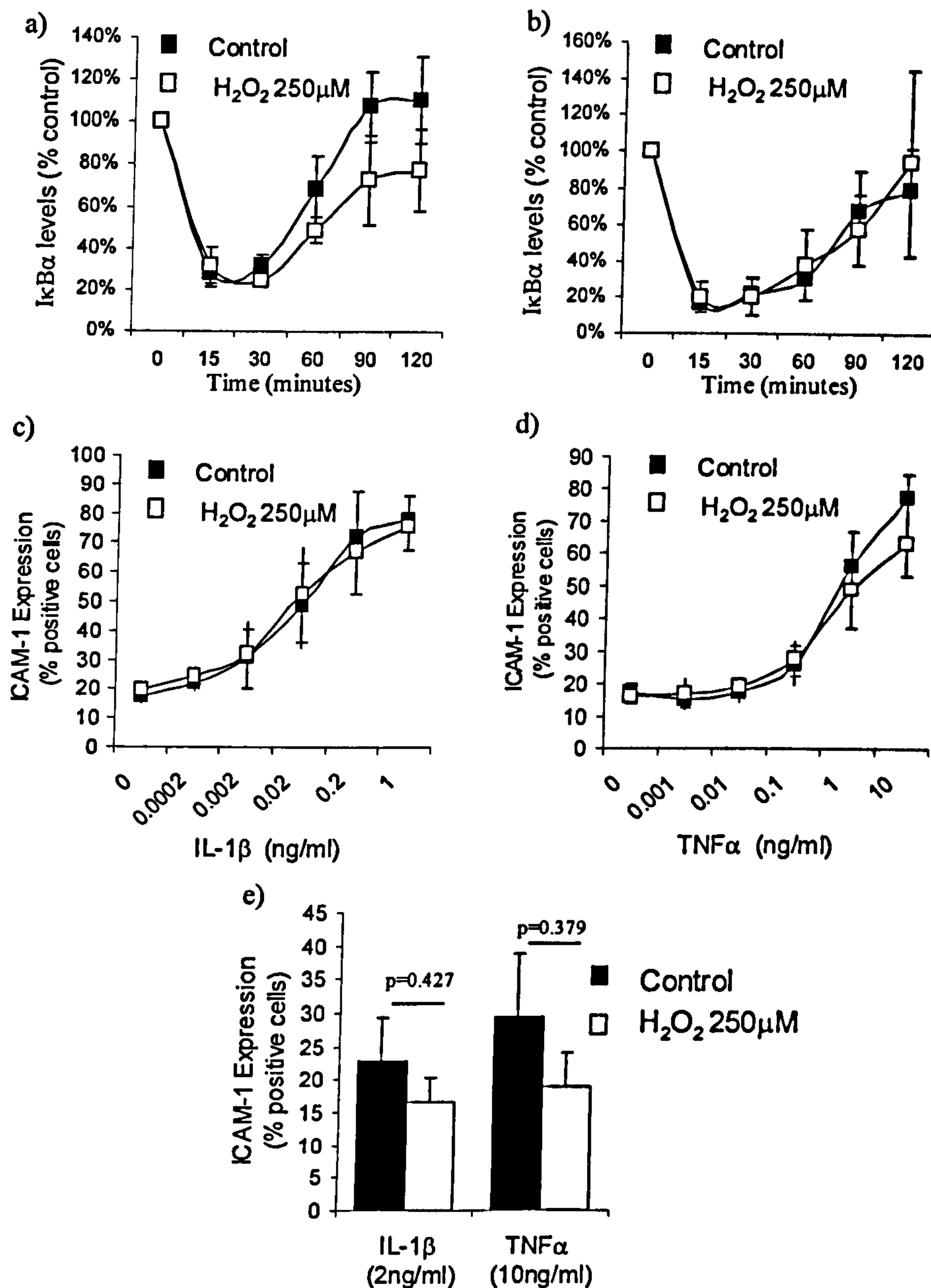


Figure 6.33. Effect of H₂O₂ on cytokines-induced NFκB activation. Time course of IκBα depletion and resynthesis induced by IL-1β 1ng/ml (a) and TNFα 10ng/ml (b) in A549 cells in the presence and absence of H₂O₂ (250μM). Dose related effects of 18 hours IL-1β (c) and TNFα (d) treatment on A549 cells ICAM-1 expression in the presence and absence of H₂O₂ (250μM). e) ICAM-1 expression on primary airway epithelial cells following 24 hours treatment with IL-1β (2ng/ml) and TNFα (10ng/ml) in the presence and absence of H₂O₂ (250μM).

6.7.9 EFFECT OF NAC ON CYTOKINE-INDUCED P38 MAPK ACTIVATION

IL-1β (1ng/ml)-induced phosphorylation of p38 MAPK peaked following 30 minutes treatment with levels increasing to $410.0 \pm 195.0\%$ of that found in control cells (Figure

6.34a). Pre-treatment with NAC (20mM) had no significant effect on the levels of p38 activation induced by 30 minutes treatment with IL-1 β (1ng/ml, $p=0.493$, $n=5$, Figure 6.34c). TNF α -induced activation of p38 MAPK peaked at 15 minutes ($289.4 \pm 60.0\%$ of control levels) before returning to control levels by 60 minutes (Figure 6.34b). Pre-treatment of the cells with NAC (20mM) caused a small decrease in the level of p38 phosphorylation induced by 15 minutes treatment with TNF α however this did not reach statistically significant levels (10ng/ml, 154.7 ± 17.5 , $p=0.128$, $n=3$, Figure 6.34d).

