MODULATION OF HUMAN NEUTROPHIL APOPTOSIS BY HYPOXIA

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DECLARATION

This thesis was composed entirely by myself on the basis of work, which, unless otherwise stated, was carried out under the supervision of Professor Edwin R. Chilvers, Dr. Andy Greening and Dr. Adriano Rossi in the Rayne Laboratory, Respiratory Medicine Unit, University of Edinburgh.

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ABSTRACT

Neutrophil apoptosis represents a major mechanism involved in the resolution of inflammation. When neutrophils die by apoptosis they retain their granule contents, but lose chemotactic and secretory responsiveness, and are phagocytosed intact by macrophages via a mechanism that does not incite an inflammatory response. While the processes involved in regulating neutrophil survival are poorly understood, there is now considerable evidence to indicate that this process is not immutable since many pro-inflammatory mediators can modulate neutrophil apoptosis. For example, under *in vitro* conditions, LPS, GM-CSF and IL-1 and IL-6 all inhibit neutrophil apoptosis, while TNFα is able to induce apoptosis in these cells. Thus it appears that such agents act not only in a priming/secretory capacity, but also affect neutrophil functional longevity by regulating apoptosis. I was interested to examine whether other physiochemical insults, in particular hypoxia, which is highly relevant to the inflamed site, could also affect this cells life span.

It was shown that incubating neutrophils under hypoxic conditions greatly enhances neutrophil survival, as assessed by morphological criteria. The inhibition of apoptosis by hypoxia was concentration-dependent and confirmed by propidium iodide-staining and annexin V binding. While neutrophils had to be hypoxic from time 0 in order for hypoxia to achieve its full protective effect, a delayed hypoxic challenge was still able to provide some inhibitory effect, even at a late stage in the apoptotic program. Hypoxic neutrophils (3-18 hours) returned to a normoxic environment underwent apoptosis at the same rate as control cells.

The anti-apoptotic effects of GM-CSF and hypoxia in neutrophils were additive, indicating these effects are discrete and act via independent mechanisms. In addition, neither of these protective effects were associated with an upregulation of the proto-oncoprotein Bcl-2. Antioxidants, glucose deprivation, heat shock or mitochondrial inhibitors were not able to mimic the protective effect of hypoxia. These results indicate

the enhanced survival of neutrophils under hypoxic conditions is specific for hypoxia, and is not sensitive to other cellular stresses such as cellular ATP depletion or inhibition of the mitochondrial respiratory chain. The hypoxic-mediated inhibition of neutrophil apoptosis was found to be sensitive to the protein synthesis inhibitor, cycloheximide, even at very low concentrations that did not influence the rate of constitutive apoptosis, suggesting that *de novo* protein synthesis is required for the inhibition and that hypoxia may be inducing upregulation of a survival protein(s).

To investigate the potential mechanisms underlying the anti-apoptotic effect of hypoxia the effect of iron chelators was investigated and found to mimic the response observed with hypoxia. This effect was concentration-dependent and, at higher concentrations, iron chelators inhibited neutrophil apoptosis to a similar extent as hypoxia. These data may implicate a role for chelatable iron in the oxygen-sensing mechanism underlying the inhibition of neutrophil apoptosis by hypoxia. A putative role of several redox sensitive transcription factors in the hypoxia-induced survival of neutrophils was also investigated. A constitutive form of NF-κB binding was found to be upregulated in response to hypoxia; however, this effect was extremely donor specific and the iron chelator, desferrioxamine, did not cause a similar enhancement. AP-1 DNA binding activity was found to be present constitutively in neutrophils and the presence of desferrioxamine or hypoxia failed to further modulate binding of this transcription factor. The transcription factor hypoxia-inducible factor-1 (HIF-1) was identified in neutrophils incubated under hypoxic conditions and in the presence of desferrioxamine but was not expressed in normoxic cells.

Finally, the role of oxidant pathways in TNFα-stimulated apoptosis in neutrophils was also investigated. It was discovered that TNFα-cytotoxicity could be abrogated by anaerobic conditions and mitochondrial inhibitors could modulate the rate of TNFα-induced apoptosis, although antioxidants and iron chelators did not exert any protective effects. Inhibition of the pro-apoptotic enzyme CPP32 (caspase 3) also protected neutrophils from TNFα-induced apoptosis. These findings implicate an oxygen-

dependent step in TNFα-mediated apoptosis and also imply that the mitochondria and caspase family of proteases may play important roles in this phenomenon.

These data suggest that hypoxia, which characterises many inflammatory sites *in vivo*, may extend the neutrophils life span by inhibiting both spontaneous apoptosis and TNFα-induced apoptosis, and this may be a contributory factor in neutrophil-mediated tissue damage. The mechanism underlying the inhibition of constitutive neutrophil apoptosis may involve the transcription factor HIF-1, although further work will be required to confirm this. The mechanism underlying TNFα-induced apoptosis appears to be largely unrelated to that controlling basal apoptosis and the data presented suggest the involvement of mitochondria and the caspase family of death-inducing proteases.

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his computer related advice and help throughout the construction of this thesis.

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ABBREVIATIONS

AEBSF	4 (2-aminoehyl) benzenesulfonyl fluoride
AIF-1	Apoptosis inducing factor-1
AP-1	Activator protein-1
ATP	Adenosine triphosphate
ARDS	Acute respiratory distress syndrome
ATF	Activating transcription factor
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
BSO	Buthionine-sulfoximine
C5a	Complement fragment 5a (anaphylotoxin)
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride
CO	Carbon monoxide
CO_2	Carbon dioxide
CHX	Cycloheximide
CP94	1, 2-diethyl-3-hydroxypyridine-4-one
DFO	Desferrioxamine
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxy-ribonucleic acid
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene glycol (B-aminoethylether)-N,N-tetraacetic acid
EPAS1	Endothelial PAS domain protein 1
EPO	Erythropoietin
ER	Endoplasmic reticulum
ERK	Extracellular signal-related kinase

FADD	Fas-associated protein with death domain
FcR	Receptor for Fc-piece of immunoglobin
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FLICE	FADD-like interleukin-1β converting enzyme
fMLP	N-formyl-methionyl-leucyl-phenylalanine
G-CSF	Granulocyte-colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GRP	Glucose-regulated proteins
HAP	Hypoxia-associated proteins
HBEC-CM	Human bronchial epithelial cell conditioned medium
HBSI	HEPES (20mM)-buffered saline containing 50 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM AEBSF)
HBSS	Hanks' balanced salt solution
HEPES	N-2-hydroxyethylpiperazine-N-ethane sulphonic acid
HIF-1	Hypoxia-induced factor-1
HL-60	Human leukaemic cell line
H_2O_2	Hydrogen peroxide
Hsp	Heat shock protein
HSTF	Heat shock transcription factor
ICAM-1	Intercellular cell adhesion molecule-1
ICE	Interleukin-1β converting enzyme
IFNγ	Interferon-γ
IκB	NF-κB inhibitory proteins
IKK	IκB kinase
IL-1	Interleukin-1
IL-3	Interleukin-3
IL-6	Interleukin-6
IL-8	Interleukin-8

iNOS	Inducible nitric oxide synthase
Iscove's MDM	Iscove's Modified Dulbecco's medium
JNK	c-Jun N-terminal kinase
LPS	Bacterial lipopolysaccharide
LSB	Laemmli sample buffer (2x, 0.125 M Tris-HCl, 4% SDS, 20% glycerol, 2.5 mM dithiothreitol, 0.01% bromophenol blue, 50 μg/ml leupeptin, 20 μg/ml aprotinin, amd 1 mM AEBSF, pH 6.8)
LTB_4	Leukotriene B_4
mAb	Monoclonal antibody
MACH	MORT1-associating CED homologue
MAPK	Mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKAP kinase	MAP kinase-activated protein kinase
$MgCl_2$	Magnesium chloride
MOF	Multiple organ failure
mRNA	messenger RNA
NAC	N-acetyl cysteine
NaCl	Sodium chloride
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-κB
NGF	Nerve growth factor
O_2	Oxygen
O_2^-	Superoxide anion
$OH^\bullet$	Hydroxyl radical
P_AO_2	Alveolar pO_2
P_aO_2	Arterial pO_2
PAF	Platelet-activating factor
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline

PC-12 cell line	Pheochromocytoma cell line
PCD	Programmed cell death
pCO_2	Partial pressure of CO_2
PMA	Phorbol-12-myristate 13-o-acetate
pO_2	Partial pressure of O_2
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
PT pore	Permeability transition pore
RNA	Ribonucleic acid
ROI	Reactive oxygen intermediate
ROS	Reactive oxygen species
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SOD	Superoxide dismutase
TCA	Trichloroacetic acid
TGFβ	Transforming growth factor-β
TH	Tyrosine hydroxylase
TNFα	Tumor necrosis factor-α
TNFR	Tumour necrosis factor receptor
TNFR55	55 kD TNFR
TNFR75	75 kD TNFR
TRADD	TNFR55-associated death domain protein
TRAF	TNFR associated factor
Tris	Tris[hydroxymethyl]-aminomethane
uPAR	urokinase-type plasminogen activator receptor
UV	Ultra-violet
VEGF	Vascular endothelial growth factor
$\Delta\psi_m$	Mitochondrial inner transmembrane potential

CHAPTER 1

INTRODUCTION

1.1 The Neutrophil

1.1.1 Generation of neutrophils

Neutrophils are derived from pluripotent stem cells located in the bone marrow. These stem cells are also the origin of all mature white cells, red cells and platelets. Pluripotent uncommitted stem cells differentiate into unipotent committed stem cells and neutrophils are then further differentiated from these cells via several recognisable morphological stages. These stages in neutrophil development are represented by the myeloblasts, promyelocytes and band cells, with the major increase in neutrophil number occurring at the myelocyte level. Three major haemopoietic growth factors: G-CSF, GM-CSF and IL-3 regulate neutrophil development (Metcalf and Nicola, 1983; Metcalf et al., 1986; Bot et al., 1988). During development neutrophils acquire their characteristic granules and nuclear morphology and, finally, mature polymorphonuclear neutrophils are stored in a pool in the bone marrow from which they are then released into the peripheral blood at a rate of 10^{11} cells per day (Cannistra and Griffin, 1988). The neutrophils in the vascular compartment can be further divided into a circulating pool and the so-called marginated pool of cells which exists in the spleen, liver and possibly lung (Peters, 1997) and is in dynamic equilibrium with the circulating pool.

1.1.2 Structure and contents

On electron microscopy neutrophils have a mean diameter of 7 μm and are characterised by a multilobed chromatin-dense nucleus. Like the other members of the polymorphonuclear leukocyte family, neutrophils contain a large number of intracellular granules. Variations in granule composition distinguish neutrophils from other granulocytes, namely eosinophils and basophils. Initially, neutrophil granules were classified as either peroxide-positive (azurophil or primary granules) or peroxide-negative (specific or secondary granules). More recently, differential exocytosis of granule proteins and incorporation of granule membrane receptors into

the plasma membrane suggested the presence of additional granule sub-groups, namely gelatinase/tertiary granules and secretory vesicles (Borregaard et al., 1993).

Azurophil granules are defined as peroxidase-positive granules (Bainton, 1975). They are round or oval and vary in size and electron density. Although CD63 and CD68 have been shown to reside in the azurophil granule membrane, no receptors or other functional proteins are present. The azurophil granule matrix contains the stores of most proteolytic and bactericidal proteins and the presence of myeloperoxidase (which acts on H_2O_2 generated by the NADPH oxidase to produce oxyhalides) is of prime importance for full function of the oxygen-dependent bactericidal system.

Specific granules appear as round, oval or elongated granules and are slightly larger than gelatinase granules. They are defined by their matrix content of lactoferrin. The specific granule membrane is an important source of cytochrome b_{558}, the flavo-cytochrome membrane-bound component of the NADPH oxidase (Borregaard and Tauber, 1984; Rostrosen et al., 1992). Other important protein constituents are laminin-receptors, fMLP-receptors and TNF-receptors, although, it is not yet known whether these receptors are fully functional when translocated to the plasma membrane. The number of integral membrane proteins present in the specific granules is much lower than that in the plasma and secretory vesicle membrane (see below). This may indicate that the membrane of specific granules serves a very specific function when translocated to the plasma membrane.

The existence of a separate subset of neutrophil peroxidase-negative granules, termed gelatinase or tertiary granules, has been proposed on the basis of differences in sedimentation by rate zonal centrifugation (Murphy et al., 1980; Dewald et al., 1982). These granules are characterised by the presence of gelatinase, cytochrome b_{558} and ubiquinone but not lactoferrin. While their existence as a separate entity has been questioned (Hibbs and Bainton, 1989), experiments showing that gelatinase in specific granules is complexed with another protein (neutrophil gelatinase associated lipocalin, NGAL), a feature not observed in gelatinase granules, has strengthened the

argument in favour of a distinct granule population (Kjeldson et al., 1992). Although the precise membrane composition of these granules is not known, the amount of gelatinase granule membrane that is incorporated into the plasma membrane appears to be only a fraction of that derived from peroxidase-negative granules. Thus, the significance of mobilising gelatinase granules following stimulation by inflammatory mediators appears to relate to the release of gelatinase rather than their contribution of membrane proteins.

Secretory granules were initially characterised by the presence of alkaline phosphatase, which is present on the luminal side of these vesicles (Borregaard et al., 1987; Sengelov et al., 1992b). It is now established that the secretory granule membrane also contains the adhesion molecule MAC-1 (CD11b/CD18) (Calafat et al., 1993), the chemotactic fMLP receptor (Sengelov et al., 1994), the receptor for urokinase-type plasminogen activator (uPAR) (Plesner et al., 1994) and cytochrome b_{558} (Calafat et al., 1993). In addition, from experiments on the kinetics of granule mobilisation, it also seems likely that the secretory vesicles contain Fcγ receptor III (CD16) (Tosi and Zakem, 1992), C3bi (Berger et al., 1984) and decay accelerating factor (Berger and Medof, 1987), although direct evidence to support this is still lacking. The exocytosis of secretory vesicles results in upregulation of adhesion proteins in the plasma membrane, suggesting that these vesicles play a primary role in regulating the interaction of neutrophils with endothelium (Borregaard et al., 1987; Borregard et al., 1990; Senglelov et al., 1993). The primary function of uPAR appears to be potentiation of the uPA-catalysed plasminogen activation system (Ellis et al., 1989). Thus, it is likely that concurrent translocation of uPAR may ease subsequent diapedesis, while upregulation of fMLP receptors will augment the chemotactic responsiveness of the neutrophil during its subsequent migration.

1.1.3 Role in inflammation

Inflammation is normally a highly complex and physiological protective response to tissue injury (e.g., due to bacterial infection or trauma). This process serves to destroy, dilute and partition off or remove the injurious agent and injured tissue, thereby promoting tissue repair. It has been proposed, however, that when this

crucial and normally beneficial response occurs in an uncontrolled or exaggerated manner the result is inappropriate or excessive tissue damage, which itself may result in chronic inflammation. The neutrophil is a key player in several aspects of the evolving inflammatory response and is essential for host defence. This role is epitomised by the often life threatening infections acquired by patients with neutropaenia, leukaemia or congenital diseases affecting neutrophil structure and/or function.

Circulating neutrophils are largely quiescent cells, serving a physiological monitoring role, and expressing only a limited number of low affinity receptors on their surface. During inflammation their responses are orchestrated by a variety of cytokines and chemotactic factors so that they attach to vascular endothelium, move through the vessel wall and migrate into the tissue. Once at the site of tissue infection or injury, the fully activated neutrophils are able to defend against pathogens by their ability to generate toxic oxygen metabolites and to release and synthesise several degradative enzymes and lipid-derived products. The oxidative burst and degranulation response are stimulated by a variety of organism-derived peptide agonists and cytokines produced by other inflammatory cells may act synergistically with such agonists to potentiate or 'prime' the oxidative burst. However, under certain circumstances, the excessive or inappropriate release of such histotoxic agents can also result in undesirable tissue damage and accentuate the inflammatory response. The main events and processes involved in the neutrophil's role in inflammation are discussed below.

1.1.4 Migration of neutrophils into tissues

Bi-directional interactions between endothelial cells and leukocytes are essential for the emigration of circulating neutrophils, from the blood, into inflamed tissues. The paradigm for leukocyte emigration in the systemic circulation is based on data obtained from *in vitro* studies and *in vivo* videomicroscopy of the mesenteric and other systemic microvessels (Abbassi et al., 1991; Butcher, 1992; Harlan et al., 1992; Lawrence and Springer, 1991; Ley et al., 1991; Smith et al., 1989; Von Andrian et al., 1991; Zimmerman et al., 1993). The initial step in this paradigm is a slowing of

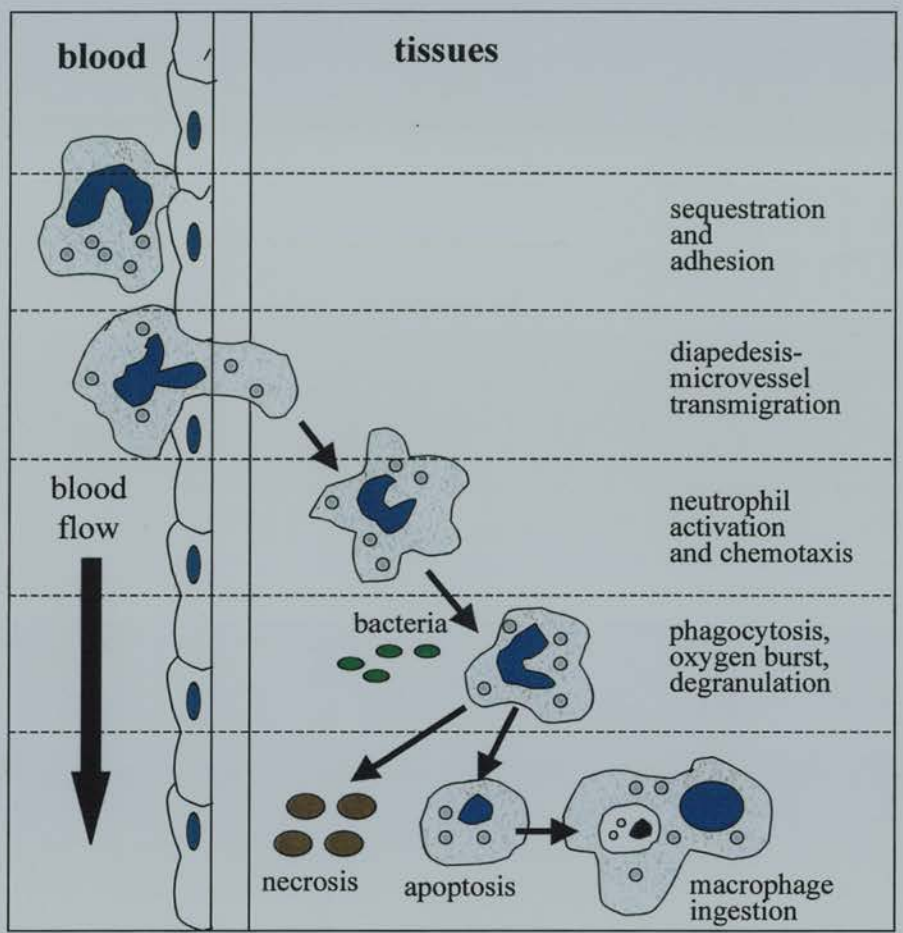

Figure 1.1.3 The role of the neutrophil in inflammation.

neutrophil transit through the injured site by reversible loose adhesion between neutrophils and endothelial cells that results in transient contacts and rolling of the neutrophil along the endothelium. This step is mediated through selectins and the details of the involvement of each of the main selectins (L-, P-, and E-selectin) has now been described. The normal distribution of L-selectin on the microvilli of neutrophils places this molecule in an ideal position for initial interactions with the endothelium (Picker et al., 1991). In the systemic circulation such interactions normally take place in the post-capillary venules but in the lung neutrophil-endothelial interactions take place largely in the capillary and, hence, alterations in neutrophil deformability may also play an important role in determining the duration of neutrophil 'hold up' in the lung.

The second step follows further activation of the neutrophils by chemotaxins, which include IL-8, platelet activating factor (PAF) and complement protein fragments (C5a). Binding of these molecules to receptors on the surface of the rolling neutrophil results in the upregulation of Mac-1 (CD11b/CD18) and the shedding of L-selectin. In the third step, leukocytes bind firmly to the endothelium through CD11/CD18-ICAM-1- or VLA-4/VCAM-1-mediated adhesion. This stable binding is required for neutrophil emigration. Finally, transmigration of neutrophils across the endothelial barrier involves an interaction between leukocyte integrins and endothelial ICAM, and between glycosylated aminoglycans on the neutrophil plasma membrane and PECAM-1, which is localised in the intercellular junctions of endothelial cells (Muller et al., 1993).

After the endothelial cell, the next 'barrier' to neutrophil emigration is the basement membrane. Although neutrophils contain an extensive repertoire of enzymes capable of degrading ECM constituents, it has been proposed that endothelial cells may themselves play the lead role of local degradation and reformation of the capillary basement membrane during neutrophil transmigration (Huber and Weiss, 1989). Similarly, neutrophils can migrate through the tight junctions of intact monolayers of epithelial cells *in vitro* without injuring the monolayer or altering its electrical resistance. During migration to the inflammatory focus, neutrophils adopt a

particular morphology, with a blunt leading front and more elongated body containing the nucleus and cytoplasmic structures including granules. The release of granules appears to occur at the blunt front end of the cell, resulting in the highest concentration of receptors at the leading edge and a gradient of receptors across the surface of the cell that are able to interact with the gradient of chemoattractant resulting in directed movement toward the site of inflammation (Nunoi et al., 1985).

1.1.5 Phagocytosis

Once a neutrophil has migrated into the tissue, its primary purpose is to recognise and destroy pathogens. Phagocytosis is a process utilised by neutrophils to ingest and clear large particles (> 0.5 μM), including infectious agents and cellular debris. Phagocytosis is initiated by the interaction of specific receptors on the surface of the neutrophil with ligands on the surface of the particle. This leads to the polymerisation of actin at the site of ingestion and the internalisation of the particle via an actin based mechanism. The receptors that participate in the ingestion and killing of microbes can be divided into those that require the target particle to be coated by serum opsonins and those that do not (Ofek et al., 1992). Non-opsonic phagocytosis allows ingestion and killing of microbes as a result of the presence of neutrophil surface receptors that recognise microbial sugar molecules. However, many microbes are most effectively killed in the presence of serum opsonins (immunoglobulin and complement fragments). Once the microbe has been internalised, the phagosome fuses with other intracellular granules to form a phagolysosome. Under normal conditions, specific granule products are released into the phagosome first and the azurophil granule products second (Bainton et al., 1971), while some specific granule products (e.g., lactoferrin; Wang-Iverson et al., 1978) and secretory vesicle (Borregard et al., 1990) products are preferentially secreted into the extracellular milieu. The formation of a phagolysosome creates a highly toxic, but confined and controlled, microenvironment capable of killing the engulfed pathogen and also prevents release of the granules microbicidal armoury into surrounding tissues.

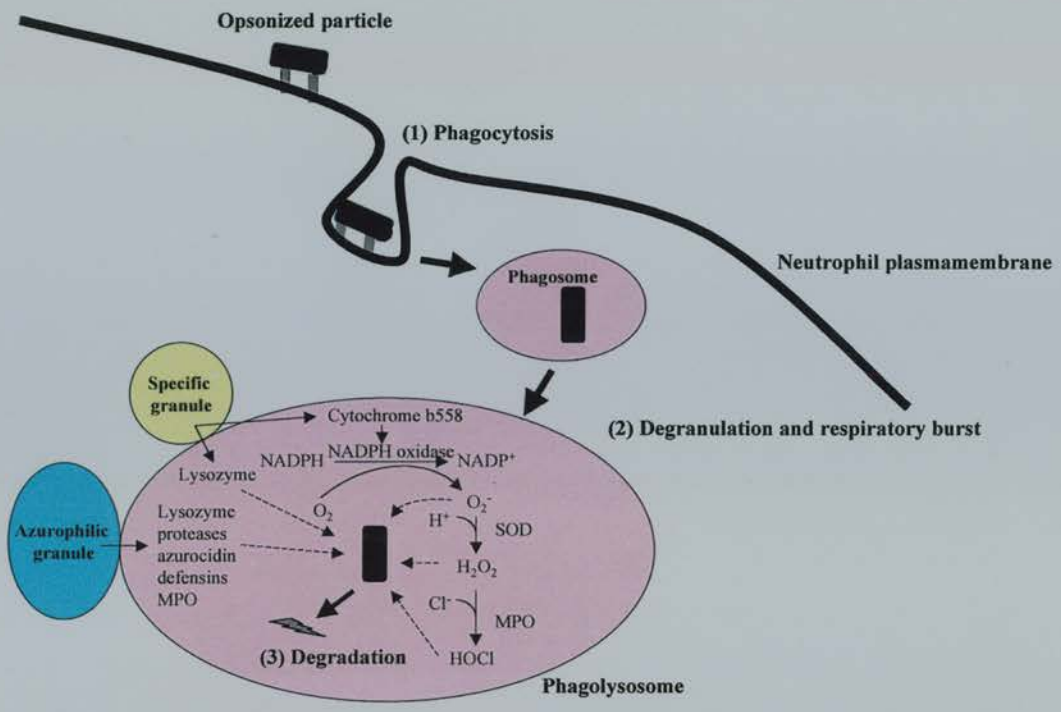

Figure 1.1.5 Schematic representation of neutrophil phagocytosis and microbial killing. (1) The recognition of opsonized agents by neutrophils at the inflammatory site initiates the process of phagocytosis. (2) Fusion and release of the granule contents into the phagosome generates a highly microbicidal environment (phagolysosome). (3) This results in the killing and degradation of the ingested agent by a combination of oxidative and non-oxidative processes. SOD = superoxide dismutase, MPO = myeloperoxidase (modified from Smith, 1994).

1.1.6 Respiratory burst

The respiratory burst, so called because of the 50- to 100- fold increase in oxygen consumption that occurs during this process, is activated concomitantly with phagocytosis and the resultant cytotoxic reactive oxygen species (ROS) work in concert with the microbicidal proteins to kill and eliminate pathogens. Activation of the respiratory burst activity occurs upon the translocation and assembly of the cytosolic components of the NADPH oxidase enzyme system ($p45^{phox}$, $p67^{phox}$, $p21^{rac}$) with the membrane-bound flavocytochrome, cytochrome b_{558}. The active enzyme complex then generates superoxide anions by the transfer of an electron from NADPH to molecular oxygen in the following reaction:

$$NADPH + H^+ + 2O_2 \rightarrow NADP^+ + 2H^+ + 2O_2^-$$

Although O_2^- may contribute to microbial killing, other more potent and effective ROS are generated from this precursor. H_2O_2 is formed by spontaneous dismutation and/or the catalytic action of superoxide dismutase (SOD). Following this, in the presence of a halide ion (usually chloride in the case of neutrophils), myeloperoxidase released from azurophil granules catalyses the conversion of H_2O_2 to oxyhalides such as hypochlorous acid (HOCl):

$$H_2O_2 + HCl \rightarrow HOCl + H_2O$$

Various groups have claimed that the hydroxyl radical ($OH^{\bullet}$), formed by the Fe^{2+}-catalyzed decomposition of H_2O_2 and singlet oxygen are also generated by neutrophils. There is considerable debate, however, as to whether these additional ROS are produced under physiological conditions. Lactoferrin may prevent Fe^{3+} being used as a Fenton catalyst, and hence the bulk of the H_2O_2 generated in these cells appears to be converted to HOCl (Weiss, 1989). Perhaps not surprisingly, neutrophils contain large reserves of antioxidants such as glutathione and ascorbate (Eaton, 1993; Voetman et al., 1980) and their ability to maintain these antioxidants in the reduced state during phagocytosis (Voetman et al., 1980) appears to prevent 'auto-oxidative' stress-mediated cell damage. However, recent reports have implied that upregulation of ROS production may play a role in the increased rate of neutrophil apoptosis following phagocytosis of opsonized particles (Coxon et al., 1996) and E. coli (Watson et al., 1996).

1.1.7 Priming

Neutrophils exist in various states of 'activation', which vary from dormant to primed to fully activated. While activation triggers the immediate expression of neutrophil microbicidal activity, the extent and nature of this response is greatly magnified if the neutrophil has initially been exposed to a (non-activating) priming agent. Indeed, the respiratory burst that occurs in response to a secretagogue agonist may be enhanced up to 20 fold by prior exposure of cells to a priming agent (Guthrie et al., 1984). Other functions upregulated by priming agents include agonist-induced degranulation (Fittschen et al., 1988) and the generation of lipid mediators (Doerfler et al., 1989; Doerfler et al., 1994). Priming agents consist of a variety of stimuli including LPS, TNFα, GM-CSF, G-CSF and a number of other cytokines (Guthrie et

al., 1984) and, except at very high concentrations, these agents are not able to elicit the effector function(s) by themselves (for recent review see Condliffe et al., 1998).

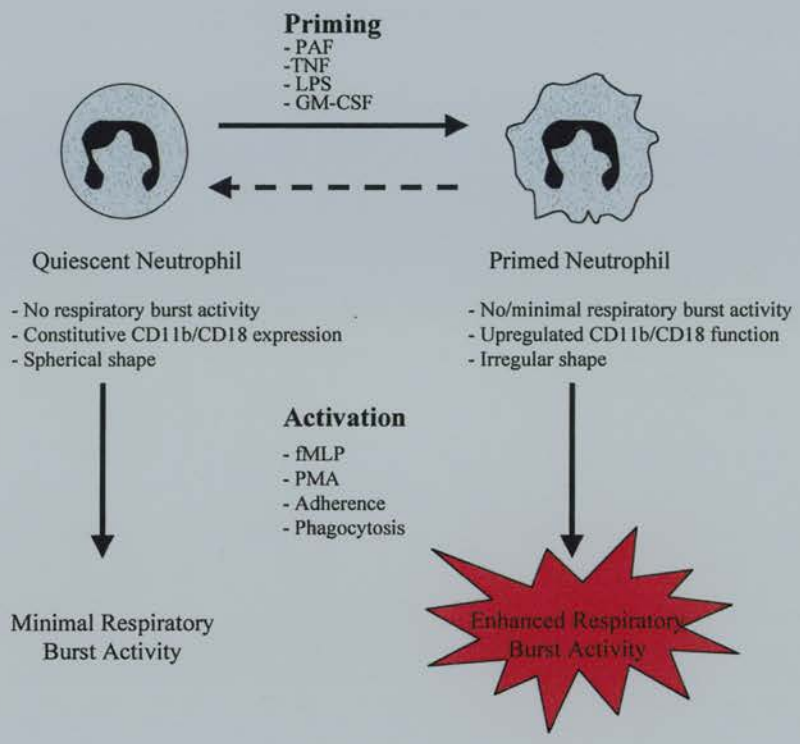

Figure 1.1.7 Schematic representation of neutrophil priming. Several pro-inflammatory mediators are able to prime neutrophils for an amplified respiratory burst to secretagogue agonists. Priming is also associated with other increased functional responses, including neutrophil shape change, adhesiveness and release of inflammatory mediators.

1.1.8 Cytokine production

It has been a long held belief that neutrophils are terminally differentiated phagocytes that arrive at the inflammatory focus pre-possessing all the weapons required to perform their primary role, i.e. that of phagocytosis and destruction of invading organisms and cellular debris. However, it is now recognised that neutrophils have a far greater synthetic capacity than originally believed and are in fact capable of producing a large number of additional proteins when appropriately stimulated. These include Interleukin (IL)-1 (Tiku et al., 1986; Lindemann et al., 1988), IL-6 (Cicco et al., 1990), IL-8 (Bazzoni et al., 1991), TNFα (Dubravek et al., 1990; Djeu et al., 1990), interferon α (Bronchud et al., 1988), C-CSF (Lindemann et al., 1989), and transforming growth factor-β (TGF-β) (Carrington et al., 1988).

Thus, it would appear that neutrophils are not solely 'pre-programmed' effector cells, but are also able to respond to environmental signals to further refine and orchestrate other aspects of the immune response at inflammatory sites.

1.1.9 Neutrophils and disease

Although neutrophils are essential to host defence, they have also been implicated in the pathogenesis of a wide variety of diseases. These include some forms of glomerulonephritis, rheumatoid arthritis, ischaemia-reperfusion injury and myocardial infarction and, in the lung, chronic bronchitis and emphysema, asthma, acute respiratory distress syndrome (ARDS) and a variety of types of interstitial lung disease. Host tissue damage may occur through several independent mechanisms. These include (i) premature neutrophil activation occurring in the intravascular space or during migration, (ii) extracellular release of neutrophil microbicidal products during 'frustrated phagocytosis' where micro-organisms are too large for full phagocytosis to occur, (iii) saturation of the normal controls preventing generalised or excessive activation or (iv) failure to terminate or resolve the acute inflammatory response. As well as playing a causative role in extending myocardial injury by releasing its histotoxic contents into areas of both necrotic and adjacent viable tissues during myocardial infarction, the neutrophil may also limit reperfusion by physically obstructing the myocardial microvasculture (Engler et al., 1986). Plugging of capillaries by aggregates of activated neutrophils may also be a relevant factor in other sites of ischaemia-reperfusion injury where neutrophil influx is thought to be triggered by substances released from damaged host cells or as a consequence of superoxide anion generation through xanthine oxidase (the superoxide radical appears to activate a latent neutrophil chemotactic factor present in the extracellular fluid) (Ricevetti et al., 1991; McCord et al., 1987). Activation of neutrophils by immune complexes in synovial fluid is also thought to contribute to the pathology of rheumatoid arthritis (Robinson et al., 1992). In addition, released ROS and granule enzymes from neutrophils have been shown to depolymerize long chain hyaluronan into smaller molecules and hence decrease its lucrative properties (Grootveld et al., 1991; Parkes et al., 1991) and inhibit the function of several other defence molecules including certain antiproteinases.

1.2 Apoptosis

Death of nucleated cells can be divided into two distinct types, necrosis (accidental death) or apoptosis (programmed cell death). Necrosis is usually a consequence of environmental insult and follows a relatively well-documented pathway (Trump et al., 1981a, b). Briefly, the cell undergoes swelling and blebbing, which rapidly becomes irreversible, and leads to the disintegration of the cell. Necrosis is characterised by damage to the plasma membrane, which leads to abnormal permeability of the cells to water and ions, and, thus, necrotic cells can be detected by their inability to exclude vital dyes such as trypan blue. In contrast, apoptosis represents a physiological form of cell death under the control of a 'cell specific' genetic program. It is widely accepted that apoptosis is crucial for the development and homeostasis of metazoan animals. For example, apoptosis serves as a prominent force in sculpting the developing organism (Granerus et al., 1995), as a major mechanism for regulating cell numbers in organs where there is rapid turnover (e.g. gut epithelium and haematopoietic system) (Raff, 1992) and as a defence mechanism to remove unwanted and potentially dangerous cells (Cohen, 1991), in particular cells that have been infected by viruses (Vaux et al., 1994) or undergone malignant transformation (Williams, 1991).