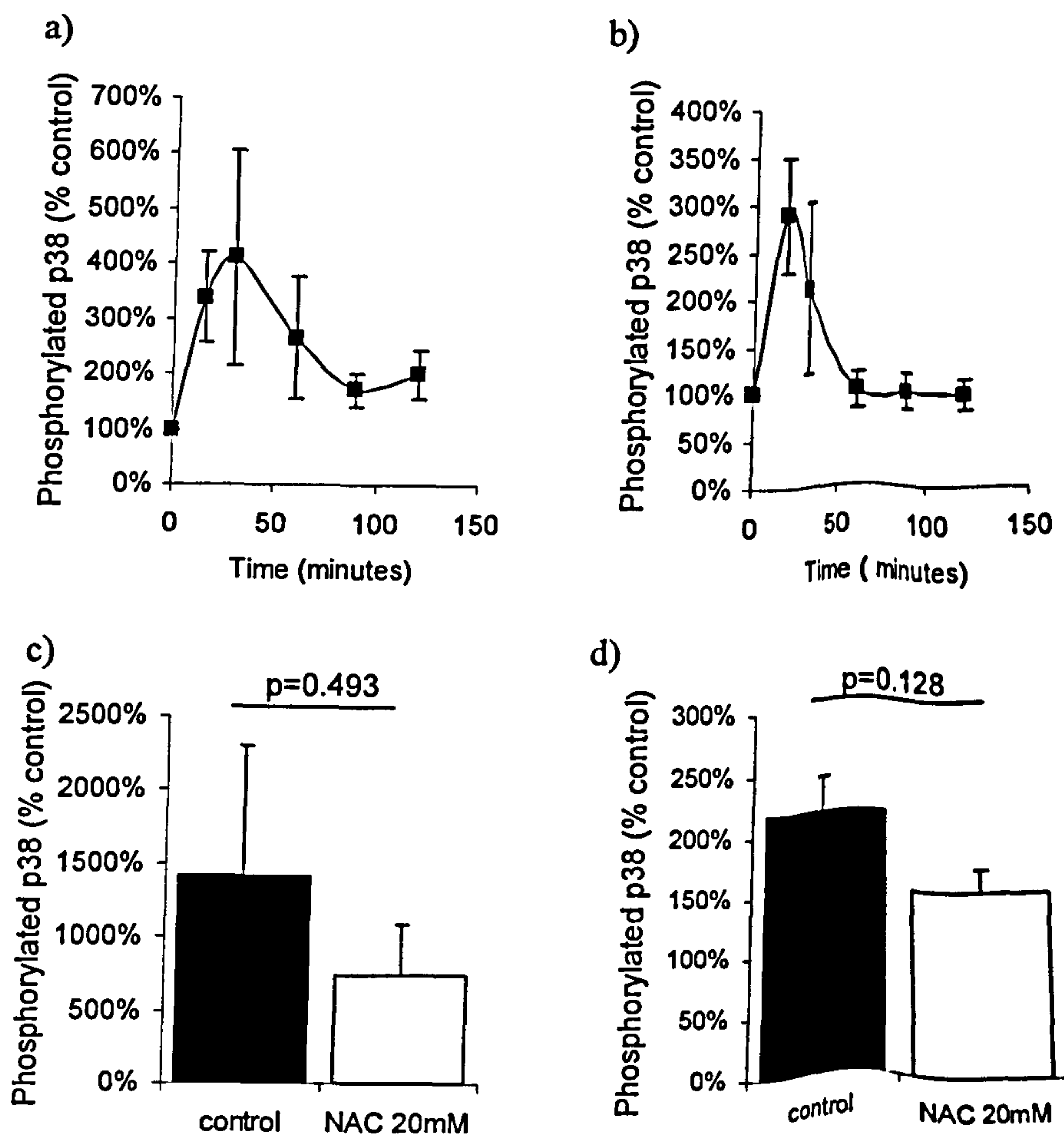


Figure 6.34. Effect of NAC on cytokine-induced p38 activation in A549 cells. Summary graphs of the optical density analysis of p38 MAPK Western blots from A549 cells treated with IL-1 β 1ng/ml (a) and TNF α 10ng/ml (b) over a 120 minute time course. Summary graphs showing the effects of NAC (20mM) on p38 phosphorylation induced by c) 30 minutes IL-1 β 1ng/ml treatment or d) 15 minutes TNF α 10ng/ml treatment of A549 cells.

6.7.10 EFFECT OF H₂O₂ ON p38 MAPK ACTIVATION

Despite the lack of effect of H₂O₂ (250 μ M) on I κ B α levels in A549 cells, in these same samples there was a marked increase in p38 MAPK activation as demonstrated by increased phosphorylation of p38, with peak response observed at 30 minutes ($272.6 \pm 17.2\%$ of control levels, $n=3$). This response was significantly attenuated by the presence of NAC (20mM, $103.1 \pm 34.2\%$ of control levels, $p=0.008$, $n=3$, Figure 6.35a). Primary airway epithelial cells in which H₂O₂ treatment failed induce a response in terms of I κ B α depletion demonstrated an increase in p38 phosphorylation with levels increasing to 283.2 ± 22.4 and $270.1 \pm 76.0\%$ of control levels following 30 and 60 minutes treatment respectively (Figure 6.35b).