Apoptotic cells were initially described with respect to their morphological criteria (Wylie et al., 1980). Cells undergoing apoptosis shrink and lose up to one third of their cytoplasmic and nuclear volume; electron micrographs show an extremely condensed cytoplasm with some organelle swelling. The plasma membrane becomes ruffled and blebbed in a way more pronounced than that seen in necrosis. The most distinct changes, however, are seen at the nuclear level, although the exact pattern varies from cell to cell. In general, the nucleus condenses and its chromatin becomes very dense, collapsing into patches, then into crescents around the nuclear envelope and, finally, into one or several dense 'pyknotic' spheres. These changes are accompanied by the process of chromatolysis, which involves the activation of endonuclease activity (Wylie, 1981). Indeed, the orchestrated fragmentation of the DNA into a 'ladder' of regular sized subunits, the result of apparently random double-stranded DNA breaks in the linker regions between nucleosomal cores, has

become a classical hallmark of apoptosis. While, larger apoptotic cells may break into fragments, termed apoptotic bodies, these remain sealed with intact osmotic gradients; smaller cells do not usually fragment. Apoptotic bodies and cells are phagocytosed rapidly by macrophages or neighbouring phagocytic cells and, indeed, the removal of the cells without the release of cellular contents is very important in the prevention of inflammation and consequent damage to healthy cells.

1.2.1 Neutrophil apoptosis

Among the leukocytes, mature human neutrophils have the shortest life span (their half life in the circulation has been estimated at 6-7 hours). It is generally accepted that neutrophils are either removed from the circulation by the reticuloendothelial system of the liver and spleen or meet their fate, under pathophysiological conditions, at the inflamed site. There is little evidence that extravasated neutrophils return to the blood stream, or that the lymphatic drainage system provides an important disposal route (Haslett and Henson, 1988). It was originally assumed that neutrophil death occurred by necrosis (Hurley, 1983). However, if acute inflammatory lesions are to be resolved and not progress to chronicity, then neutrophils must be effectively disposed of in a manner that prevents the disgorgement of granule contents, which would otherwise lead to further tissue injury and a perpetuation of the inflammatory response. Death by necrosis, with its associated cell disintegration, would not be expected to fulfill such a role and, although subsequently ignored for many years, pioneer observations by Metchnikoff (1891), suggesting that intact neutrophils were engulfed by macrophages, implied that an alternative mechanism indeed existed.

Initial studies *in vitro* demonstrated that neutrophils died rapidly in a constitutive fashion, with changes characteristic of cells undergoing apoptosis (Lopez et al., 1986). More vigorous analysis of these cells have confirmed that this process is indeed apoptosis (Savill et al., 1989a); and it has been shown that during this event neutrophils also lose CD16 (FcγRIII) expression and demonstrate a reduced ability to degranulate, generate a respiratory burst or undergo chemotaxis in response to external stimuli (Whyte et al., 1993a). Thus, apoptosis may serve to limit tissue

injury and promote resolution of inflammation. Furthermore, it is now recognised that apoptosis is the triggering event that determines the recognition and phagocytosis of intact senescent neutrophils by macrophages (Savill et al., 1989a and b) and there is now clear evidence to support a role *in vivo* for apoptosis in the clearance of neutrophils from tissues in a variety of acute inflammatory disorders and their experimental models (Grigg et al., 1991; Cox et al., 1995). It is also noteworthy that macrophages phagocytose apoptotic neutrophils using a mechanism involving a novel recognition system that does not trigger macrophage release of pro-inflammatory mediators. This system utilises the integrin $\alpha v\beta 3$ (the vitronectin receptor) and CD36 (thrombosondin receptor) (Savill et al., 1990; Savill et al., 1992b). Finally, it is apparent that semi-professional phagocytes, such as fibroblasts and mesangial cells, also have the capacity to recognise and ingest apoptotic neutrophils *in vitro* although to a lesser extent than macrophages (Savill et al., 1992a; Hall et al., 1994). Thus, it is now evident that neutrophil apoptosis is a major process, essential for the successful resolution of granulocyte inflammation *in vivo*.

Although neutrophils appear to be 'pre-programmed' or committed to death via apoptosis, it is clear that the life span and functional longevity of these cells can be modulated significantly by a number of exogenous agents. These include pro-inflammatory cytokines, such as G-CSF, GM-CSF, IFN-γ, IL-8 and IL-2, bacterial products LPS and fMLP, chemotactic peptides e.g. C5a, anti-inflammatory glucocorticoids and calcium ionophores, all of which have been shown to inhibit neutrophil apoptosis (Cox et al., 1992; Colotta et al., 1992a; Lee et al., 1993; Leuenroth et al., 1998; Cox, 1996; Whyte et al., 1993b). TNFα has been reported to have a bi-phasic effect, inducing apoptosis at early times while inhibiting neutrophil death at late time points (Murray et al., 1997).

1.2.2 Molecular and genetic regulation of apoptosis

Apoptosis is widely regarded as a suicidal response since the dying cell appears to be an active participant in this process. Earlier studies in non-haemotopoeitic cells showing that chemical inhibition of protein or RNA synthesis delay or abrogate apoptosis (Wyllie et al., 1984; Cohen and Duke, 1984; Sellins and Cohen, 1987)

provided early evidence to support this view and led to the proposal that transcription and translation of specific genes is a requirement for death via apoptosis. However, later studies found that inhibitors of macromolecular synthesis had no effect and could even induce apoptosis in other systems, including constitutive apoptosis in neutrophils (Waring, 1990; Martin et al., 1990; Whyte et al., 1997). These latter observations may be explained by the presence of constitutive inhibitors of execution pathways in these cells. However, it was the recent identification and investigation of the molecular components of the apoptotic machinery that has provided the strongest support for the widespread involvement of an active, genetically determined cell death program.

Genetic analysis of the nematode *Caenorhabditis elegans* revealed three gene products essential for selective apoptotic cell deletion during worm development. These are CED-3 and CED-4, which promote apoptosis, with a third gene, CED-9, able to prevent their action (Ellis and Horvitz, 1986; Hengartner et al., 1992). This mechanism of apoptosis has proven to be remarkably conserved, albeit with greater complexity, in mammals. Bcl-2, the gene activated by chromosome translocation in human follicular lymphoma, was the first mammalian 'anti-apoptotic' regulator discovered (Tsujimoto et al., 1984). To date, at least 15 Bcl-2 family members have been identified in mammalian cells (Adams and Cory, 1998). The products of the mammalian Bcl-2 gene family are related to CED-9 but include two subgroups of proteins that either inhibit or promote apoptosis (Adams and Cory, 1998). The nematode CED-3 gene product was found to be homologous to a new family of mammalian proteases, the caspases (Yuan et al., 1993). These are a family of cysteine proteases, which cleave certain proteins after specific aspartic acid residues. They are synthesised as zymogens and are activated through cleavage that can be auto-catalytic or catalysed by other 'ICE-like' proteases (Kumar and Harvey, 1995). Indeed, there is *in vivo* evidence of a caspase catalytic cascade (Enari et al., 1996).

Members of this family of proteases are thought to play a major role in mammalian cell apoptosis and certain viral proteins or synthetic peptides have been shown to block apoptosis by acting as caspase inhibitors (Nicholson et al., 1995; Tewari and

Dixit, 1995; Xue and Horvitz, 1995; Fearnhead et al., 1995). CED-4 and its mammalian homologue, Apaf-1, have recently been recognised as adapters for facilitating the auto-catalysis, which can initiate activation of the caspase cascade (Zou et al., 1997). In addition, the caspase cascade can be activated by external stimuli via so called death receptors. These belong to the tumour necrosis factor/nerve growth factor (TNF/NGF) receptor gene superfamily, which is defined by the presence of a number of similar, cysteine-rich extracellular domains linked through a single transmembrane domain to a cytoplasmic death effector domain (Smith et al., 1994; Gruss and Dower, 1995; Tartaglia et al., 1993). Although the death domains can enable death receptors to engage the cell's apoptotic machinery, in some instances they also trigger a set of signals that serve distinct and even anti-apoptotic functions. One of the best characterised death receptors is the TNFR1 (also called p55 or CD120a), which is activated by its corresponding ligand TNFα. Upon binding TNFα the TNFR1 trimerizes, inducing association of the receptors' intracellular death domains. Subsequently, an adapter termed TRADD (TNFR-associated death domain) binds to the receptor death domain (Hsu et al., 1995) and functions as a platform adapter that recruits several additional signalling molecules to the activated receptor. Of these, FADD (Fas-associated death domain) couples the TNF1-TRADD complex to activation of caspase-8, thereby initiating apoptosis (Chinnaiyan et al., 1996; Varfolomeev et al., 1996).

1.2.3 Reactive oxygen species and apoptosis

A central role for reactive oxygen species (ROS) in apoptosis has been proposed based on a number of observations in different cell types: (1) the addition of ROS or the depletion of endogenous antioxidants has been shown to induce apoptosis (Lennon et al., 1991; Sandstrom et al., 1994b; Hockenbery et al., 1993), (2) apoptosis can sometimes be inhibited by the addition of endogenous or exogenous antioxidants (Wolfe et al., 1994; Ramakrishnan et al., 1993; Sandstrom et al., 1994a), (3) apoptosis has been demonstrated to be associated with increases in intracellular ROS levels (Heller and Kronke, 1994), (4) the nematode gene ced-9 is part of a bi-cistronic gene co-encoding a protein similar to cytochrome b_{560} of complex II of the mitochondrial respiratory chain, indicating that ced-9 might have redox or ROS-

regulatory functions (Hengartner and Horvitz, 1994), (5) Bcl-2 is located to organelles known to participate in redox reactions (Nakai et al., 1993) and, finally, (6) increased synthesis of Bcl-2 was shown to block the increased ROS levels associated with apoptosis (Hockenbery et al., 1993; Kane et al., 1993).

However, findings demonstrating that cells incubated in near-anaerobic conditions (where ROS production would be negligible) can still undergo apoptosis has argued against a ubiquitous and critical need for ROS generation during apoptosis (Jacobson and Raff, 1995). In addition, hypoxia itself has been shown to induce apoptosis in certain cells, an effect that can be inhibited by Bcl-2 and Bcl-XL (Shimisu et al., 1995). Thus, Bcl-2 can inhibit apoptosis in the absence of ROS suggesting that whatever antioxidant or ROS inhibiting properties Bcl-2 may possess, they are not crucial for the anti-apoptotic effect of this protein. Additional evidence arguing against a universal role for ROS in driving apoptosis came from experimental systems where antioxidants were clearly not capable of blocking all modes of apoptosis (Jacobson and Raff, 1995; Schulze-Osthoff et al., 1994). Furthermore, antioxidants and ROS scavengers undoubtedly serve many other functions that may interfere indirectly with the molecules required for apoptosis. For example, many antioxidants are reducing agents and, as such, might modify sulphydryl groups of proteins. An example of one such protein is the transcription factor AP-1 where DNA-binding activity is regulated by a redox mechanism. Metal ion chelators were also commonly used as antioxidants in these studies; however, various additional cellular enzymes require these ions to function and, thus, can be inhibited by such compounds.

This evidence argues strongly against a central role for ROS in mediating apoptosis; however, it would seem that ROS are capable of activating apoptosis, at least in certain cell types. Hence, apoptosis can be induced by treating cells either (i) directly with ROS, such as H_2O_2 or lipid peroxides (Lennon et al., 1991; Sandstrom et al., 1994b); (ii) with agents that promote intracellular ROS formation by inhibiting endogenous antioxidant functions (for example inhibition of catalase or depletion of intracellular glutathione) or (iii) with compounds that enhance ROS production (e.g.

transition metals or excess glucose) (Hockenbery et al., 1993; Ratan et al., 1994; Donnini et al., 1996). ROS have also been implicated in several physiological forms of cell death. For example, ROS have been proposed to play a role in TNFα-induced cytotoxicity. TNFα can kill by either apoptosis or necrosis depending on the concentration of TNFα, the cell environment and the cell type. TNFα-induced cell death can be inhibited by antioxidants, mitochondrial inhibitors and anaerobic culture conditions (Laster et al., 1988; Chang et al., 1992; Schulze-Osthoff et al., 1992). However, at least some of these effects appear to be due to an effect on cell necrosis rather than apoptosis.

1.2.4 The role of mitochondria in apoptosis

Several investigators have hypothesised that the origins of mitochondria and aerobic metabolism in eukaryotes formed the basis for the evolution of 'active' cell death and that apoptosis was developed as a mechanism to remove ROS-overproducing or ROS damaged cells (Frade and Michaelidis, 1997). Although such a hypothesis is virtually impossible to verify, a central role for mitochondria as mediators of apoptosis has been established in many systems. Not only are mitochondria a major source of ROS, they are also the site of electron transport, oxidative phosphorylation and adenosine triphosphate production and it is now recognised that these organelles contain a number of pro-apoptotic proteins. It is likely that each of these properties interrelate and together play a role in resulting cellular apoptosis. Disruption of electron transport has long been recognised as an early feature of cell death (Scaife, 1966; Garcia-Ruiz et al., 1997). One predicted consequence of electron transport failure would be a drop in ATP production; this has been observed during apoptosis, but often occurs quite late in the process (Bossy-Wetzel et al., 1998). Thus, although loss of mitochondrial ATP production may contribute towards apoptotic killing in some cells, it is unlikely that this is a general mechanism for the induction of apoptosis.

The collapse of the mitochondrial inner transmembrane potential ($\Delta\psi_m$) has been proposed to play a key role in apoptosis (see Kroemer et al., 1998 for recent review). Dissipation of the $\Delta\psi_m$ is mediated through the opening of a large conductance

channel known as the mitochondrial permeability transition (PT) pore. A series of drugs known for their PT-inhibitory potential, such as cyclosporin A or bongkrekic acid, inhibit the pre-apoptotic collapse and, in many systems, are effective inhibitors of receptor or stress-induced apoptosis. A further argument in favour of the implication of PT in apoptosis regulation is the finding that Bcl-2 functions as an endogenous PT inhibitor. This PT-protective effect by Bcl-2 has been observed in whole cells as well as in isolated mitochondria.

A critical role of the mitochondria in mediating apoptosis has been implied further by studies using a cell-free system in which the presence of mitochondria was essential for constitutive, Bcl-2 inhibitable nuclear morphological changes and DNA fragmentation (Newmeyer et al., 1994). Following this, studies in a second cell-free system found cytochrome c (released from the mitochondria during cytosol preparation) was required for activation of the caspase cascade by deoxyadenosine triphosphate (Liu et al., 1996). There is now evidence to suggest that cytochrome c (also termed Apaf-2) is released from the mitochondria during apoptosis both *in vitro* and *in vivo* and this release may be blocked by the presence of Bcl-2 located on these organelles (Yang et al., 1997; Kluck et al., 1997). Cytosolic cytochrome c forms an essential part of the vertebrate apoptosome, which is composed of cytochrome c, dATP, Apaf-1 and pro-caspase 9 (Li et al., 1997). This results in activation of caspase 9, which in turn is able to activate a cascade of lower 'effector' caspases and thus induce apoptosis. A second factor, apoptosis inducing factor (AIF)-1, is also released from mitochondria and this can also stimulate the proteolytic activation of caspase 3 (CPP32) (Susin et al., 1996; Susin et al., 1997).

1.3 Oxygen levels in the body

1.3.1 The oxygen cascade

The pO_2 of dry air at sea level is 21.2 kPa. However, this partial pressure is in no way physiological when compared to the pO_2 present at the cellular level. Oxygen moves down a partial pressure gradient from air, through the respiratory tract, the alveolus, pulmonary circulation, the systemic capillaries and the cell. While the pO_2 differs significantly within different parts of the body and even within particular

organs, it reaches the lowest level within the mitochondria where it is consumed. The steps by which the pO_2 decreases from air to the mitochondria are known as the oxygen cascade (see figure 1.3.1) and are of great practical importance. Any one step in the cascade may be increased under pathological conditions and this can lead to tissue hypoxia. The highest pO_2 value in the body is that in the alveoli and, although this P_AO_2 (Alveolar pO_2) may vary depending on the subject, the mean value for alveolar pO_2 in healthy adults is 13.6 kPa. The next highest pO_2 value is the P_aO_2 (arterial pO_2). This value is dependent upon the alveolar pO_2 and shows a modest but progressive decrease with age. Marshall and Whyche (1972) have suggested the following relationship in subjects breathing air: Mean arterial pO_2 = 13.6 – 0.044 (age in years) kPa. While the standard pO_2 value for mixed venous blood in the pulmonary artery is 5.3 kPa, this is again subject to variation.

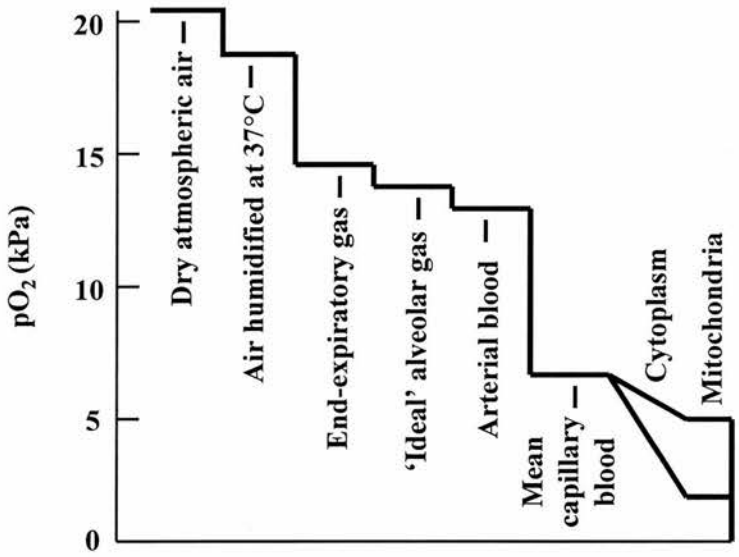

Figure 1.3.1 The oxygen cascade. The pO_2 falls from the level in ambient air down to the level in mitochondria, which is the site of utilisation. However, cellular oxygen tension varies depending on the cell's position relative to the capillaries (Adapted from Nunn, 1993).