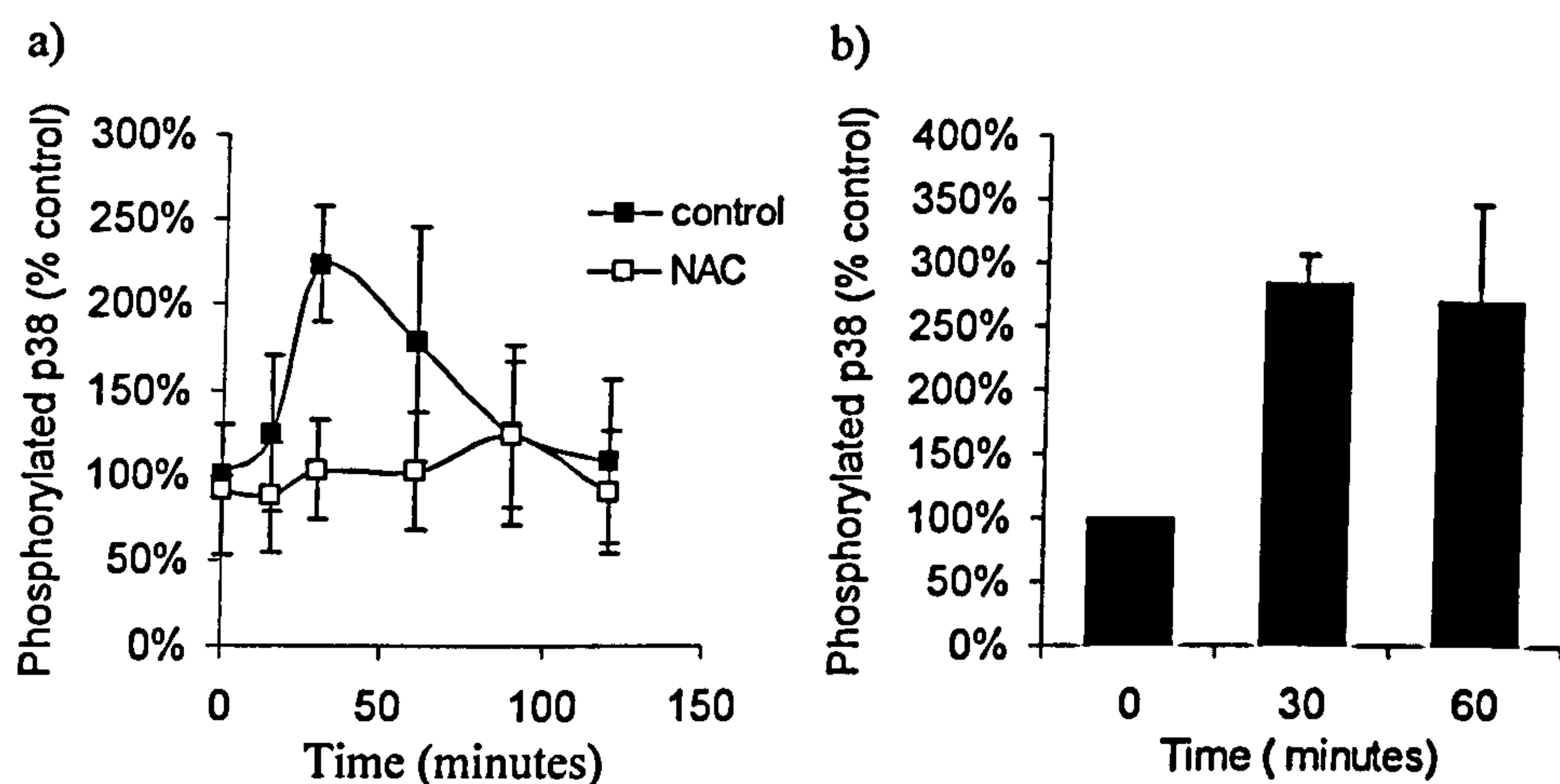


Figure 6.35. Effect of H₂O₂ on p38 activation in pulmonary epithelial cells. a) Time course of p38 phosphorylation induced by treatment of A549 cells with H₂O₂ (250 μ M) over a 120 minute time course in the presence and absence of NAC (20mM). b) Effect of H₂O₂ (250mM, 30 and 60 minutes treatment) on p38 activation in primary epithelial cells.

6.8 DISCUSSION

The data presented above demonstrates that, in human cultured pulmonary artery endothelial cells, vascular smooth muscle cells and pulmonary epithelial cells (primary airway and A549) -

- Treatment with NAC increases intracellular anti-oxidant defences as demonstrated by increased intracellular thiols and inhibition of H₂O₂-induced oxidation.
- Treatment with H₂O₂ increases intracellular oxidative stress as demonstrated by a reduction of intracellular thiol concentration and increased DCFH oxidation.

- Treatment with either IL-1 β or TNF α does not alter intracellular thiol concentrations or oxidise DCFH.
- Cytokine-induced I κ B α depletion, NF κ B p65 nuclear translocation and I κ B α resynthesis are not affected by pre-treatment with NAC.
- Treatment with H₂O₂ has no effect on I κ B α levels or nuclear NF κ B p65 over the time course studied.
- Treatment with H₂O₂ induces the activation of p38 MAPK in an NAC sensitive manner.

In addition

- In endothelial cells and to a lesser extent in A549 cells p38 activation induced by TNF α but not IL-1 β can be attenuated by NAC.
- In endothelial cells E-selectin expression induced by TNF α but not IL-1 β is attenuated by pre-treatment with NAC.
- In A549 cells and SMC H₂O₂ treatment is not capable of inducing ICAM-1 expression.
- In A549 cells H₂O₂ has no effect on IL-1 β or TNF α -induced I κ B α depletion, I κ B α resynthesis or ICAM-1 expression.

Taken together these data suggest that in the cell types studied NF κ B is not an oxidative stress responsive transcription factor. However in some cell types other pathways that are responsive to changes in the cellular redox environment, such as p38 MAPK may co-operate with NF κ B to induce gene transcription.

The proposal that NF κ B is an oxidative stress responsive transcription factor could have important implications for a pivotal role of NF κ B in mechanism underlying the inflammatory response following cardiac surgery and in heart failure in which there is an apparent redox imbalance towards oxidative stress. Two studies have linked the degree of oxidative stress during CPB to the activation of NF κ B demonstrating the importance of both these events in the complications following such surgical procedures. The study by Fan et al demonstrated that administration of an anti-oxidant prior to reperfusion of an ischaemic heart attenuated NF κ B and AP-1 activation, ICAM-1 expression and neutrophil accumulation (Fan et al., 2002). In addition, a study by

Cargnoni et al demonstrated that the shift of myocardial sulphhydryl pools towards oxidation, (i.e. oxidative stress) acts as a cellular redox switch for NFκB activation, which only occurs when a certain degree of oxidation is achieved (Cargnoni et al., 2002).

As such the effects of anti-oxidant and pro-oxidant conditions on NFκB activation in relevant vascular and pulmonary cells were examined in order to establish the potential contribution of disordered redox balance in controlling NFκB activity in these settings. In addition, the effects on cell viability, intracellular anti-oxidant thiols and oxidation also were determined. As a control p38 MAP kinase activation (phosphorylation) was monitored.

6.8.1 CELL VIABILITY

Endothelial cells appeared to be especially sensitive to H₂O₂ showing a dramatic induction of cell death over a five hour time course, an effect that was significant at 250μM H₂O₂ and resulted in less than 30% of the cells remaining viable in response to 1mM. This effect of H₂O₂ was completely abolished by increasing the intracellular antioxidant defences by pre-treating the cells with NAC.

In contrast, A549 cells appeared to be more resistant to these toxic effects of H₂O₂ and a reduction in cell viability was only evident after 18 hours of treatment with 1mM H₂O₂. Under such circumstances there were still more than 80% of the cells capable of efficient mitochondria dehydrogenase activity. However, following 18 hours treatment with NAC there was extensive cell death with only one quarter of the cells remaining viable. Shorter treatment (3 hours) of these cells with either H₂O₂ or NAC did not appear to effect cell viability.

Smooth muscle cells appeared to be somewhat less resistant to H₂O₂ than the epithelial cells however were not as sensitive as the endothelial cells. Similar to A549 cells NAC appeared to be extremely toxic to the SMC over the 18 hour time course but no such effect was observed over 3 hours. It has not been investigated if the reduced viability of the cells under the conditions used here are as a result of cell necrosis or apoptosis although previous studies suggest that the latter is the mechanism responsible. Tsai et al previously demonstrated that NAC can induce human smooth muscle apoptosis (Tsai et al., 1996). In contrast, endothelial cells appear to be resistant to this action of NAC. Indeed, NAC has been shown to inhibit endothelial cell apoptosis induced by a number

of different stimuli including oxidative stress, $\text{TNF}\alpha$ and $\text{TGF}\beta$ (Aluigi et al., 2000; Dimmeler and Zeiher, 2000). Studies have suggested that the role of ROS on vascular cell injury and survival will depend on the particular ROS and on the concentration. In endothelial cells pro-oxidant conditions are generally observed to result in apoptosis (Dimmeler and Zeiher, 2000; Taniyama and Griending, 2003). This is consistent with the data presented in this current study demonstrating the extensive death of these cells in response to H_2O_2 treatment. However, in vascular SMC data in this regard are often conflicting. Prolonged exposure of vascular SMC to high concentrations of ROS leads to cell death (Fiorani et al., 1995; Johnson et al., 1996; Li et al., 1997). Interestingly, exposure of SMC to relatively low levels of oxidant stress for short periods can promote cell survival and induce proliferative responses. Indeed ROS may mediate the proliferative responses to PDGF (Sundaresan et al., 1995) and angiotensin II (Ushio-Fukai et al., 1998; Zafari et al., 1998). Moreover, the species of oxidant used appears to be important in determining the fate of the cell; superoxide induces cell growth whereas H_2O_2 leads to cell death (Li et al., 1997) supporting, at least in part, the data in this thesis suggesting that prolonged exposure of the smooth muscle cells to high doses of H_2O_2 leads to cell death.

Two distinct sub-populations of A549 cells have been identified, with respect to predisposition to cell death, that are classed as either susceptible or resistant. In addition, differences in the intracellular levels of several anti-oxidant components between these two clones have also been identified (Watanabe et al., 2002). The relative proportions of these cell clones in the current study are unknown, however the study by Watanabe also demonstrated that both clones were relatively resistant to H_2O_2 -induced cell death even at high concentration (2mM) and that the death that did occur under such conditions was caspase independent suggesting necrotic rather than apoptotic mechanisms. Consistent with this the A549 cells in the current study demonstrated only low levels of cell death in response to H_2O_2 even following prolonged exposure to high concentrations.

The reasons for these differences in sensitivity to H_2O_2 may be related to the level of intracellular anti-oxidant defences and thus the ability of H_2O_2 to achieve varying degrees of intracellular oxidative stress.

6.8.2 INTRACELLULAR ACID SOLUBLE THIOLS

Endothelial cells appear to have much lower concentration of intracellular acid soluble thiols than either the SMC or the A549 cells and these low levels were significantly reduced by treatment with H_2O_2 even at $250\mu\text{M}$. Pre-treatment with NAC substantially increased intracellular thiol content of these cells and prevented the oxidant-induced reduction. In A549 cells, however, which were the most resistant to H_2O_2 -induced cell death, the intracellular thiol content was around three times higher than that in the endothelial cells and remained substantially higher even after addition of exogenous H_2O_2 .

There appears to be an inverse relationship between the basal concentration of intracellular acid soluble thiols (endothelial cells < SMC < A549 cells) and sensitivity to oxidant mediated cell death (endothelial cells > SMC > A549 cells). Indeed there is evidence to suggest that GSH decrease is often associated with the apoptotic program (Filomeni et al., 2002).

Neither $\text{TNF}\alpha$ nor $\text{IL-1}\beta$ demonstrated the ability to alter intracellular thiol concentrations under the conditions used to initiate $\text{NF}\kappa\text{B}$ activation. This data suggests that these cytokines cannot induce a significant intracellular oxidant stress in these cell types.

However, it should be noted that quantifying intracellular acid soluble thiols in this manner is not a definitive measure of intracellular anti-oxidant capacity and has not been intended to be so in this study. Many other factors contribute to the anti-oxidant power of the cell such as enzymes, vitamins and protein bound thiols. Indeed the use of such a measurement in this study was made simply as a guide to the effects of exogenously added oxidants and anti-oxidants on first line antioxidant defences of the cell (GSH) and to establish whether the desired effect was being achieved (increased intracellular oxidative stress or enhanced GSH concentration).

6.8.3 INTRACELLULAR OXIDATION

Intracellular oxidative stress was monitored using the oxidant sensitive dye DCFH which, when oxidised forms the fluorescent compound DCF. The level of fluorescence in the cells can then be quantified as an indication of intracellular oxidant stress. The differences between the three cell types with respect to anti-oxidant defences (6.8.2) was also reflected in the greater intensity of DCF fluorescence in the endothelial

compared to the other two cell types under both control condition and H_2O_2 stimulated conditions. This data suggests that a greater intracellular oxidative stress exists in the endothelial cells compared to the smooth muscle cells or the epithelial cells following identical treatment with H_2O_2 . Neither $\text{TNF}\alpha$ nor $\text{IL-1}\beta$ demonstrated the ability to induce the oxidation of intracellular DCFH under the conditions used to initiate $\text{NF}\kappa\text{B}$ activation supporting the conclusion drawn in section 6.8.2 that these cytokines cannot induce a significant intracellular oxidant stress in these cell types.