1.3.2 Regulation of normal oxygen tensions in the tissues

The primary site of O_2 utilisation is at the tissue level, where cells require O_2 for the generation of the high energy phosphate bonds (ATP), which support cellular aerobic biochemical function. Thus, it would seem that the most meaningful pO_2 value would be that within the tissues. The overall extraction ratio of O_2 from capillary

blood is about 25% in healthy adults at rest but may increase to 70-80% during maximal exercise in well trained athletes. The relationship between pO_2 and % saturation of haemoglobin (the oxygen-haemoglobin dissociation curve) is non-linear. This sigmoidal curve is defined by the O_2 concentration at which 50% of the haemoglobin is saturated (P_{50}) - normally 3.5 kPa. However, various physiochemical factors (increased temperature, increased pCO_2, decreased pH and increased 2,3 diphosphoglycerate concentration) shift the capillary oxygen-haemoglobin dissociation curve to the right and, thus, cause enhanced release of O_2 to tissues and improved O_2 availability. Chemical release from haemoglobin is followed by diffusion through the endothelium and subsequently through the tissues to its site of utilisation in the mitochondria, although diffusion may be potentially aided by protoplasmic streaming. Diffusion paths are much longer in the tissues than in the lung. In well vascularized tissue, such as the brain, each capillary serves a cellular zone with a radius of about 20 μm; this contrasts to distances of approximately 200 μm in skeletal muscle and greater still in fat and cartilage. Krogh and Erlangen (1959) outlined the determinants of tissue oxygen tension by suggesting a cylindrical model to represent the volume of tissue served by a single capillary: the oxygen concentration (px) at the point x within a tissue depends upon the distance (x) from the point to the nearest capillary and also on the intercapillary distance (2R). The oxygen tension also depends on the radius of the capillary (r), the rate of oxygen consumption by the tissue (m) and the diffusion coefficient of oxygen in the tissue (d). The mathematical formulation based on these factors is as follows:

$$Px = Po - m/d(R^2/2\ \log x/r - x^2 - r^2/4)$$

In which Px is the oxygen tension in question and Po is the oxygen tension in the capillary directly opposite point x. Although this focuses on oxygen transport mediated by single capillaries, oxygenation of tissues as a whole is extrapolated from findings on oxygen exchange in these vessels. This analysis is clearly simplistic for complex organs and is under review (Intaglietta et al., 1996), not least because capillaries are not the sole source of oxygen and it is becoming increasingly apparent

that arterioles are a significant equivalent source, with venules serving as sinks for capillary and arteriolar oxygen.

1.3.3 Measurement of tissue pO_2

A number of methods can be used to estimate tissue pO_2 directly. (1) One of the most frequently employed methods utilises polarographic electrodes. The electrodes consist of a cell formed by a silver anode and a platinum cathode, both in contact with an electrolyte in dilute solution. When a potential difference is applied to the cell, a current is passed which is directly proportional to the pO_2 of the electrolyte in the region of the cathode. At the end of the electrode is a thin membrane, which separates the electrolyte from the sample and is gas permeable. Hence, the electrolyte is able to equilibrate rapidly with the sample and, thus, the current passed by the cell is proportional to the pO_2 of the sample. Electrodes may be inserted directly into tissue or placed or attached on or to the surface of an organ to indicate the pO_2. (2) A different approach is to implant a silastic tonometer (gas permeable silastic tubing) within the tissue by means of a wide bore needle. The tube is then perfused with deoxygenated saline solution and the pO_2 of the fluid inside the tonometer is then measured after a suitable equilibration period. (3) The oxygenation status of the tissue may be determined biochemically by the use of transmission spectroscopy in the near infrared (700-1000nm), where tissues are relatively translucent. At present, however, the path length is limited, although successful measurements of cardiac and cerebral tissue pO_2 values have been obtained. (4) An indication of tissue pO_2 may also be obtained by measuring the venous pO_2 of blood draining a particular tissue. However, the significance of this index is lost when there are shunts that enable arterial blood to mix with blood draining the tissue. (5) Finally, polarographic estimation of skin pO_2 has been advocated as a non-invasive method of determination of pO_2. A polargraphic electrode is applied to the skin, which must be heated to at least 44°C to maximise cutaneous blood flow (Severinghaus, 1981). The resultant pO_2 does not equal arterial pO_2 in all circumstances, but maybe used as an indirect determination of the overall oxygenation state of a patient.

From such methods it is evident that tissue pO_2 varies from one organ to another, with the tissue pO_2 being dependent, not only on arterial pO_2, but also on the ratio of tissue oxygen consumption to perfusion. However, as predicted by the Krogh model (see section 1.3.2), even greater difficulties arise where variations in tissue pO_2 exist in different parts of the same organ. These are mainly due to regional variations in tissue perfusion and oxygen consumption. Within an organ there are invariably some areas occupying more favourable sites towards the arterial ends of capillaries, while others must accept oxygen from the more distal ends of these blood vessels. Thus, unlike arterial and mixed venous pO_2, tissue pO_2 is not a uniform or fixed entity and, consequently, there is variation in reported pO_2 values for the same tissue. For example, reported skeletal muscle pO_2 values range from 0 - 13.1 kPa, with mean values varying from 2.13 ± 0.53 kPa to 5.87 kPa ± 0.27 (Kunze, 1969). Likewise, in the liver steep intercellular gradients exist along the sinuoids from the portal to the central regions of the liver lobules (Sies, 1977; Ji et al., 1982). As demonstrated by the use of micro-needle electrodes, the actual pO_2 around an individual hepatocyte in the liver ranges between 0.13 and 8 kPa, with mean values around 2.9 kPa (De Groot and Noll, 1987).

Tissue	60 μm diameter electrode pO_2 (kPa)	330 μm diameter electrode pO_2 (kPa)
Liver	1.73 ± 0.27	2.27 ± 0.4
Kidney	2.67± 0.27	3.73 ± 0.53
Intraperitoneal	6.13 ± 0.8	6.53 ± 0.53
Subcutaneous	3.07 ± 0.4	4.13 ± 0.27
Brain	4.93 ± 0.8	4.4 ± 0.27
CSF subarachnoid space	4.67 ± 0.93	4.67 ± 0.27
Spleen	2.27 ± 0.27	3.07 ± 0.27

Table 1.3.3 Effect of electrode size on pO_2 measurements in rat tissue. Results from Jamieson and Van Den Brenk (1964) showing that electrodes with larger diameters tend to give higher tissue pO_2 values. These differences have been attributed to tissue damage caused during electrode insertion. See text for more detail.

Further variation has also been attributed to the method used to measure tissue pO_2. Jamieson and Van Den Brenk (1964) demonstrated that the physical trauma produced by electrode insertion causes quantitative estimations of pO_2 that outweigh errors of calibration (see table 1.3.3). When electrodes were inserted into the liver, kidney cortex or spleen, macroscopic bruising was immediately apparent. As smaller electrodes tend to give somewhat lower values of tissue pO_2, it appears that any bleeding that occurs paradoxically gives rise to a higher value of pO_2 than initially present in the tissue. Alteration of O_2 consumption in surrounding damaged cells will also contribute to tissue pO_2 changes.

1.3.4 Causes and consequences of tissue hypoxia

Significant hypoxia is best regarded as a deficiency of O_2 at the tissue level, and approximately 8 decades ago Barcroft (1920) introduced a classification of the forms of hypoxia that, with modifications and minor name changes, remains in use today (West, 1990). Hypoxia has been divided into 4 types: (1) hypoxic hypoxia, in which the pO_2 of the arterial blood is reduced; (2) anaemic hypoxia, in which the arterial pO_2 is normal but the amount of haemoglobin available to carry O_2 is reduced; (3) stagnant or ischaemic hypoxia, in which the blood flow to a tissue is so low that adequate O_2 is not delivered, despite a normal pO_2 and haemoglobin concentration; and (4) histotoxic hypoxia, in which the amount of O_2 delivered to a tissue is adequate but, because of the action of a toxic agent, the tissue can not make use of the O_2 supplied. Tissue hypoxia features prominently in a number of pathological conditions including tissue injury, lung disease, myocardial infarction, rheumatoid arthritis, sepsis and hypovolaemic shock.

The impositions of the effects of injury on most of the determinants shown in the Krogh model equation predict a decrease in tissue oxygenation. The intercapillary distance is increased, as is the rate of O_2 utilisation by accumulation of O_2-consuming leukocytes and the formation of granulation tissue. The blood flow is also reduced in the centre of the wound because of thrombosis of the microcirculation in that area. Wound pO_2 levels have been measured by Niinikoski et al (1972) and Hunt et al., (1967). These groups reported values of 0.8 and 1.5 kPa

respectively when measuring wound 'dead space' using an implanted tonometer. Since the dead space is generally the area farthest removed from the capillary, this tension probably represents the lowest average extracellular oxygen tension within the wound. Niinikoski and co-workers (1972) also investigated the oxygen gradients around damaged tissue using microelectrodes. Using this method, the pO_2 of the fluid filling the dead space was very low and persistently showed tensions between 0 and 0.4 kPa. At the point the electrode moved from the dead space to the new tissue, the pO_2 varied between 0.7 and 2 kPa. This region was occupied predominantly by cells resembling macrophages. In the area occupied by fibroblasts, i.e. closer to the distal capillaries, the pO_2 was 2.7 to 4 kPa. The oxygen gradient of this area was extremely steep, from 10 to 12 kPa directly over the capillary to a mean of 2.7 kPa in the fibroblast region 50 μm from the vessel.

Tissue hypoxia is an essential feature of hypovolaemic shock. In traumatised patients gross injury can induce irreversible changes and inhibit wound healing and increase susceptibility to infection. Correction of cardiac index and arterial pO_2 does not necessarily ensure a normal tissue pO_2 and, therefore, arterial pO_2 tension provides an inadequate index of peripheral tissue oxygenation. In patients with multiple injury and hypovolaemic shock the tissue pO_2 remains depressed for several days. Niinikoski (1977) investigated the effect of hypovolaemic shock on myocardial and skeletal muscle tissue oxygenation. They found that profound decreases of myocardial oxygenation occurred, even in moderate hypovolaemia (control myocardial pO_2 = 4 kPa vs hypovolaemic pO_2 = 2 kPa); they also found that occlusion of the left anterior descending coronary artery lowered myocardial tissue pO_2 even further to 1.3 kPa. Similarly, in skeletal muscle, control pO_2 values of 4.1 ± 0.7 dropped to 0.3 - 0.7 kPa following haemorrhage. Hypovolaemia also resulted in a complete cessation of blood flow in distal capillaries and a fully anoxic environment at the wound edge (Silver, 1973).

Multiple organ failure appears to be a major factor determining the poor outcome of patients with acute respiratory distress syndrome (ARDS). One mechanism that has been proposed to contribute to multiple organ failure is regional hypoxia in tissues

rendered particularly vulnerable by the high metabolic demands of ARDS. Profound arterial hypoxaemia is a diagnostic criterion of ARDS, and is largely caused by ventilation-perfusion mismatch and right to left shunting (Petty and Fowler, 1982; Wallace and Spence, 1983; Dantzker et al., 1979), which presumably results from blood flow through under- or non- ventilated areas of lung. Furthermore, ARDS also appears to be associated with peripheral gas exchange abnormalities manifested as an abnormal relationship between systemic oxygen uptake and systemic oxygen delivery. This may be explained by a combination of a greater basal metabolic requirement and a decreased ability of the tissues to extract oxygen from the blood. Due to the impracticalities of directly measuring tissue pO_2 in patients with ARDS metabolic markers of anaerobic metabolism have been monitored instead and, in several studies, have shown elevated blood lactate concentrations in conditions such as sepsis and ARDS (Gilbert et al., 1986; Vincent et al., 1990). The products of adenosine monophosphate (AMP) degradation, namely inosine, hypoxanthine, xanthine and uric acid have also been proposed as metabolic markers of tissue hypoxia and Grum et al. (1985) noted an increase of these metabolites in the venous blood of critically ill patients with ARDS.

As in ARDS, tissue oxygen requirements rise above normal in patients with sepsis. However, it is not known whether the tissue dysfunction observed in sepsis results from cellular hypoxia or deranged intracellular oxygen metabolism. The tissue hypoxia associated with sepsis may result from inadequate oxygen delivery to 'at risk' areas, consequent upon disturbed microvascular control or from abnormal extraction of oxygen, despite adequate perfusion. Sepsis is believed to disrupt microcirculatory flow and nutrient exchange and, in addition, cause intravascular leukocyte aggregation, abnormal red cell deformability, increased microvascular permeability, interstitial protein loss and tissue oedema. These changes are promoted by proinflammatory mediators and modulated by the function and integrity of the endothelium (Curzen et al., 1994). Indeed, Sair et al. (1996), measuring skeletal muscle pO_2 in a rat model of endotoxaemia using implantable tissue electrodes, found a significantly lower tissue pO_2 in endotoxaemic rats compared to control

animals (3.2 kPa vs 6.7 kPa) when the arterial blood pO_2 in sham and endotoxin treated animals was not significantly different.

The rheumatoid synovium has been widely studied as an example of a pathological hypoxic environment. This occurs due to a combination of increased O_2 utilisation by metabolically active tissue (oxygen consumption by rheumatoid synovial membrane has been reported to be twenty times that of normal synovium (Dingle and Page-Thomas, 1956)) and an inadequate, or impaired, supply of oxygen to the tissue. When Falchuk and co-workers (1970) analysed synovial fluid samples from 30 patients with a variety of conditions, the lowest pO_2 values were found in those with the most active and severe rheumatoid disease. Similarly, Lund-Oleson (1970) measured the oxygen tension in 103 joint aspirates obtained from patients with rheumatoid arthritis (RA) (n =85) and a variety of other conditions (n=18). The lowest oxygen tensions were found in the rheumatoid group (mean 3.5 kPa). Treuhaft and McCarty (1971) analysed 55 synovial fluid samples and, in general, found lower pO_2 values in fluids obtained from patients with inflammatory arthritis and this has been confirmed subsequently by other workers (Goetzl et al., 1971). While the measurement of oxygen tension in aspirated synovial fluid does not necessarily reflect the situation in the synovium, when O_2 tensions were measured directly in the knee synovium and peri-articular tissues of patients with RA and osteoarthritis, as well as normal subjects, the results confirmed that the lowest pO_2 tensions were found in the rheumatoid group (mean control value = 6.7 kPa vs mean RA patients = 3.8 kPa) (Ellis et al., 1994).

When flow through a coronary artery is reduced the part of the myocardium that it supplies becomes hypoxic; if the myocardial ischaemia is severe and prolonged, irreversible changes occur in the muscle and the result is myocardial infarction. Several groups have investigated myocardial pO_2 using animal models of myocardial infarction. Feola and colleagues (1979) reported that occlusion of the left anterior descending coronary (LAD) artery in dogs produced a rapid and significant drop in myocardial pO_2 to 0.33 ± 0.13 kPa compared to control values of 1.9 ± 0.4 kPa. Likewise, in a second study using pigs, Walfridsson and co-workers (1985) found

that myocardial pO_2 dropped rapidly following LAD occlusion; in the representative experiment shown, tissue pO_2 levels dropped from 6-8 kPa to 0 kPa in most myocardial regions measured. Finally, a study by Faithful et al. (1996) using pigs ventilated with 100% oxygen gave mean control myocardial oxygen tensions of 15 ± 1.1 kPa with a reduction following LAD occlusion to 8 kPa and 10 kPa in the ischaemic area and ischaemic border respectively.

The measurement of absolute tissue pO_2 values in such disease states is invaluable in furthering understanding of these conditions. Moreover, these values would contribute in the design of suitable *in vitro* systems in which to study individual cell responses to similar levels of hypoxia. At present, the measurement of the extent of tissue hypoxia in these pathological conditions is hampered, not only by natural variations in tissue pO_2 within individual organs and limitations in methodology as discussed in section 1.3.3, but also by the impracticalities of studying critically ill patients and the need for invasive monitoring. Thus, studies are predominantly restricted to those using animal models. However, such experiments demonstrate that pathological conditions can result in profound tissue hypoxia and suggest that tissue pO_2 values can be reduced to levels only slightly above zero.

1.4 Hypoxia and signalling

The primary role of oxygen in most eukaryotic cells is to insure an adequate energy supply via aerobic metabolism. Therefore, the ability to detect a reduced oxygen tension in the environment and adapt to O_2 deprivation is an essential requirement of most complex cells and organisms. Mammals respond to a drop in environmental O_2 (hypoxic hypoxia) with both short and long term adaptive mechanisms that are designed to maintain O_2-delivery to the tissues at a near-normal or stably reduced level. The immediate response of the body to hypoxic hypoxia is usually a compensatory increase in respiratory rate. This is initiated by the activation of arterial O_2 chemoreceptors, which are located strategically near the carotid arteries. In these organs, a decrease in arterial pO_2 is translated into afferent nerve discharges, stimulating the brain stem respiratory centres to produce hyperventilation (McDonald, 1981). A second short term response is initiated by acute local hypoxia

and involves vasodilatation of most systemic arteries in order to increase blood flow and, thus, O_2 delivery to hypoxic tissues. In contrast, hypoxia causes constriction of pulmonary resistance arteries, thereby diverting blood towards better ventilated alveoli (Wadsworth, 1994). Longer term responses to hypoxia include an increase in the production of red blood cells and the growth of new blood vessels. Red blood cell production is under the control of the glycoprotein hormone erythropoietin, and expression and secretion of erythropoietin by the kidney and liver is enhanced dramatically by chronic hypoxia. By this mechanism, the production of red blood cells is increased and the O_2 carrying capacity of the blood enhanced accordingly. A second response to chronic hypoxia is angiogenesis, or the growth of new vasculature. This process plays a critical role during development and wound healing and provides increased blood flow to previously poorly vascularized or hypoxic areas. This process appears to be mediated by vascular endothelial growth factor (VEGF), a mitogenic growth factor highly specific for endothelial cells (Shweiki et al., 1992).