The use of this dye for the measurement of intracellular oxidant stress is not without its pitfalls that should be borne in mind when interpreting such data. DCFH is a probe capable of detecting a broad range of oxidising reactions that may be increased during intracellular oxidant stress but cannot differentiate between specific oxidising species. For such specificity with this method the use of specific inhibitors of ROS generating systems are required (Hempel et al., 1999). Furthermore, recent reports have demonstrated that DCFH is oxidised in processes that do not involve the generation of ROS (Kalinich et al., 1997; Ohashi et al., 2002). Photo-irradiation incidental to spectrofluorometric or fluorescence microscopic observation has also been reported to oxidise DCFH. This is a serious drawback for the use of DCFH as an indicator of oxidative stress since excitation of DCF at wavelengths shown to result in fluorescence of DCFH is necessary to determine the level of DCF fluorescence and thus photo-irradiation is unavoidable (Afzal et al., 2003). In addition, there is also evidence for the generation of ROS during the generation of DCF from DCFH by peroxidase (Rota et al., 1999). With this in mind the fluorescence generated by the treatment of cells is always compared to a time match control (cells loaded with dye with no further treatment) and the results are considered only as an indication of the relative levels of intracellular oxidative stress.

6.8.4 $\text{NF}\kappa\text{B}$ ACTIVATION

6.8.4.1 $\text{I}\kappa\text{B}\alpha$ depletion

It has been proposed that IKK activation is the point at which diverse signalling events converge in the activation of $\text{NF}\kappa\text{B}$ and that this is a crucial step. In this respect all activators of this transcription factor would be expected to induce the serine phosphorylation of $\text{I}\kappa\text{B}$ with the subsequent ubiquitination and degradation. As demonstrated in chapter 4 this event is rapidly induced by $\text{TNF}\alpha$ and $\text{IL-1}\beta$ in all the

cell types tested. Increasing intracellular antioxidant thiols by the addition of NAC (20 mM) to the cells 30 minutes prior to the addition of cytokines had no effect on the profile of cytokine-induced I κ B α depletion in any of the cells tested. In addition treatment of cells with H₂O₂ under conditions already observed to increase intracellular oxidation and reduce intracellular thiols also failed to influence I κ B α depletion. Taken together these data suggest that ROS are not acting as second messengers in the signal transduction pathway of either IL-1 β or TNF α with respect to I κ B α degradation and that increased oxidative stress is not an efficient activator of NF κ B in these cells under the conditions used.

However, several recent studies have suggested that exposure to oxidative stress activates NF κ B through a pathway that involves phosphorylation of I κ B α on tyrosine 42 in a manner that is independent of IKK activity and serine phosphorylation. Such a modification of the I κ B α protein has been shown to induce its dissociation from the NF κ B dimer without degradation (Byun et al., 2002; Canty, Jr. et al., 1999; Fan et al., 2003; Janssen-Heininger et al., 1999).

The importance of tyrosine phosphorylation over serine phosphorylation of I κ B α in response to oxidative stress has been further substantiated by Schoonbroodt et al who demonstrated that while mutation of ser³² and ser³⁶ of I κ B α inhibited NF κ B activity in response to IL-1 β or PMA it had no effect on H₂O₂-induced NF κ B activation. However, mutation of tyr⁴² of I κ B α abolished NF κ B activation by H₂O₂, while it had no effect on activation by IL-1 β or PMA-ionomycin. This phosphorylation of I κ B α was independent of IKK activity and in contrast to the studies by Canty, Janssen-Heininger and Byun described above, tyrosine phosphorylation of I κ B α was followed by proteasome dependant degradation (Mukhopadhyay et al., 2000; Schoonbroodt et al., 2000).

These studies demonstrate that in some systems I κ B α depletion may not be a reliable measure of NF κ B activation and that NF κ B nuclear translocation may occur in the presence of intact I κ B α . This does not appear to be the situation in human vascular SMC as immunoprecipitation studies demonstrated that I κ B α protein remained bound to NF κ B p65 throughout the period of H₂O₂ treatment.

6.8.5 EFFECT OF REDOX MODULATION ON NFκB p65 NUCLEAR TRANSLOCATION

Irrespective of whether oxidative stress induces IκBα depletion or not one critical event in the activation of NFκB is the ability of the liberated transcription factor to enter the nucleus.

In all four cell types used (EC, vascular SMC and both primary airway epithelial and A549 epithelial cells) nuclear NFκB p65 protein or NFκB/DNA binding activity of nuclear proteins were similar to that observed in resting, unstimulated cells following treatment with H₂O₂. This data suggests that the lack of effect of increased oxidative stress with respect to IκBα depletion is not simply due to the use of alternative pathways and that H₂O₂ is indeed not capable of inducing NFκB activity in these cells under the conditions used.

However, in saying this it cannot be ruled out that NFκB dimers composed of Rel proteins other than p65 are activated in this system by H₂O₂. While most studies have investigated effects of oxidative stress on NFκB p65, some investigators have demonstrated DNA binding activity of other Rel proteins (p50:p65 and p50:RelB heterodimers, p50:p50 homodimers and c-Rel containing dimers) in response to pro-oxidant condition (Shi et al., 2003; Zhou et al., 2001). In human micro-vascular EC H₂O₂ induced the DNA binding of NFκB complexes that were composed predominantly of p65 likely forming heterodimers with p50 and c-Rel. Interestingly though, in that study H₂O₂-induced NFκB DNA binding to the ICAM-1 promoter did not induce promoter activity and it remained transcriptionally silent suggesting that there is a lack of additional signals that are required to induce modifications to p65 (phosphorylation, acetylation) to confer transcriptional competencies of NFκB (True et al., 2000).

In the human cells used in this study neither TNFα nor IL-1β-induced nuclear translocation of NFκB p65 was affected by pre-treatment of the cells with the antioxidant NAC. This supports the IκBα data that suggests that signalling events leading to IκBα depletion in response to either of these cytokines are not dependant on the generation of an oxidative stress and further suggests that mechanisms responsible for the nuclear uptake of NFκB also do not rely on pro-oxidant condition.

6.8.6 EFFECT OF REDOX MODULATION ON NFκB DRIVEN GENE INDUCTION

As discussed in previous chapters the presence of NFκB sub-units in the cell nuclei does not automatically allow one to assume that efficient gene transactivation will follow. Indeed the orchestration of a number of events is required to achieve transcription factor-driven gene activation. Thus the presence of NFκB p65 protein in the nucleus of cells treated with cytokines in the presence of NAC does not imply that anti-oxidant conditions have no effect on NFκB activity. To this end IκBα resynthesis and NFκB-driven adhesion molecule expression were monitored in response to IL-1β and TNFα in the presence of anti-oxidant treatment. The profiles of IL-1β and TNFα-induced IκBα depletion and resynthesis were not altered by the presence of NAC demonstrating efficient NFκB driven transcription of the IκBα gene induced under these reducing conditions. However, increased E-selectin expression induced by TNFα but not IL-1β was inhibited by the presence of NAC. The exact mechanism responsible for this observation is unclear although several possibilities can be ruled out given other results described in this chapter.