Characterisation of the cellular mechanisms whereby such O_2-sensitive cells modulate gene expression in response to hypoxia has been greatly facilitated by the development of suitable cell lines. These include the pheochromocytoma (PC-12) cell line, which serves as an *in vitro* model for the carotid body type I cells (Czyzk-Krzeska et al., 1992; Czyzk-Krzeska et al., 1994), and the fetal hepatoma cell lines Hep3B and HepG2, which have provided an important system for the detailed molecular study of EPO gene regulation by hypoxia (Fandrey and Bunn, 1993). Recently, however, it has become clear that the concept of 'O_2-sensing' is not limited to a few highly specialised O_2 sensitive cells. All aerobic cells are capable of detecting O_2 deprivation and responding by adapting certain cellular functions that, in many cases, involves changes in gene expression. The molecular mechanisms underpinning these effects of hypoxia have many similarities but vary depending on the cell type and function, as well as the degree and duration of O_2 deprivation.

1.4.1 Hypoxic regulation of gene expression

In general, cells reduce their total RNA synthesis by 50-70% under hypoxic conditions. However, an increasing number of genes are being identified whose transcription is markedly augmented when cells lack oxygen. Hyperventilation, caused by stimulation of the carotid body by hypoxia, is associated with an increase in the activity of tyrosine hydroxylase (TH), the rate limiting enzyme in dopamine biosynthesis in glomus cells. This augmented TH activity is regulated at the mRNA level in carotid body type I cells (Czyzk-Krzeska et al., 1992). This finding agrees with subsequent studies, which have shown that hypoxia increases TH expression in the PC-12 cell line by increasing transcription and stabilising the mRNA transcript of TH (Czyzk-Krzeska et al., 1994). Likewise, erythropoietin (EPO) production in Hep3B cells has been shown to be induced directly by hypoxia and, again, this effect is due to increased transcription, as well as increased stability of EPO mRNA (Goldberg et al., 1991).

In addition to EPO and TH, several other gene products are now recognised to be positively regulated by hypoxia. Perhaps unsurprisingly, this group of genes includes a number of enzymes involved in anaerobic metabolism; these consist of several glycolytic enzymes (e.g. phosphoglycate kinase-1, lactate dehydrogenase A, aldolase A and C, phosphofructase L and C, pyruvate kinase M and enolase A) and a number of glucose transporters (GLUT-1, GLUT-2 and GLUT-3) (Wood and Ratcliffe, 1997). In contrast, hypoxia has been reported to decrease expression of the mitochondrial respiratory enzymes (Murphy et al., 1984). Other hypoxia-regulated genes are involved in inflammation, tumourogenesis, coagulation and cell growth. Of the hypoxia-induced growth factors vascular endothelial growth factor (VEGF) has been studied in most detail, but this group also includes platelet derived growth factor B chain (PDGF-B) (Kourembanas et al., 1990), placental growth factor and transforming growth factor β-1 (TGFβ-1) (Wood and Ratcliffe, 1997). Hypoxic regulation of VEGF expression occurs in many tissues and cell lines and this growth factor has become widely recognised as a major angiogenic factor stimulating endothelial cell mitogenesis and capillary proliferation (Shweiki et al., 1992). Hypoxia has been shown to increase transforming growth factor (TGF)-β1

expression in human dermal fibroblasts and it has been proposed that this response may contribute to enhanced collagen synthesis and the process of wound healing (Falanga et al., 1991; Falanga et al., 1993; Falanga and Kirsner, 1993). Human umbilical vein endothelial cells (HUVEC) have been shown to increase expression of interleukin (IL)-1α after hypoxic exposure and thereby enhance adherence of leukocytes during subsequent re-oxygenation owing to IL-1α dependent up-regulation of endothelial cell-leukocyte adhesion molecule and intercellular adhesion molecule (ICAM-1) (Schreeniwas et al., 1992). Release of IL-8 from hypoxic HUVEC (Karakurum et al., 1994) and induction of procoagulative and 'oedemogenic factors' in cultured bovine endothelial cells (Ogawa et al., 1990; Ogawa et al., 1991) has also been reported. It is also noteworthy that several candidate vasoconstrictors and growth factors (e.g. endothelin (ET)-1, PDGF) are increased in the lungs of hypoxic animals (Cai et al., 1996; Muramatsu et al., 1997). Finally, macrophages have been reported to produce enhanced levels of inflammatory mediators in response to hypoxia; these include TNFα, IL-6 (Albina et al., 1995), macrophage inflammatory protein-1 (MIP-α) (VanOtteren et al., 1995) and IL-1 (Hempel et al., 1996). Collectively, these results support the direct regulation of several gene products in vascular tissue by hypoxia that may contribute to the vascular pathologies and coagulative disorders associated with ischaemia-reperfusion injury and other cardiovascular pathophysiologies.

1.4.2 Stress proteins

A generalised response to many cellular stresses is the induction of a prescribed set of proteins, the so-called stress proteins. These are classically sub-divided into two families, the heat shock proteins (HSPs) and the glucose-regulated proteins (GRPs). Their names are derived from the stimuli traditionally known to induce them. HSPs were first described following exposure of cells to increased environmental temperatures and GRPs are classically induced following glucose deprivation. However, most cell types studied to date, including Chinese hamster ovary cells, mouse mammary tumour cells, rat embryo cells, rat fibroblasts, myogenic cells and cardiac and brain tissue, also induce HSP and/or GRP synthesis in response to hypoxia (Sciandra et al., 1984; Heacock and Sutherland, 1986; Roll et al., 1991;

Dwyer et al., 1989; Knowlton et al., 1991). Although their precise function is, as yet, unclear, these proteins appear to play a role in maturation and/or repair of nascent or damaged proteins (Becker and Craig, 1994; Hightower, 1991) and it is thought that induction of these proteins may help cells survive otherwise lethal stress. GRP and HSP expression has also been linked to certain forms of drug resistance. For example, chronic anoxia, 2-deoxyglucose (both known GRPs inducers) and heat shock can all lead to a cellular resistance to adriamycin and these findings may be relevant to the increased resistance of hypoxic tumour regions to chemotherapy (Shen et al., 1987).

More recently, a third group of stress proteins termed hypoxia-associated proteins (HAPs) have been identified (Zimmerman et al., 1991; Graven et al., 1993). As their name suggests, these proteins are specifically induced by hypoxia. They appear distinct from HSPs and GRPs and cannot be induced by heat shock or glucose deprivation. To date, HAP expression appears to be restricted to endothelial cells where it is thought to contribute to endothelial cell hypoxic tolerance (Graven et al., 1995). However, direct evidence for this is still required.

1.4.3 Oxygen sensing

The mechanism(s) by which mammalian cells sense hypoxia remains obscure. However, several models have been proposed and these are discussed in this section. There are two main pathways by which oxygen may interact with biological systems. Firstly, oxygen may act as an electron acceptor in redox reactions, for example its role as the terminal electron acceptor in the mitochondrial respiratory chain. Secondly, oxygen may react with specific 'ligands'. However, because of oxygen's unique chemical properties, there are a limited repertoire of molecules to which it can combine. Probably the best known oxygen ligands are the haem proteins exemplified by the oxygen carrying protein haemoglobin. Thus, it is not surprising that a similar haem protein based mechanism has been sought in order to explain mammalian oxygen sensing. The main evidence supporting a role for such a protein comes from experiments utilising carbon monoxide (CO). CO reacts specifically with the O_2 binding site of haem-proteins, substituting for O_2, and has been shown to

inhibit hypoxia-induced expression of erythropoietin (EPO) and vascular endothelial growth factor (VEGF) and has a parallel effect on O_2-sensing in the carotid body (Goldberg et al., 1988; Goldberg and Schneider, 1994). Further evidence comes from studies showing that the haem synthesis inhibitor, 4,6-dioxoheptanoic acid, produced a reduction in EPO production in response to hypoxia (Goldberg et al., 1988). Goldberg and co-workers (1988) proposed that the putative haem-protein sensor might switch from an oxygenated to a deoxygenated state like haemoglobin allowing close control of gene expression depending upon the cellular oxygen tension. A haem-based O_2 sensor system has been found in *Rhizobium meliloti*, a bacterium whose nitrogen-fixation genes are regulated by the ambient O_2 tension (Gilles-Gonzalez et al., 1991). In this system the O_2-sensor FIXL is an integral membrane haem containing kinase whose activity is modulated by O_2 binding. In its deoxy-conformation the kinase phosphorylates the transcription factor FIXJ, which turns on a cascade of transcriptionally controlled events leading to the expression of gene products engaged in nitrogen fixation. However, no such molecule has been identified in mammals and subsequent observations that EPO gene regulation can also be upregulated by iron chelators (Wang and Semenza, 1993) are hard to accommodate using this model as haem iron is non chelatable.

Other lines of evidence are also inconsistent with a haem-protein based O_2-sensing model and have suggested an alternative mechanism based on intracellular ROS levels. The sequence of the oxygen sensor in *Rhizobium meliloti* was found to share some homology with a human cytochrome P_{450}, a member of the *b*-type haem protein family (Lois et al., 1993), and Fandrey and co-workers have suggested that such a molecule may be important to mammalian oxygen sensing. This group found that inhibitors of cytochrome P_{450} reduced, and enhancers of this enzyme increased, EPO-production (Fandrey et al., 1990). More recently, spectrophotometric analysis demonstrated the presence of a *b*-type cytochrome in hepatoma (Gorlach et al., 1993) and type I carotid body cells (Cross et al., 1990). Further work showing that iodonium compounds exert powerful inhibitory effects on the hypoxic induction of gene expression suggest the involvement of a flavoprotein oxidoreductase (Gleadle et al., 1995b) and haem proteins in the group of b-cytochromes include such

enzymes, for example, NADPH-oxidase. This enzyme catalyses the production of H_2O_2 from molecular O_2. Experiments showing that EPO induction by hypoxia could be aborted by the addition of H_2O_2 or by an inhibitor of catalase suggested that H_2O_2 may play a role in the regulation of hypoxia-responsive genes (Fandrey et al., 1994). The effect of iron chelators in this model can be explained by a decrease in iron-catalysed production of ROS. However, the failure of many other antioxidants agents to mimic the effects of hypoxia on gene expression is not consistent with this theory. In addition, the ability of certain transition metals to induce EPO, and other hypoxia-induced gene expression (Goldberg et al., 1988) is also hard to explain given that these ions are more likely to have pro-oxidant actions. It is also very unlikely that NADPH oxidase itself is involved as patients with genetic subtypes of chronic granulomatous disease, characterised by the absence or abnormality of one of the subunits of this enzyme, have no evidence of disordered oxygen sensing (Wenger et al., 1995).

1.4.4 Redox sensitive transcription factors

The mechanisms by which cells may sense changes in oxygen concentration remain unclear. However, oxygen-dependent induction of hypoxic-sensitive genes depends, to a large extent, on an increase in their transcriptional rate and a number of transcriptional activators involved in the up-regulation of genes by hypoxia have now been identified. The most widespread mechanism identified in regulating mammalian hypoxic gene expression involves interaction of, the recently identified, hypoxia-inducible factor-1 (HIF-1) with specific cis-acting elements within promoters and enhancers of target genes (for recent review see Ratcliffe et al., 1998). This transcription factor was initially described in relation to its role in EPO gene expression in hepatoma cell lines. In response to hypoxia HIF-1 was shown to bind to an enhancer element containing the sequence 5' TACGTGCT-3' (Semenza and Wang, 1992), which lies 3' to the EPO gene. HIF-1 is a heterodimer consisting of the subunits HIF-1α and HIF-1β; both these proteins have now been purified, and details of their characterisation and regulation are discussed in Chapter 5. Because of the highly tissue specific expression of EPO, it was initially thought that the O_2-sensitive mechanism regulating its transcriptional control would be restricted to the

cells in the kidney and liver which produce EPO. However, expression of reporter genes containing the hypoxic-responsive cis-acting sequence from the EPO gene has subsequently been identified in a wide variety of cell types (Maxwell et al., 1993). Thus, it appears that HIF-1 may represent part of a universal oxygen sensing mechanism. In accordance with this, the binding sequence for HIF-1 has been identified in a number of other hypoxia-induced genes. Such genes include those encoding the glycolytic enzymes aldolase phosphoglycerate kinase, phosphofructokinase and lactate dehydrogenase (Semenza et al., 1994; Firth et al., 1994), VEGF (Forthsythe et al., 1996), tyrosine hydroxylase (TH) (Norris and Millhorn, 1995), GLUT-1 (Ebert et al., 1995), iNOS (Melillo et al., 1995) and HO-1 (Lee et al., 1997). In all these cases, binding of HIF-1 to its consensus sequence appears to be necessary, if not sufficient, for hypoxic regulation of transcription. Indeed, further analysis has suggested that other transcription factors beside HIF-1 may also be required, or at least have the potential, to enhance the hypoxic induction of some genes. For example, the transcriptional activator p300 has been reported to increase the response of the EPO enhancer to hypoxia, in a HIF-1 dependent manner (Arany et al., 1996). The TH enhancer region, as well as containing HIF-1 binding sites, also includes AP-1 and AP-2 binding sites. Indeed, binding of AP-1 to its respective sites appears to be critical for hypoxic induction of the TH gene. Hypoxia has also been demonstrated to induce binding of a c-fos and JunB dimer to the AP-1 element in PC-12 cells and mutation of the AP-1 site in a TH reporter gene construct was sufficient to prevent hypoxic stimulation of TH gene activity (Norris and Milhorn, 1995). A second hypoxia-regulated gene known to contain the AP-1 site in its promoter region is TGF-β and preliminary experiments using a TGF-β promoter-CAT reporter construct suggest that the AP-1 binding site is involved in hypoxic induction (Helfman and Falanga, 1993). AP-1 binding has also been proposed to play a role in the hypoxic upregulation of IL-8 by human macrophages (N. Hirani, unpublished data). A further candidate for gene redox regulation is NF-κB: nuclear extracts from hypoxic HUVEC have been shown to contain proteins, which bind to a NF-κB site on the IL-8 promoter (Karakurum et al., 1994). However, the role of NF-κB binding in hypoxia has been questioned as it is also activated by pro-oxidant conditions and is down regulated by antioxidants (Meyer et al., 1993).

1.5 AIMS

The principal aim of this thesis was to determine the potential for hypoxia to regulate human neutrophil apoptosis *in vitro*.

(1) Preliminary studies focused on assessing the rate of spontaneous neutrophil apoptosis under varying oxygen tensions. Having established that hypoxia caused a marked enhancement of neutrophil longevity by inhibiting apoptosis, subsequent experiments were designed to investigate the kinetics of this inhibition.

(2) A study of the intracellular signalling pathway(s) underlying the inhibition of neutrophil apoptosis by hypoxia was undertaken and the potential involvement of several redox sensitive transcription factors explored.

(3) The putative involvement of an oxidant based pathway in mediating TNFα-induced apoptosis in neutrophils was investigated and the role of the caspase family of proteases in this effect studied.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

The following reagents were obtained from R&D Systems Europe Ltd (Oxon, UK): TNFα was dissolved in PBS at 10 μg/ml and stored at -80°C; mouse IgG_1 anti-human TNFR55 mAb was dissolved in PBS at 500 μg/ml and stored at -20°C.

The following reagents were obtained from Genzyme Diagnostics (Kent, UK): rat IgG_{2b} anti-human TNFR75 mAb (1 mg/ml in PBS) was stored at 4°C; GM-CSF (1000 U/ml in PBS) was stored at -70°C.

The following reagents were obtained from Gibco Life Technologies (Paisley, Scotland, UK): Iscove's Dulbecco's modified Eagles medium, without supplements with L-glutamine (Iscove's MDM); culture supplements penicillin (50 U/ml)/streptomysin (50 U/ml); TRIZOL® reagent; trypsin-EDTA (1x) in HBSS without calcium and magnesium; L-glutamine (200 mM); 10% SDS; Hepes buffer solution (1M); Iscove's MDM without D-glucose or sodium pyruvate (made to order); dialysed fetal calf serum and 30% (w/v) acrylamide/bis solution.

The following reagents were purchased from Promega (Southampton, UK): HeLa nuclear extract (approximately 5 mg/ml protein); T4 polynucleotide kinase; T4 polynucleotide kinase 10x buffer; gel shift binding 5x buffer and AP-1 and NF-κB consensus oligonucleotides.

Sodium citrate solution (3.8%) was purchased from Phoenix Pharmaceuticals Ltd. (Glouster, UK). Calcium chloride was obtained from Martindale Pharmaceuticals Ltd. (Essex, UK). Desferrioxamine mesylate was purchased from CIBA Pharmaceutical Company (New Jersey, USA). Hsp70 mouse monoclonal IgG was obtained from StressGen Biotechnology's Corporation (Canada). Z-DEVD-FMK was purchased from Calbiochen-Novabiochem Corporation (Nottingham, UK) and

Hybond C nitrocellulose membrane from Amersham Life Science Ltd. (Cheshire, UK). Horse radish peroxidase-conjugated, goat polyclonal, anti-mouse, secondary antibody, MAC387 monoclonal antibody, anti-human Bcl-2 IGG_1 (clone 124) mouse monoclonal antibody and FITC-conjugated goat anti-mouse Ig polyclonal antibodies were obtained from DAKO (Bucks, UK). MOPC21 IGG_1 antibody was a gift from Dr I Dransfield (Respiratory Medicine Unit, University of Edinburgh). Consensus oligonucleotides for HIF-1 were gifts from Professor P. Ratcliffe (Institute of Molecular Medicine, Oxford, UK). CP94 was a gift from Professor R.C. Hider (King's College, London, UK).

All other reagents were purchased from Sigma Chemical Company (Poole, Dorset, UK) and stored according to the manufacturer's instructions. Dextran-500 (MW 500,000) was dissolved in sterile 0.9% saline (6% w/v) and stored at 4°C. Rotenone was dissolved (100 μg/ml) in chloroform prior to dilution (1 μg/ml) in Iscove's MDM and stored at -20°C. Antimycin A and dinitrophenol were initially dissolved in ethanol (50 mg/ml) before being diluted in Iscove's MDM (10x stock) and stored at -20°C.

2.2 METHODS

2.2.1 NEUTROPHIL PREPARATION

Human neutrophils were purified from the peripheral blood of healthy human volunteers according to the method of Haslett et al., (1985). Neutrophil isolation was performed at room temperature, under sterile conditions and using endotoxin-free reagents and plasticware (Falcon, Oxford, UK). Freshly drawn venous blood was collected into 50 ml polypropylene tubes, anticoagulated (4 ml 3.8% sodium citrate/36 ml blood) and centrifuged (300g, 20 min). The platelet-rich plasma supernatant was aspirated and centrifuged (2500g, 20 min) for production of platelet-poor plasma (PPP) or used to prepare autologous serum in glass tubes by the addition of $CaCl_2$ (final concentration 20 μM) at 37°C. 5 ml of 6% dextran (MW 500,000, 37°C) was added to pelleted cells from the initial centrifugation and 0.9% saline (pre-warmed to 37°C) added to increase the volume to 50 ml per tube. The tubes were mixed gently and allowed to stand for 30-40 min at room temperature until the majority of erythrocytes had sedimented. The leukocyte-rich plasma was aspirated, centrifuged (300g, 6 min) and the supernatant discarded. The resulting leukocyte pellet was resuspended in 2 ml PPP and underlayered with 2 ml 42% and 2 ml 51% plasma-Percoll (freshly prepared from 90% Percoll in 0.9% saline, and PPP). The gradients were centrifuged (275g, 10 min) and polymorphonuclear cells harvested from the 42%/51% Percoll interface. Mononuclear cells sedimented at the PPP/42% Percoll interface. Purified cells were washed sequentially in 40 ml PPP, 40 ml Dulbecco's phosphate buffered saline (PBS) without added cations, and 40 ml PBS with $CaCl_2$ and $MgCl_2$ and the cell concentration adjusted following haemocytometer counts. Cell purity was assessed using May-Grünwald-Giemsa staining of methanol fixed cytocentrifuge preparations; mononuclear cell contamination was routinely <0.1%. The above density gradient centrifugation method does not separate neutrophilic from eosinophilic or basophilic granulocytes, however harvested polymorphonuclear cells generally consisted of <3% eosinophils, and basophils were rarely seen. Preparations containing >5% eosinophils were discarded. Cell viability was assessed by trypan blue exclusion and was routinely >99%. The typical yield for this isolation method was 300-600 x 10^6 polymorphonuclear cells/240 ml whole blood.

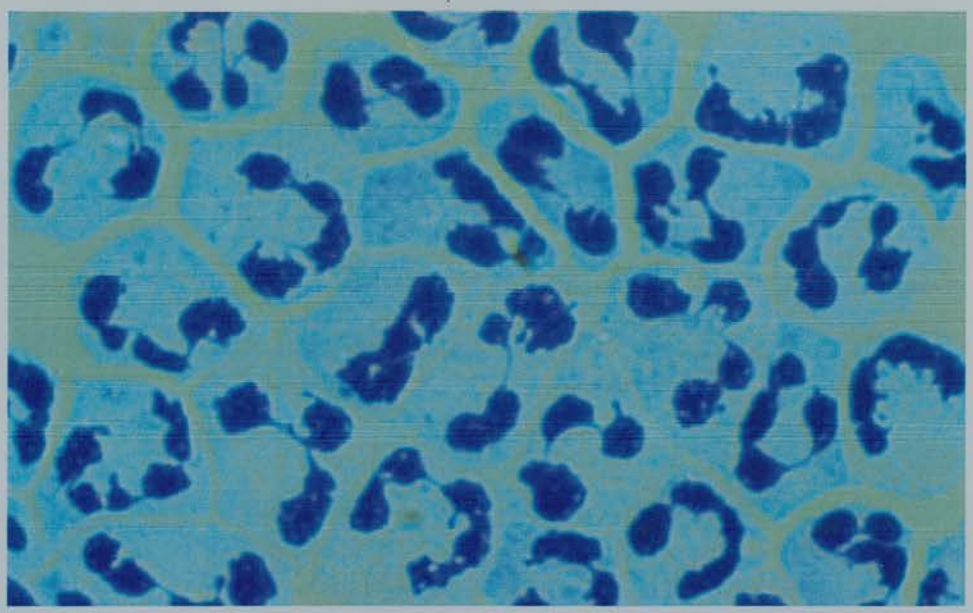

Figure 2.2.1 May/Grünwald/Giemsa-stained cytospin preparation of human neutrophils isolated on plasma/Percoll gradients. Freshly drawn, anticoagulated human blood was subjected to sedimentation with 6% dextran prior to centrifugation through discontinuous 42%/51% plasma/Percoll gradients as described in section 2.2.1. Polymorphonuclear cells were harvested from the 42%/52% Percoll interface and washed sequentially in PPP, calcium and magnesium free PBS and PBS with calcium and magnesium. 100 µl of the resulting cell suspension was spun (300g, 3 min) onto a glass slide and the resulting cytoprep was stained with May-Grünwald-Giemsa.

2.2.2 CELL CULTURE

2.2.2.1 Neutrophil culture

Freshly isolated neutrophils were routinely suspended at a density of 5 x 10^6/ml in Iscove's modified Dulbecco's medium (MDM) supplemented with 10% autologous PRP derived serum, 50 U/ml penicillin, and 50 U/ml streptomycin ('monofeed'). In certain experiments where pre-incubation of monofeed was required, i.e. when the medium needed to be fully deoxygenated prior to incubation with cells, heat inactivated (56°C, 1 hour) human AB serum was used in place of autologous serum. Unless otherwise stated, 6.75 x 10^5 neutrophils were cultured in a final volume of 150 µl in flat-bottomed 96-well Falcon flexiwell plates (Becton-Dickinson, UK) (at 37°C in a humidified, 5% CO_2 atmosphere) for the time periods indicated. Reagents to be examined in this assay system were diluted to 10x the final concentration required in Iscove's MDM.

2.2.2.2 HeLa cell culture

HeLa cells (European Tissue Culture Collection, Portan Down, UK) were grown in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal calf serum, 1% glutamine, 50 U/ml penicillin, and 50 U/ml streptomycin. Cells were used when 90-100% confluent and serially passaged when confluent. Prior to passaging, cells were washed with PBS without calcium and magnesium and harvested by incubating with 1x trypsin/EDTA (3 min, 37°C). Residual trypsin was inactivated by the addition of DMEM supplemented with 10% fetal calf serum and the cells pelleted (300g, 5 min), resuspended in fresh medium and subdivided into fresh cell culture flasks (162 cm^2, Costar, NY, USA).

2.2.2.3 Hypoxic incubation

In initial experiments, the flexiwell plates containing neutrophils were placed in air-tight plastic boxes and sealed with sleek (Smith and Nephew, UK). The boxes were flushed through (15 min) with either a gas mixture containing 2.5% O_2, to provide a hypoxic environment, or a gas mixture containing 21% O_2, to provide a normoxic (control) environment. Both gas mixtures contained 5% carbon dioxide, with the balance made up with nitrogen. The boxes containing the neutrophils were then

incubated at 37°C for the time periods detailed. More severe hypoxic conditions were achieved by placing neutrophils in an MK3 anaerobic incubator (0% O_2, 10% CO_2) (Don Whitley Scientific Ltd., UK) or a NAPCO hypoxic incubator (0.1% O_2, 5% CO_2) (Precision Scientific, Inc., USA). In order to ascertain the exact pO_2/pCO_2 of the cell culture medium and to ensure that these levels remained constant through out the experiments, the pO_2, pCO_2 and $[H^+]$ of the culture medium was measured using an automated blood-gas analyser (model ABL-330, Radiometer Copenhagen) and/or an oxygen electrode (World Precision Instruments Inc., USA). Equilibration of the oxygen concentration in the medium with the prescribed atmospheric oxygen levels took approximately 30 min with the boxes and ≤ 1 hour with the anaerobic incubators.

2.2.3 Assessment of neutrophil apoptosis

2.2.3.1 Assessment of neutrophil apoptosis by morphological criteria

Neutrophil apoptosis was assessed morphologically according to the method of Savill et al., (1989a). Cells were gently resuspended and 100 µl of cell suspension (approximately 5 x 10^5 neutrophils) harvested from each well, cytocentrifuged (300g, 3 min) and the resulting slide preparations air dried, fixed and stained with May-Grünwald-Giemsa. Cell recovery was measured in parallel using a haemocytometer and cell viability assessed by trypan blue exclusion. Cell morphology was examined by oil immersion light microscopy using a x100 objective, and apoptotic neutrophils defined as cells containing one or more darkly stained pyknotic nuclei (see figure 2.2.3). For each condition examined, slides were prepared from triplicate incubations and, after coding, a total of ≥ 500 neutrophils counted over a minimum of five randomly selected high power fields, with the observer blinded to the assay conditions.

2.2.3.2 Assessment of neutrophil apoptosis by annexin V binding

To substantiate the data obtained using the above morphological criteria, apoptosis was also assessed by flow cytometry using FITC-labelled recombinant human annexin V that binds to phosphatidylserine exposed on the surface of apoptotic cells. Stock annexin V was diluted 1:200 with binding buffer (Bender, MedSystems,

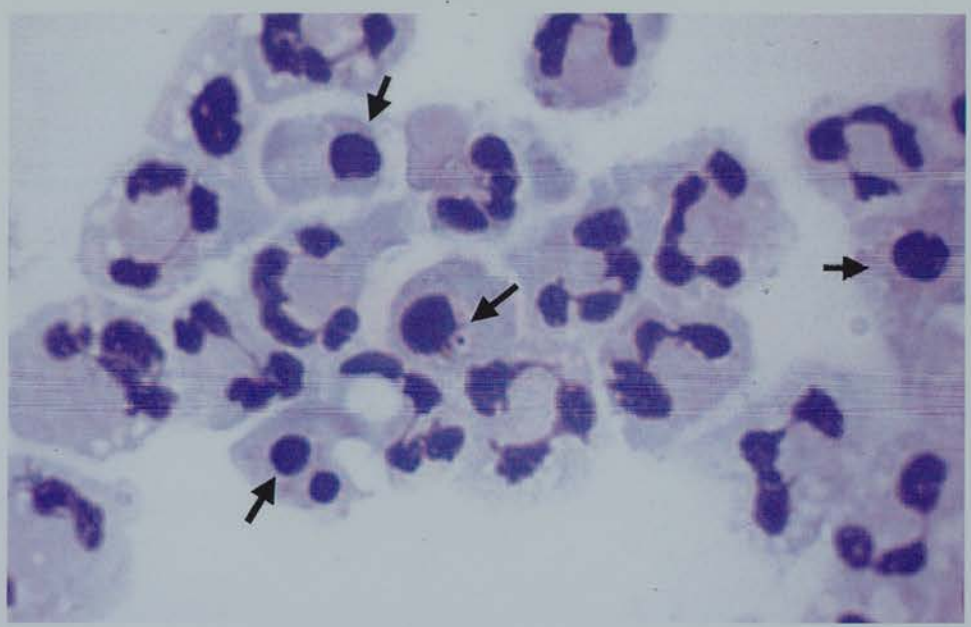

Figure 2.2.3.1 Assessment of neutrophil apoptosis by morphological criteria. Human neutrophils were cultured in serum-supplemented Iscove's MDM for the time period of interest; cells were resuspended and 100 μl (approximately 5 x 10^5) neutrophils harvested from each well, cytocentrifuged (300g, 3 min) and the resulting slide preparations air dried, fixed and stained with May-Grünwald-Giemsa. Cell morphology was assessed by x100 oil immersion light microscopy and apoptotic neutrophils defined as cells containing one or more darkly stained pyknotic nuclei (arrowed).

Vienna, Austria) and then added (25µl) to 75 µl of the recovered cell samples. Following a 10 min incubation at 4°C, these samples were fixed by the addition of 100 µl of 3% paraformaldehyde, prior to analysis using an EPICS Profile II flow cytometer (Coulter Electronics, Luton, UK). Mean fluorescence values were determined from a minimum of 5000 cells.

2.2.3.3 Assessment of neutrophil apoptosis by propidium iodide staining

DNA fragmentation occurring during apoptosis was quantified by flow cytometric analysis of permeabilized cells stained with the intercalating fluorescent dye, propidium iodide, using a modification of the method of Nicoletti et al. (1991). Cells (6.75×10^5) were fixed in 100 µl ice-cold 70% ethanol at 4°C for 10 min, washed 3x (220g, 1 min) in PBS (4°C) and resuspended in 30 µl PBS and 30 µl RNase (1 mg/ml). After gentle mixing, 60 µl of propidium iodide (100 µg/ml) was added and the samples incubated in the dark (25°C, 15 min). Cells were then analysed immediately using an EPICS Profile II (Coulter Electronics, Luton, UK). Mean fluorescence values from a minimum of 5000 cells were determined.

2.2.4 IMMUNOBLOTTING OF HSP 70 IN HUMAN NEUTROPHILS

2.2.4.1 Heat shock protocol

Aliquots of 10^7 freshly isolated neutrophils were suspended in 2 ml monofeed and transferred to sterile 6 well plates (Costar, USA). The cells were then heat or cold shocked by incubation at 42°C or 4°C respectively for 1-2 hours as indicated, before being returned to a control (37°C) environment. Hypoxic (0% O_2, 37°C) and normoxic (21% O_2, 37°C) cells were incubated in parallel. At the appropriate times neutrophils were harvested into 2 ml Eppendorfs, pelleted (7,500g, 1 min) and washed twice in PBS without calcium and magnesium.

A number of lysis methods were employed to extract neutrophil proteins as inconsistent results and Hsp 70 proteolysis were found when standard lysis methods were utilised (figures 2.2.4.2, 3 and 4). These problems occurred despite inclusion of a broad spectrum of protease inhibitors and may reflect the major protease content of neutrophils.

2.2.4.2 Extraction of proteins from neutrophils using Triton X-100 lysis buffer

200 µl of lysis buffer (10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, pH 7.4) containing protease inhibitors (500 mM e-amino-n-caproic acid, 50 µg/ml antipain, 50 µg/ml chymostatin, 50 µg/ml leupeptin, 50 µg/ml pepstatin A, 50 mM AEBSF) was added to the PBS washed, pelleted neutrophils (see 2.2.4.1) and the cells thoroughly resuspended by repeat pipetting. The samples were then incubated on ice for 10 min before being centrifuged (10,000g, 4°C, 10 min). The resulting supernatants were stored at -80°C prior to analysis. Protein concentration was ascertained using the BCA protein assay (section 2.2.4.6) and protein concentrations standardised. Prior to electrophoresis, 5 µl of 4x sample buffer (50 mM Tris base, 10% glycerol, 2% SDS, 0.1% and 100 mM dithiothreitol, pH 6.8) was added to 15 µl of lysate and samples boiled for 5 min, 95°C.

2.2.4.3 Extraction of proteins from neutrophils using boiling SDS

Washed cell pellets were resuspended in 20 mM Tris (pH 7.4, 200 µl) and an equal volume of boiling SDS (2%) added. Samples were then vortexed and incubated at 95°C for 5 min before being placed on ice to cool and centrifuged (10,000g, 4°C, 10 min). The resulting supernatants were stored and subsequently treated as in section 2.2.4.2.

2.2.4.4 Extraction of proteins from neutrophils using TCA

After the treatments described in section 2.2.4.1 neutrophils were harvested into 12 ml of ice-cold HEPES (20 mM, pH 7.5)-buffered saline containing protease inhibitors (50 µg/ml leupeptin, 20 µg/ml aprotinin and 1 mM AEBSF) ('HBSI'), and neutrophils collected by centrifugation (7,500g, 1 min, 4°C). Cell pellets were then immediately resuspended in 0.5 ml HBSI plus 0.5 ml 20% TCA, vortex mixed, and incubated for 10 min at 4°C. TCA precipitated material was sedimented (10,000g, 10 min, 4°C) and lipids extracted by washing the resulting pellet twice in 1 ml ice-cold ethanol. Following sedimentation (10,000g, 10 min, 4°C), protein extracts were taken up in 10 µl dH_2O, resuspended in 190 µl boiling (2x) Laemmli sample buffer (LSB: 0.125 M Tris-HCl, 4% SDS, 20% glycerol, 2.5 mM dithiothreitol, 0.01%

bromophenol blue, 50 μg/ml leupeptin, 20 μg/ml aprotinin and 1 mM AEBSF, pH 6.8) and boiled for 5 min in a heated water bath (Grant, UK). Cooled samples were then incubated with 10 mM iodoacetamide for 20 min (25°C), centrifuged (14,000g, 5 min, 25°C) and supernatants stored at 4°C prior to analysis.

2.2.4.5 Extraction of proteins from neutrophils using Laemmli sample buffer

The neutrophil pellets (10^7 cells) (obtained after harvesting cells into ice-cold HBSI and centrifugation; see 2.2.4.4) were taken up in 25 μl HBSI, immediately resuspended in 175 μl boiling LSB and boiled for 20 min. Following centrifugation (14,000g, 5 min, 25°C), supernatants were again stored at 4°C prior to electrophoresis.