NAC has been reported to interfere with the interaction of TNFα with its receptors thus inhibiting the cellular effects of this cytokine (Hayakawa et al., 2003). However, in all the cell types studied in this thesis, IκBα depletion and resynthesis induced by TNFα in the presence of NAC was identical to that induced by TNFα alone ruling out this as an inhibitory mechanism of NAC on TNFα-induced E-selectin expression.

Another study has demonstrated that NAC can inhibit the activity of the 26S proteasome. It is highly unlikely that this is the mechanism of actions responsible for the observation in this current study for several reasons. TNFα-induced IκBα depletion and nuclear accumulation of NFκB p65 were unaffected by such treatment, however, the results presented in chapter 4 clearly demonstrate that these events are inhibited by inhibition of proteasome activity with MG132. In addition this is an event that is common to many NFκB inducing stimuli and would thus also affect IL-1β-induced E-selectin expression.

NFκB p65 phosphorylation at ser⁵³⁶ has been shown to be inhibited by NAC suggesting another possible mechanism of action of NAC (Schubert et al., 2002). However, this again is a common feature of NFκB activation that is required for IL-1β as well as TNFα-induced NFκB transactivation (Sizemore et al., 2002). In addition IκBα gene

induction would likely be affected if this were the mechanism whereby NAC were inhibiting TNF α -induced E-selectin expression.

In contrast to I κ B α , E-selectin expression is delayed following NF κ B nuclear translocation suggesting the possible requirement for modifications of chromatin structure and the formation of an initial transcription factor complex that directs the hyperacetylation of the promoter making it is accessible to NF κ B. In this regard, although NF κ B activation is essential for the transcription of the E-selectin gene, the coordinated activity of a number of other signalling events and factors are also likely to be required. Since IL-1 β -induced E-selectin expression was unaffected by the presence of NAC implies that the effect of NAC is not a general inhibitory one at the E-selectin promoter or on NF κ B. It is most likely that the effect of NAC on TNF α -induced E-selectin expression is due to the inhibition of a redox sensitive signalling pathway that is induced by TNF α , in parallel to the NF κ B activation pathway, which is required for successful NF κ B transcriptional activity of the E-selectin gene.

In these cells, TNF α but not IL-1 β -induced p38 activation was inhibited by NAC suggesting that this may be the anti-oxidant sensitive step in TNF α -induced E-selectin expression. A recent study by Sacconi et al demonstrated that recruitment of NF κ B to a subset of genes was regulated by p38. In their model, p38 acted by enhancing the accessibility of NF κ B binding sites found in these promoters through the activation of a H3 kinase and the subsequent phosphorylation and phosphoacetylation of H3. This orchestrates the chromatin modifications that leads to enhanced accessibility of NF κ B sites or enhances the binding of transcription factors required for co-operative recruitment of NF κ B to chromatin. However, this same study identified NF κ B dependent genes that do not require p38 dependent phosphorylation of H3 for successful recruitment of NF κ B to the promoter and gene induction. H3 at the I κ B α gene promoter is rapidly phosphorylated in a p38 independent manner again demonstrating that I κ B α gene induction is required to be fast and ubiquitous upon NF κ B activation and does not require the apparently sophisticated control of other genes. In such a manner this may confer selective NF κ B driven gene expression through the induction of additional signalling pathways depending on the stimuli (Sacconi et al., 2002). Given the inhibitory effect of NAC on TNF α -induced p38 activation in A549 cells it would be interesting to investigate whether TNF α -induced ICAM-1 expression also possess an anti-oxidant sensitive component and the involvement p38 MAPK pathway. However,

recent studies suggest that p38 activity is not required for such adhesion molecule expression (Holden et al., 2004).

The role of p38 in TNF α -induced E-selectin expression in this present study is purely speculative and cannot be concluded without additional studies aimed at specifically inhibiting p38 activation. However, this data does further supports the differences in TNF α and IL-1 β with respect to NF κ B mediated gene transcription highlighting that, although both cytokines activate NF κ B, other accessory factors and/or signalling pathways may be required for successful gene transcription that are distinct for each cytokine and distinct sets of gene promoters.

6.8.7 EFFECT OF H₂O₂ ON TNF α AND IL-1 β -INDUCED NF κ B ACTIVATION

It is now apparent that the redox environment of different cellular compartments is crucial for the efficient induction of many genes by transcription factors. As such a pro-oxidant environment in the cytosol may promote or induce the activation of a signal transduction pathway or transcription factor but in the nucleus such an environment may reduce or prevent transcription factors binding to DNA and /or gene transactivation.

It appears that in this regard NF κ B is no exception and the reduction of a critical cysteine residue in the DNA binding domain of NF κ B p50 is essential for its interaction with DNA and hence gene transactivation. Pro-oxidant condition in the nucleus can oxidise this residue and prevent interaction with DNA (Hirota et al., 1999b; Matthews et al., 1992; Takeuchi et al., 2000).

In addition several recent papers have shown that increased intracellular oxidative stress can inhibit TNF α -induced NF κ B activation by blocking IKK activity (Byun et al., 2002; Korn et al., 2001; Torrie et al., 2001). It has been suggested that IKK contains a cysteine sulphydryl group that is critical for enzyme activity and is susceptible to oxidative stress (Jeon et al., 2000; Kapahi et al., 2000).

On the other hand, Jaspers et al demonstrated that H₂O₂ could increase IKK activity and the phosphorylation and ubiquitination of I κ B α in normal human bronchial epithelial cells. However, proteolytic degradation of I κ B α was reduced thus inhibiting the NF κ B nuclear translocation and DNA binding activity and IL-8 transcription. This data suggests that H₂O₂ inhibits proteolytic degradation of polyubiquitinated proteins by the 26S proteasome (Jaspers et al., 2001).

In this regard the effects of H_2O_2 treatment on $\text{TNF}\alpha$ and $\text{IL-1}\beta$ -induced $\text{I}\kappa\text{B}\alpha$ depletion and resynthesis and ICAM-1 expression were investigated in A549 cells. Neither $\text{IL-1}\beta$ nor $\text{TNF}\alpha$ -induced $\text{I}\kappa\text{B}\alpha$ depletion or resynthesis, or ICAM-1 expression in these cells was altered by the pro-oxidant conditions induced by H_2O_2 . This data suggests that none of the above mechanisms of H_2O_2 -induced inhibition of $\text{NF}\kappa\text{B}$ activity occur in these cells.

6.9 CONCLUSION

$\text{NF}\kappa\text{B}$ activation is not affected by altering the redox state of cells used in this study. However co-operating pathways that are required for the successful transactivation of a subset of $\text{NF}\kappa\text{B}$ responsive genes are sensitive to inhibition by anti-oxidants when activated by specific stimuli. This data also demonstrates that some aspects of $\text{TNF}\alpha$ signalling pathways are sensitive to inhibition by the anti-oxidant NAC. However whether this inhibitory action of NAC is due to its anti-oxidant activity and the requirement for $\text{TNF}\alpha$ -induced generation of ROS remains to be established.

Chapter Seven: CONCLUSIONS AND FUTURE WORK

The Results described and discussed in this thesis have provided novel insights into the complex nature of the systemic inflammatory cytokine response that is associated with heart failure and cardiac surgery, with particular reference to the potential effect on the transcription factor NF κ B and endothelial cell activation.

* * *

The results of the heart failure study in this thesis have demonstrated that despite increased TNF α concentrations and reduced sTNFR/TNF α in the systemic circulation of patients with severely decompensated heart failure (LVAD patients) there was no evidence that the plasma from these patients possessed a net inflammatory bioactivity capable of inducing inflammatory gene expression in endothelial cells *in vitro*. Interestingly, the plasma from these patients did possess the activity to initiate the pathway of NF κ B activation (I κ B α depletion) suggesting the presence of soluble factors that can effectively control potential inflammatory consequences of NF κ B activation. Indeed there was an increase in the circulating levels of a number of anti-inflammatory molecules. However, no single factor was identified as being responsible for the observed bioactivity of the plasma to modulate either I κ B α depletion or E-selectin expression. In patients with stable end stage heart failure the plasma bioactivity to induce I κ B α depletion was similar to that observed in the LVAD patients despite lower concentrations of TNF α and certain anti-inflammatory factors and increased sTNFR/TNF α . However, the plasma from these patients did possess a bioactivity to induce E-selectin expression on endothelial cells *in vitro*.

These data go against the original hypothesis of this study in which the increased pro-inflammatory cytokines observed in the circulation of deteriorating heart failure patients were expected to induce NF κ B activation resulting in inflammatory gene expression and endothelial cell activation. In such a manner these pro-inflammatory cytokines would drive the systemic inflammatory response and contribute to disease progression and adverse clinical outcome. As such the inflammatory consequences of NF κ B activation observed in the *in vitro* bioassay appear to be controlled at least in terms of E-selectin expression. This observed control of NF κ B driven inflammatory gene expression may be either as a result of the presence of factors capable of inhibiting NF κ B or co-operating pathways or may be due to the lack of activation of essential co-operating factors or pathways.

In agreement with this current *in vitro* study, NFκB activity (nuclear NFκB and NFκB DNA binding activity) in the failing myocardium has also been shown to occur independently of altered TNFα expression suggesting that *in vivo* other stimuli are also responsible (Frantz et al., 2003). It is, however, still unclear as to the role of NFκB in heart failure and which genes are induced by this transcription factor under these circumstances. Interestingly, attenuation of LV remodelling and improved cardiac function have been associated with a significant increase in myocardial NFκB activation (Frantz et al., 2003). The authors of that study attributed to NFκB a beneficial effect against progressive LV remodelling and dilatation, which is consistent with the pro-survival, anti-apoptotic role of NFκB (Bergmann et al., 2001). It would therefore be interesting to determine the bioactivity of the plasma of heart failure patients to induce the expression of other NFκB-dependant genes with the hypothesis that NFκB activation in heart failure is beneficial and provides a compensatory anti-apoptotic, pro-survival mechanism that is dysregulated during disease progression.

With this in mind it would also be important to monitor changes in the circulating cytokines profile and plasma bioactivity, with relation to specific protective and inflammatory NFκB-regulated genes, in the heart failure patients after implantation of the LVAD during the process of reverse remodelling and improved clinical status. In doing so one might establish which genes drive the direction of the disease.

Altering the redox balance of a cells environment appears to be a mechanism whereby specific NFκB dependant gene activation can be modulated in a stimuli and cell type specific manner (Chapter 6). Increased oxidative stress and reduced anti-oxidant defences have been implicated in the progression of heart failure and a number of clinically used heart failure therapies have been reported to have anti-oxidant properties. Although this current study found no evidence for a universal role of reactive oxygen species in activation of NFκB *in vitro* it does demonstrate that specific signalling events can be modulated by anti-oxidants (6.5.5.3, 6.5.7). Thus, the anti-oxidant properties of some heart failure therapies may contribute to their efficacy by inhibiting specific signalling events which contribute to disease progression whilst preserving certain beneficial functions of increased cytokines and neurohormones. Such anti-oxidant therapies may therefore be of greater therapeutic benefit than approaches aimed at inhibiting all the activities of specific molecules, such as NFκB and TNFα.

The results of the current study investigating the changes that occur in the circulation of patients following cardiac surgery have demonstrated that uncomplicated coronary artery bypass surgery does not routinely activate a systemic pro-inflammatory cytokine cascade or increase the inflammatory bioactivity in the plasma of these patients. On the contrary, the predominant and consistent response in the systemic circulation of these patients in the post-operative period was an anti-inflammatory one capable of reducing the response of blood components to endotoxin challenge and inhibiting pro-inflammatory endothelial cell activation *in vitro* (3.3.5, 5.6.4). Furthermore, the anti-inflammatory cytokine response to cardiac surgery occurred irrespective of the use of cardiopulmonary bypass and the associated global pulmonary and myocardial ischaemia and reperfusion.

As with the heart failure study this data goes against the original hypothesis that cardiac surgery induces a systemic inflammatory response with increased circulating pro-inflammatory cytokines capable of activating NFκB and inflammatory gene expression that can adversely affect the clinical outcome of these patients.