2.2.4.6 Assessment of protein concentrations

Protein concentrations were quantified using a BCA protein assay (Pierce, IL, USA). This assay is based on the ability of protein present in the test samples to cause a reduction of Cu^{2+} to Cu^{+} and bicinchoninic acid (BCA) to chelate Cu^{+} forming a purple compound which can be measured using spectrophotometry (562 nm) (Smith et al., 1985). Samples were diluted 1 in 10 in dH_2O and 10 μl incubated with 200 μl of test solution (30 min, 37°C) in 96 well plates prior to analysis using an automated plate reader (MR5000, Dynatech, UK). Samples were assayed in triplicate and standard curves formed using pre-made BSA standards.

2.2.4.7 Separation of protein extracts by polyacrylamide gel electrophoresis (PAGE) and electroblotting onto nitrocellulose membranes

20 μl of each sample and 10 μl of pre-boiled (5 min, 100°C) molecular weight markers (Rainbow markers, Biorad, CA) were loaded into individual lanes of a 10% Tris-glycine polyacrylamide gel. PAGE was then conducted (Mini Protean II apparatus, Biorad, CA) in running buffer (50 mM Tris base, 0.1% SDS, 250 mM glycine) at a current of 30 mA for approximately 2 hours. Gels run under identical, parallel conditions were either (i) stained for total protein content with Coomassie brilliant blue (0.25% plus 10% glacial acetic acid-v/v) for 4 hours followed by 4 x 4 hour washes in 40% methanol/10% glacial acetic acid; or (ii) used for protein

transfer onto nitrocellulose membranes (Hybond C, Amersham, UK) (400 mA, 4 hours) using Protein II transfer apparatus, (Biorad, CA) filled with transfer buffer (25 mM Tris base, 210 mM glycine, 20% v/v methanol).

2.2.4.8 Detection of Hsp 70 in neutrophils

Following protein transfer to nitrocellulose membranes, non-specific binding sites were blocked overnight in 50 ml blocking buffer (150 mM NaCl, 20 mM Tris-HCl, 0.02% Tween-20, 5% powdered fat-free milk (Marvel), pH 7.4) on a rocking platform at 4°C. Membranes were then incubated at 25°C for 2 hours in 50 ml blocking buffer containing mouse monoclonal anti-Hsp 70 antibody (Stressgen, Canada). This was followed by 3 sequential washes (30 min, 25°C) in TBS Tween (150 mM NaCl, 20 mM Tris-HCl, 0.02% Tween 20, pH 7.4) and incubation with a horse radish peroxidase-conjugated, goat polyclonal, anti-mouse, secondary antibody (diluted 1:10,000) (DAKO, Bucks, UK) in blocking buffer, and three further washes in TBS Tween. Immuno-labelled proteins were then detected by enhanced chemiluminescence (ECL, Amersham, UK) and exposure to photographic film (20 s, Kodak XAR).

When neutrophil lysates, prepared using a standard triton-X100 based lysis buffer (section 2.2.4.2), were separated with SDS-PAGE and the western blots developed using an anti-Hsp 70 antibody, two bands, both with molecular weights < 34 kDa, were observed (figure 2.2.4.2) suggesting that severe protein degradation had occurred during the neutrophil lysis. Two bands were also observed when neutrophils were lysed in boiling SDS (figure 2.2.4.3). The slower migrating band had a molecular weight of 72 kDa and probably represented the full sized Hsp 70 protein, while the faster moving band was again thought to represent a proteolytic cleavage product of Hsp 70. When neutrophils were lysed using either TCA (section 2.2.4.4) or Laemmli sample buffer (section 2.2.4.5) only a single band (72 kDa) was observed when the western blots were developed with anti-Hsp 70 antibody; however, in subsequent experiments (n = 3) the Laemmli sample buffer method gave inconsistent results (see figure 4.2.6B). In accordance with these findings, analysis of the Coomassie stained gels (figure 2.2.4.4) showed that TCA-extracted samples

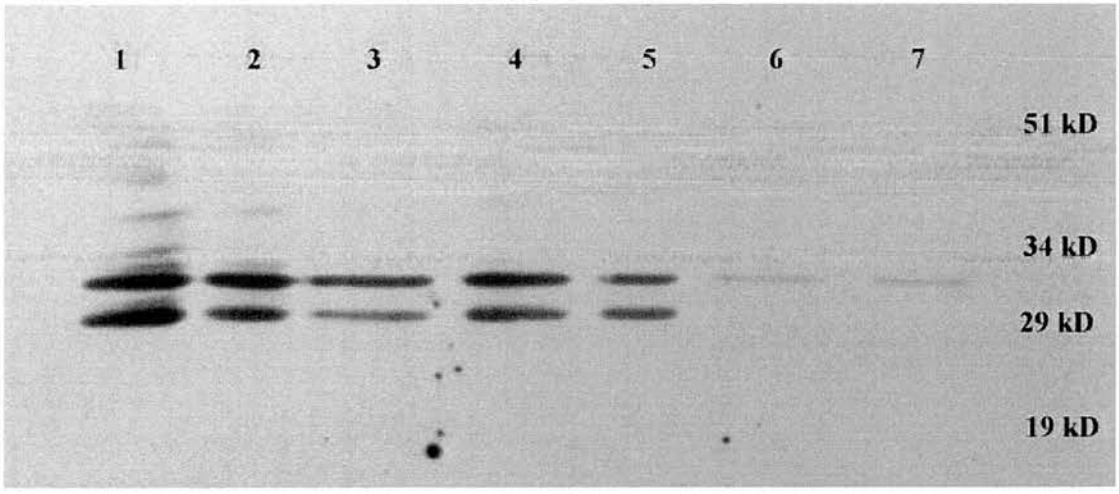

Figure 2.2.4.2 Neutrophil lysates (30 μg total protein per lane), prepared using a standard triton-X100 lysis buffer supplemented with protease inhibitors, and separated by 10% SDS-PAGE. The western blot was developed with an antibody to Hsp 70 (see section 2.2.4.8). No protein band of 72 kDa was observed, however, two protein bands were identified with molecular weights of approximately 30 and 33 kDa. These results suggest that proteolytic cleavage of Hsp 70 occurred during cell lysis. Lane 1 contains untreated neutrophils lysed at time 0. Lanes 2, 4, and 6 show control cells incubated at 37°C for 1, 4, and 20 hours respectively, while lanes 3, 5 and 7 show parallel cells which were heat shocked for 1 hour at 42°C before being returned to control (37°C) incubation conditions.

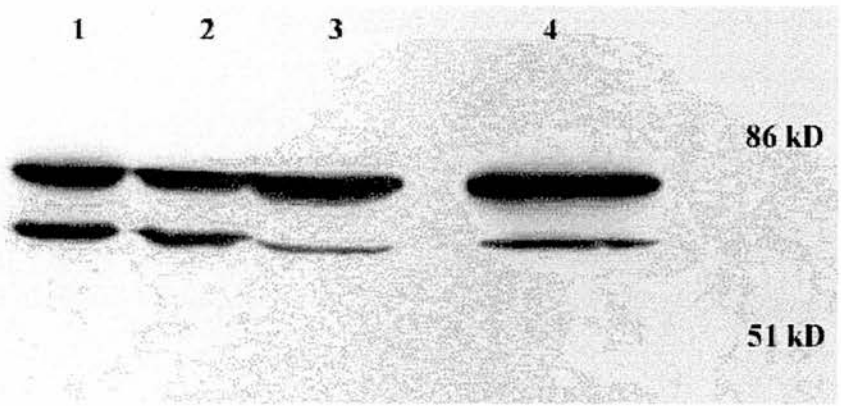

Figure 2.2.4.3 Neutrophil lysates (30 μg total protein per lane) prepared using boiling SDS and separated by 10% SDS-PAGE. The western blot was developed with an antibody to Hsp 70 (see section 2.2.4.8). Two protein bands were identified; the slower migrating band had a molecular weight of 72 kDa and probably represents Hsp 70 protein, while the faster migrating band (MW 70 kDa) was thought to be a proteolytic cleavage product of this protein. Lane 1 contains control cells (37°C, 4 hours). Parallel cells were either heat shocked (42°C, 60 min, lane 2) or cold shocked (4°C, 60 min, lane 3) prior to incubation at 37°C for a further 3 hours and lane 4 contains hypoxic cells (0% oxygen, 37°C, 4 hours).

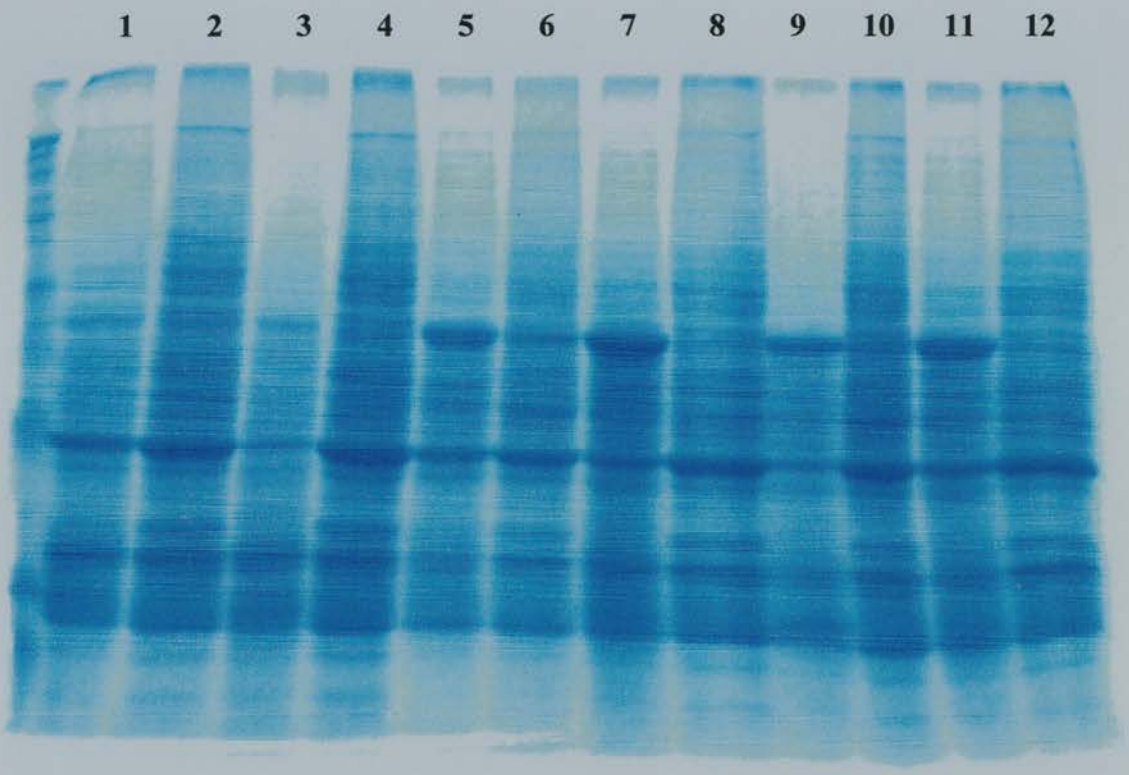

Figure 2.2.4.4 Comparison of TCA and Laemmli sample buffer methods of neutrophil protein extraction. After isolation neutrophils were either lysed immediately (time 0; lanes 1, 2, 3 and 4) or cultured for 4 hours at 37°C (control; lanes 5, 6, 7 and 8). Parallel cells were heat shocked for 1 hour at 42°C before being returned to 37°C for 3 hours prior to lysing (lanes 9, 10, 11 and 12). Neutrophil lysates were prepared by either direct lysis in boiling Laemmli sample buffer (odd numbered lanes) or a TCA based lysis method (even numbered lanes) and separated by 10% SDS-PAGE. Gels were stained for total protein content with Coomassie brilliant blue. Improved protein extraction was noted with the TCA based extraction method; in particular the preservation of high molecular weight proteins. Identical data was obtained in 2 additional experiments.

contained greater numbers of prominent protein bands than samples extracted by the Laemmli sample buffer method. Therefore, the TCA extraction protocol was selected for all further studies.

2.2.5 IMMUNOCYTOCHEMISTRY

To substantiate the data obtained using the above western blot method, Hsp 70 expression in human neutrophils was also assessed by immunocytochemistry. This method was also used to investigate the presence of the Bcl-2 oncoprotein in human neutrophils. After appropriate times in culture, cells (1-2 x 10^5) were transferred into round bottomed 96-well polypropylene plates and permeabilized and fixed with 0.01% (w/v) L-α-lysophosphatidylcholine in PBS containing 3.7% (v/v) formaldehyde (50 µl) for 15 min at 37°C. The cells were then washed twice with PBS containing 0.5% (w/v) BSA and labelled with pre-determined saturating concentrations of primary antibody (see below) for 1 hour at 4°C. Samples were subsequently washed with PBS/0.5% (w/v) BSA before the addition of FITC-conjugated secondary antibody (1 hour, 4°C). The cells were then analysed using an EPICS Profile II flow cytometer (Coulter Electronics, Luton, U.K.). A minimum of 3000 events were collected and analysed per sample. Appropriate negative and positive controls were prepared with each sample, and the data presented as mean fluorescence relative to the negative control.

Saturating concentrations of antibody was determined by titration: the mouse anti-human Hsp 70 monoclonal antibody was titrated against HL-60 cells. Controls included MAC 387 (DAKO, Bucks, UK), a monoclonal antibody, which recognises the myeloid cell antigen p8,14 present in neutrophils (positive control to ensure efficient permeabilization; mean fluorescence 25.1 ± 8, n=7) and MOPC21 control antibody (kindly donated by Dr I. Dransfield, University of Edinburgh) as a negative control.

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2.2.6 IDENTIFICATION OF HIF-1α IN NEUTROPHILS

2.2.6.1 RNA analysis

2.2.6.1a Isolation of total cell RNA

Freshly isolated neutrophils (30 x 10^6) were washed twice in PBS without calcium and magnesium and the resulting cell pellet immediately lysed in 6 ml of TRIZOL® reagent (5 min, 25°C). After the addition of 1.2 ml of chloroform, samples were vortex mixed until they took on a cloudy appearance and then incubated at room temperature for 2-3 min. On centrifugation (12,000g, 4°C, 15 min), the mixture separated into 3 phases, with the RNA present in the upper aqueous phase. This aqueous phase was transferred into sterile tubes and RNA precipitated by the addition of 3 ml of isopropanol. The samples were subsequently incubated for 10 minutes at room temperature or, alternatively overnight at -20°C, and the RNA pelleted by centrifugation (12,000g, 4°C, 10 min). The supernatant was discarded, 6 ml of ethanol (75%) added, and the samples vortexed mixed. Following this, samples were centrifuged (7,500g, 4°C) for 5 minutes and the supernatant removed taking care not to disturb the pellet. The pellet was then allowed to air dry for 20-30 minutes prior to being redissolved in a minimum amount of 0.5% SDS. Samples were stored at -80°C prior to analysis. The RNA content of samples was quantified using a RNA/DNA calculator (Pharmacia Biotech, Herts., UK); 4 μl of sample was diluted with 996 μl dH_2O prior to measurement of the 260/280 ratio from which the RNA concentration was ascertained.

2.2.6.1b RNase protection assay

RNase protection assays were performed by Dr M. Wiesener (Wellcome Trust Centre for Human Genetics, Oxford, UK). 40 μg of total neutrophil RNA was subjected to hybridisation with ^{32}P-labelled riboprobes for HIF-1α and EPAS-1. These were generated using an SP6 or T7 RNA polymerase; the templates used yielded riboprobes of distinct sizes: 221 bases for EPAS-1 (nucleotides 2542 to 2762, Genbank accession no. U81984) and 268 bases for HIF-1α (nucleotides 760 to 1028, Genbank accession no.U22431). Hybridisation was performed at 60°C in 80% formamide, 40 mM Pipes, (pH 6.4), 400 mM NaCl and 1 mM EDTA, and RNase

digestion performed at 20°C for 30 minutes. The protected fragments were then subjected to denaturing PAGE using 8% gels.

2.2.6.2 Protein analysis

2.2.6.2a Extraction of proteins from neutrophils using TRIZOL® reagent

Neutrophils (10 x 10^6) were resuspended at a density of 5 x 10^6/ml in monofeed, which had been previously incubated overnight in either normoxic (21% O_2) or hypoxic (0% O_2) conditions. A third condition consisted of normoxic cells treated with desferrioxamine (final concentration 1 mM). Cells were cultured at a final volume of 2 ml in 6 well plates and were then incubated for 3-4 hours in either hypoxic or normoxic environments. After incubation, neutrophils were harvested rapidly into a large volume of ice cold PBS (in order to minimise degradation of HIF-1) and collected by centrifugation (7,500g, 1 min, 4°C). The resulting pellet was washed twice in ice cold PBS before being lysed in 2 ml of TRIZOL® reagent and subsequently treated as described in section 2.2.6.1a up to the point when samples were phase partitioned using chloroform. At this stage the upper aqueous phase was removed leaving an upper interphase containing DNA and lipid soluble proteins and a lower organic phase containing the remaining protein. To this, 100% ethanol (0.3 ml per 1 ml TRIZOL used initially) was added and the samples mixed by inverting the tubes. The samples were subsequently incubated for 2-3 min at room temperature and then centrifuged (2,000g, 4°C) for 5 min. The resulting pellet, which contained the DNA was discarded, while the protein containing supernatant was transferred to clean tubes. To these, isopropanol (1.5 ml per 1 ml TRIZOL used for the initial lysis) was added, and the samples incubated for 10 min at room temperature before being centrifuged (12,000g, 4°C) for a further 10 min. The resulting protein pellets were then washed 3x in 0.3 M guanidine hydrochloride in 95% ethanol (2 ml per 1 ml TRIZOL used for the initial lysis), centrifuging at 7,500g (4°C) for 5 min between each wash. After the final wash the pellets were resuspended in 100% ethanol, incubated (20 min, 25°C), and the protein collected by centrifugation (7,500g, 5 min, 4°C). The protein pellets were then vacuum dried for 10 min and dissolved in 50 µl 1% SDS (50°C, 30 min). Finally, samples were

centrifuged (10,000g, 10 min, 4°C) and the resulting supernatants stored at -80°C. Protein concentrations were assessed as described in section 2.2.4.6.