It should be noted that all the patients undergoing cardiac surgery in this study were low risk patients with good ventricular function and no evidence of pre-existing systemic inflammation. All of these patients experienced an uneventful post-operative period, with no evidence of clinically relevant post-operative systemic inflammatory response compromising major organ function. In this regard the circulating cytokine profile and bioactivity are probably characteristic of no adverse reaction to surgery. This may relate to the lack of pro-inflammatory responses or the efficacy of the anti-inflammatory cytokines to maintain homeostasis.

As a logical extension of these studies it would be important to characterise the specific changes in circulating cytokine profile and bioactivity to induce NFκB activation that occur during the course of cardiac surgery that characterise an adverse outcome and the development of clinically significant events. In doing so one might gain a better understanding of the mechanisms responsible for the development of such events and provide a novel basis for effective preventative intervention.

One particular patient group where these issues might be critical are those who present for cardiac surgery with impaired ventricular function or heart failure. It is recognised that patients with poor ventricular function are at higher risk of post-operative complication and that they exhibit an altered pre-operative cytokine profile (3.2.5). It is

however, unclear whether the response of these patients to surgery is merely a reflection of poor ventricular and hemodynamic performance or involves active inflammatory components such as their inflammatory cytokine profile and the ability to induce or inhibit NFκB activity.

The relationship between the pre-operative cytokine profile of the patient and the response of these patients to cardiac surgery also remains largely unknown. Pre-operative cytokine profile has not been considered as a predetermining risk factor for post-operative morbidity, but given the individual variation in both pro- and anti-inflammatory cytokines, it may be that patients with low anti-inflammatory functional reserve are at greater risk for peri-operative complications.

Although the current studies focused on plasma bioactivity and utilised NFκB activation as a biomarker of inflammatory events, it will be important to correlate these *in vitro* responses to NFκB activation *in vivo*. These studies could involve analysis of NFκB activation in circulating leucocytes and in various tissues recovered during the course of cardiac surgery. Preliminary studies in this thesis demonstrating NFκB activation in circulating monocytes of patients undergoing CPB suggest that the anti-inflammatory events observed in these patients may effectively control potential adverse effects of such NFκB activation *in vivo*.

* * *

In conclusion this thesis has explored a fascinating area of translational research at the interface of clinical problems associated with heart failure and cardiac surgery and molecular regulation of NFκB in isolated human cells. These studies have highlighted the significant anti-inflammatory component accumulated in the plasma of patients with severe heart failure and in patients following cardiac surgery. This is an important recognition since both syndromes are generally considered to involve pro-inflammatory pathogenesis. The *in vitro* results appear to question the physiological consequences of elevated individual cytokine levels by demonstrating a lack of net pro-inflammatory bioactivity in the circulation of these patients that is capable of driving NFκB-dependant inflammatory events in the vasculature. In this respect, relevant bioassay measurements may provide additional insight into disease mechanisms above that gained from considering individual cytokine concentrations in isolation. Further exploration of these issues *in vivo* and elucidation of the exact role of observed NFκB activity in the

myocardium and other tissues of such patient will be an exciting and hopefully fruitful area of future research.

Finally, uncovering the main events in the molecular mechanisms of NF κ B activation has been one of the greatest achievements of recent inflammatory research. The focus on the molecular signalling in human cells in this thesis has highlighted important cell specific mechanisms, which increase knowledge in this important area and may have important clinical and therapeutic implications.

APPENDIX I

BUFFERS AND SOLUTIONS FOR PROTEIN ANALYSIS BY SDS-PAGE AND WESTERN BLOTTING

All buffers and solutions were made up in MilliQ double distilled, ultra pure water unless otherwise stated.

SDS LYSIS BUFFER

1% sodium dodecyl sulphate (electrophoresis grade) in MilliQ water containing protease inhibitors (0.5mM PMSF, 2.5µg/ml aprotinin, 2.5µg/ml pepstatin A, 2.5µg/ml leupeptin) and phosphatase inhibitors (50mM NaF, 1µM Na₃VO₄, 20mM β-glycerol phosphate, 10µM sodium molybdate)

NUCLEAR ISOLATION BUFFER A

10mM HEPES (pH 7.9), 1.5mM MgCl₂, 10mM KCl, 0.1% nonidet-p40 (NP-40), 0.5mM dithiothreitol (DTT), protease inhibitors as for SDS Lysis Buffer (section 0.)

NUCLEAR ISOLATION BUFFER B

20mM HEPES (pH7.9), 25% glycerol, 1.5mM MgCl₂ , 420mM NaCl, 0.2mM EDTA, 0.5mM DTT, protease inhibitors as for SDS Lysis Buffer (Section 0.)

IMMUNOPRECIPITATION LYSIS BUFFER

50mM TRIS HCl, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, protease inhibitors as for SDS Lysis Buffer (section 0.), phosphatase inhibitors as for SDS Lysis Buffer (section 0.)

EMSA BUFFER C

20mM HEPES (pH7.9), 50mM KCl, 0.5mM DTT, 0.2mM EDTA, 0.5mM PMSF

TRIS-BORATE-EDTA (TBE) BUFFER (10X)

890mM Tris Base, 890mM Boric acid, 25mM EDTA

LAEMMLI BUFFER

0.164M Tris (pH 6.8), 3.25% SDS, 25% v/v Glycerol, 0.0015 % Bromo phenol blue

POLYACRYLAMIDE RESOLVING GEL 10%

37.5:1 Acrylamide:bis-acrylamide solutions, 375mM Tris Buffer pH 8.8, 1% w/v SDS, 0.02% v/v NNN'-tetramethyl-1,2-diaminoethane (TEMED), 0.5% w/v Ammonium peroxodisulphate (APS, freshly prepared).

POLYACRYLAMIDE STACKING GEL 3%

37.5:1 Acrylamide:bis-acrylamide solutions, 125mM Tris Buffer pH 6.8, 1% w/v SDS, 0.1% v/v TEMED, 0.5% w/v APS (freshly prepared)

RUNNING BUFFER

25mM Tris base, 192mM Glycine, 0.1% w/v SDS

BLOTTING BUFFER

20mM Tris base, 150mM Glycine

STRIPPING BUFFER

100mM 2-mercaptoethanol, 2% SDS, 62.5mM Tris-HCl pH 6.7

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