2.2.6.2b Immunoblot analysis

Western blots for HIF-1α were performed by Dr M. Wiesener (Wellcome Trust Centre for Human Genetics, Oxford, UK). Protein extracts (80 μg) were separated using SDS/6% polyacrylamide gels and transferred to Immobilon P (Millipore) overnight in 10 mmol/L Tris, 100 mmol/L glycine, 10% methanol and 0.05% SDS. Membranes were blocked with PBS containing 5% fat-free dried milk and 0.1% Tween 20. For HIF-1α detection, mAb 28b was used at 4 μg/ml. This antibody was from a fusion following immunisation with a GST fusion protein including amino acids 329 to 530 of human HIF-1α. For EPAS-1, 190b supernatant was diluted 1:4 (for characterisation of these antibodies see Wiesener et al., 1998). Antibody binding was detected using horse radish peroxidase (HRP)-conjugated goat anti-mouse Igs (DAKO, Glostrup, Denmark) at 1:2,000 and enhanced chemiluminescence (SuperSignal Ultra; Pierce, Rockford, IL). After analysis, membranes were stained with Ponceau S to verify equal protein loading and transfer.

2.2.6.3 Electrophoretic mobility shift assay for HIF-1

2.2.6.3a Preparation of nuclear extracts

Neutrophils were prepared, cultured under normoxic or hypoxic conditions for 4 hours and harvested into pre-weighed 15 ml conical polyproplylene tubes as described in section 2.2.6.2a. HeLa cells were used as a positive control, and treated in an identical fashion to the neutrophils. At the beginning of the experiment, the HeLa medium was replaced by 25 ml of medium which had either been incubated overnight in a 0% O_2 (hypoxic), 21% O_2 (normoxic) or normoxic medium containing desferrioxamine (100 μM). The HeLa cells were then incubated under these conditions for 4 hours and the incubations terminated by the addition of a large volume of ice cold PBS and placing the flasks on ice. The cells were then washed again with ice cold PBS before being harvested into 5 ml of PBS using a cell scraper and transferred into pre-weighed 15 ml conical tubes. Both HeLa cells and neutrophils were then pelleted (1,000g, 5 min, 4°C) and the supernatants carefully

removed using a pulled Pasteur pipette. The pellet was then washed briefly in ice cold buffer A (see table 2.2.6.3a) before being weighed, resuspended in 4 packed cell volumes of buffer A (4°C) and incubated for 10 min on ice. The cells were then lysed using a dounce homogenizer (30 strokes on ice), and cell lysis verified by microscopy using trypan blue. The lysed cells were then transferred to a second weighed 15 ml conical tube, the nuclei pelleted (3,000g, 5 min, 4°C) and the supernatant again carefully removed with the aid of a pulled Pasteur pipette. The nuclear pellets were weighed and resuspended in 3 packed cell volumes of buffer C before being transferred to 2 ml Eppendorfs and mixed on a rotator (4°C) for 30 min. The nuclear debris was then pelleted (13,000g, 30 min, 4°C) and the resulting supernatant dialysed against 500 ml of buffer D (4°C) for 2 hours with one change of dialysis buffer. The dialysed extracts were aliquoted, snap frozen (liquid nitrogen) and stored at -80°C. Protein concentrations were ascertained using the method described in section 2.2.4.6.

Buffer A (Hypotonic)*
10 mM Tris-HCl (pH 7.8)
1.5 mM $MgCl_2$
10 mM KCl

Buffer C (Hypertonic)*
20 mM Tris-HCl (pH 7.8)
1.5 mM $MgCl_2$
420 mM KCl
20 % glycerol

Buffer D (Dialysis)#
20 mM Tris-HCl (pH 7.8)
100 mM KCl
0.2 mM EDTA
20 % glycerol

Table 2.2.6.3a Buffers used for isolation of nuclear proteins. *Buffers A and C were additionally supplemented, immediately prior to use, with the following protease inhibitors (final concentration): ABSEF (0.5 mM); aprotinin (1 μg/ml); leupeptin (1 μg/ml); pepstatin A (1 μg/ml); sodium vandate (1 mM); benzamidine (0.5 mM); levamisole (2 mM); β-glycerophosphate (10 mM) and DTT (0.5 mM). #Buffer D was supplemented immediately prior to use with (final concentration): sodium vandate (1 mM); β-glycerophosphate (10 mM) and DTT (0.5 mM).

2.2.6.3b Labelling of HIF-1 probe with polynucleotide kinase

Oligonucleotides derived from the mouse erythropoietin 3' enhancer were used as probes or competitors in the EMSAs. These were kindly donated by Prof. P. J. Ratcliffe (Institute of Molecular Medicine, Oxford, UK) and their sequences are shown in figure 2.2.6.2b. The wild type probe contained the HIF-1 binding site, while the binding site in the mutated probe was modified to abolish HIF-1 binding. Probes were purified by polyacrylamide gel electrophoresis prior to labelling. Single-stranded oligonucleotide (100 ng/µl) was incubated with 2 µl 10x kinase buffer, 11 µl dH2O, 4 µl [γ-^{32}P] ATP (3,000Ci/mmol at 10 mCi/ml) and 2 µl polynucleotide kinase for 2-3 hours at 37°C. The reaction was stopped by the addition of 5 µl 500 mM EDTA, and 75 µl dH_2O added. Labelled oligonucleotides were then loaded onto prespun Sephadex G25-containing spin columns and centrifuged at 13,000g for 1 min. The probe was then eluted with 100 µl of TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) by centrifugation (1 min, 13,000g). Following this, 10 µl NaAc (pH 8.0) and 330 µl of ethanol were added to precipitate the probe and the oligonucleotide pelleted (10,000g, 4°C, 40 min). The oligonucleotide pellet was then washed in 85% ethanol before being resuspended in 40 µl TE buffer (pH 8.0) and annealed with a four fold excess of the unlabelled complementary strand (30 min, 37°C). Unlabelled oligonucleotides were annealed in molar equivalent quantities. Finally, the double stranded probe was diluted 1 in 20 in TE buffer to give a final concentration of 0.1 ng/µl and stored at -20°C prior to use.

EPO WT 24mer			
EPO 108	5'	GCC, CTA, CGT, GCT, GCC, TCG, CAT, GGC	3'
EPO 109	3'	CGG, GAT, GCA, CGA, CGG, AGC, GTA, CCG	5'
EPO Mut3 24mer			
EPO 110	5'	GCC, CTA, ATG, TCT, GCC, TCG, CAT, GGC	3'
EPO 111	3'	CGG, GAT, TAC, AGA, CGG, AGC, GTA, CCG	5'

Figure 2.2.6.2b Sequences of probes used for HIF-1 gel shift assays. All probes were kindly donated by Prof. P. J. Ratcliffe, Institute of Molecular Medicine, Oxford, UK.

2.2.6.3c Binding reactions and electrophoresis conditions for HIF-1 EMSAs

Binding reaction mixtures (final volume 20 µl) contained 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 5% glycerol, 0.03% NP40, 1 mM sodium vanadate, 150-300 ng sonnicated poly dIdC and 0.1 µg labelled probe (see 2.2.6.2b). The binding reactions were performed as follows in Eppendorf tubes at room temperature: 5 µl of 5x binding buffer (25 mM Tris-HCl (pH 7.5), 125 mM KCl, 5 mM $MgCl_2$, 4.75 mM EDTA, 25 mM DTT and 0.15% NP40) and poly dIdC were initially placed in the tubes followed by the nuclear extract (5 µg total protein). The samples were then mixed and incubated at room temperature for 5 min before the addition of 1 µl labelled probe and (if required) unlabelled competitor. After a further 10 min incubation, reaction products were loaded on to a pre-run and pre-cooled 5% polyacrylamide gel. Gels were run on a Biorad Protean II system at 280 V for approximately 3 hours at 4-10°C. The running buffer used consisted of 0.3x TBE (final concentrations, 27 mM Tris base, 27 mM boric acid, 0.06 mM EDTA, pH 7.3) at room temperature and was supplemented with 0.03% NP40. Finally, the run gels were transferred to Whatman paper and dried under a vacuum (80°C, 30 min) before being exposed to film (Biomax MR, Kodak) overnight at -80°C.

2.2.7 Electrophoretic Mobility Shift Assay for NF-κB and AP-1

2.2.7.1 Preparation of nuclear extracts

Neutrophils (20–30 x 10^6) were cultured at a density of 10^7cells/ml in either hypoxic monofeed (pre-incubated overnight in 0% O_2), normoxic monofeed, or normoxic monofeed containing desferrioxamine (1 mM). Cells were then incubated in either 21% O_2 or 0% O_2, as appropriate, for 0.5 to 6 hours. At the stated time points cells were harvested immediately into ice cold PBS and pelleted by centrifugation (3000g, 3 min) at 4°C. The supernatants were discarded and the cell pellets resuspended in 0.4 ml ice cold solution A (see table 2.2.7.1), transferred to Eppendorf tubes and incubated on ice for 15 min. Following the incubation, 15 µl of solution B was added and the samples vortex mixed for 30 s prior to centrifugation (13,000g, 4°C, 1 min). The resulting supernatant was removed, 50 µl of ice cold solution C added to the nuclear pellet and the samples mixed by pipetting. The samples were then placed

on a rotating table at 4°C for 20 min prior to centrifugation (13,000g, 5 min, 4°C). The supernatant containing the nuclear protein was removed, aliquoted and stored at -80°C. Protein concentrations were determined as described previously in section 2.2.4.6.

Solution A (Hypotonic)
10 mM HEPES (pH 7.8, buffered with KOH)
10 mM KCl
2 mM $MgCl_2$
1 mM DTT
0.1 mM EDTA

Solution B
10% Nonident p40 in dH_2O

Solution C (Hypertonic)
50 mM HEPES pH 7.8
50 mM KCl
300 mM NaCl
0.1 mM EDTA
1 mM DTT
10% sterile glycerol

Table 2.2.7.1 Solutions used for isolation of nuclear proteins. Solutions A and C were made up at 2x concentration and stored at –20°C. Immediately prior to use these solutions were additionally supplemented with the following protease inhibitors (final concentration): ABSEF (0.5 mM); aprotinin (1 μg/ml); leupeptin (1 μg/ml); pepstatin A (1 μg/ml); sodium vandate (1 mM); benzamidine (0.5 mM); levamisole (2 mM) and β-glycerophosphate (10 mM).

2.2.7.2 Labelling of consensus NF-κB and AP-1 oligonucleotide probes

The double stranded oligonucleotides (see figure 2.2.7.2 for sequences) were labelled with γ-^{32}P in the following reaction: 2 μl consensus oligonucleotide (1.75 pmol/μl), 1 μl T4 polynucleotide kinase 10x buffer (Promega), 1μl T4 polynucleotide kinase (5-10 U/μl), 5 μl dH_2O and 1 μl [γ-^{32}P] ATP (3,000 Ci/mmol at 10 mCi/ml) were added in a sterile Eppendorf tube and incubated together for 10 min at 37°C. The reaction was terminated by the addition of 1 μl of 0.5 M EDTA and the volume increased to 100 μl with TE buffer. The labelled probe was then stored at -20°C prior to use.

AP-1

5' -CGC TTG ATG AGT CAG CCG GAA- 3'

3' -GCG AAC TAC TCA GTC GGC CTT- 5'

NF-κB

5' -AGT TGA GGG GAC TTT CCC AGG C 3'

3' -TCA ACT CCC CTG AAA GGG TCC G 5'

Figure 2.2.7.2 Sequences of probes for the transcription factors AP-1 and NF-κB used in gel shift assays. Both probes were purchased from Promega (Southampton, UK).

2.2.7.3 Binding reactions and electrophoresis conditions for NF-κB and AP-1 EMSAs

The following binding reactions were performed in sterile Eppendorf tubes: (1) positive control, 5 μl dH_2O, 2 μl 5x gel shift buffer (Promega, Southampton, UK), 2 μl HeLa nuclear extract (Promega, Southampton, UK); (2) negative control, 7 μl dH_2O, 2 μl 5x gel shift buffer; (3) sample tubes, 4 μl dH_2O, 2 μl gel shift buffer and 3 μl of nuclear extract (4 μg total protein). All tubes were then incubated for 5-10 min at room temperature prior to the addition of 1 μl of labelled target consensus oligonucleotide. The binding reactions were then incubated for a further 20 min at room temperature before termination of the reactions with 1 μl 10x gel-loading buffer (20% glycerol, 0.01% bromophenol blue, 5 mM EDTA). The reaction products were immediately loaded onto pre-run 8% polyacrylamide gels and run in 0.5% TBE at 150 V for 2-3 hours. The gels were then transferred to Whatman paper and dried under a vacuum for 30 minutes at 80°C. Finally, the dried gels were exposed to film (BIO-MAX MR, Kodak) overnight at -80°C.

2.2.8 Flow cytometric analysis of TNFR55 and TNFR75 expression in human neutrophils

Expression of TNFR55 and TNFR75 in human neutrophils was studied by indirect immunofluorescence with primary antibody directed against TNFR55 or TNFR75 and FITC-conjugated secondary antibody detected using an EPICs Profile II.

At the appropriate times neutrophils were harvested and transferred to pre-chilled U-bottomed flexiwell plates (Becton-Dickinson, UK), washed (220g, 1.5 min, 4°C) in 100 μl ice-cold wash buffer (PBS containing 0.2% BSA and 0.1% sodium azide) and resuspended in 40 μl of a saturating concentration of mouse anti-human TNFR55, TNFR75 (R&D Systems, UK), or CD2 mAb as a negative control (UCHT-1 clone IgG1; SAPU, Carluke, UK). After a 30 min incubation on ice, the cells were washed twice and incubated with 40 μl FITC-conjugated goat anti-mouse immunoglobin (Dako, Buckinghamshire, UK diluted 1 in 40 with PBS/BSA azide buffer). After washing, samples were analysed using an EPICS Profile II (Coulter Electronics, Luton, UK) and mean fluorescence from a minimum of 3000 cells determined. Samples were processed with the help of Dr J. Murray (University of Edinburgh).

2.2.9 Data analysis

Results are reported either as pooled data from a series of n separate experiments (mean ± SEM) or as individual representative experiments (mean ± SD, 3 replicates/condition). Statistical significance was assessed by the students t-test or, for multiple comparisons, a one way analysis of variance followed by the Student-Newman-Keuls post test.

CHAPTER 3

REGULATION OF NEUTROPHIL APOPTOSIS BY HYPOXIA

3.1 INTRODUCTION

Neutrophils are the most abundant and shortest lived of all the leukocytes. Their primary role is to defend the host against infection. However, as phagocytic cells, neutrophils contain powerful degradative granule enzymes and toxic catonic proteins, and inappropriate release of these histotoxic contents has been shown to cause host tissue damage (Weiss, 1989). It is thus imperative that neutrophil function is tightly regulated and this is achieved predominantly through two distinct processes, namely priming and apoptosis. Priming upregulates the secretagogue capacity of the neutrophil resulting in a hyperresponsive cell, which has the potential to cause tissue damage if inappropriately stimulated. On the other hand apoptosis, a process associated with maintenance of membrane integrity, results in a functionally downregulated cell, which can be phagocytosed intact by macrophages and certain other phagocytes (Savill et al., 1989a and b, 1990b, 1992a; Haslett et al., 1989; Whyte et al., 1993a). The observation that engulfment of apoptotic neutrophils by macrophages does not trigger a pro-inflammatory macrophage response (Meagher et al., 1992) predicts that this process plays an important role in the safe disposal of neutrophils from an inflamed focus and will thus promote the resolution of inflammation. This view is supported by experiments showing the occurrence of this process *in vivo*, for example, in endotoxin-induced lung injury (Cox et al., 1995) and the neonatal respiratory distress syndrome (Grigg et al., 1991).

However, although these regulatory processes exist they are not infallible. It is now recognised that neutrophils play a role in the pathogenesis of many inflammatory diseases that, in the lung, include chronic bronchitis, emphysema (Janoff, 1985), asthma and adult respiratory distress syndrome (Stevens and Raffin, 1984), and, in other tissues, glomerulonephritis (Holdsworth and Bellomo, 1984), rheumatoid arthritis and reperfusion injury following myocardial infarction (Allan et al., 1985). In these conditions neutrophil accumulation correlates, and is associated, with the

tissue destruction and fibrosis, which can severely compromise organ function. It is noteworthy that in the majority of these diseases the site of inflammation, and hence neutrophil accumulation, will be relatively hypoxic. This is due to many factors including reduced blood flow to the site of inflammation, increased local oxygen requirements, and, in the case of pulmonary disease, reduced gas exchange due to lung damage. Furthermore, tissue hypoxia following haemorrhage and trauma is thought to be a possible initiating factor of the generalised inflammatory response seen after shock.

The neutrophil has a circulating half life of 6-8 hours *in vivo* (Fliedner et al., 1964) and undergoes constitutive apoptosis *in vitro* with a half-time of 12-18 hours (Savill et al., 1989a and 1992; Grigg et al., 1991; Whyte et al., 1993a). However, a number of agents are capable of extending the neutrophil's life-span (Colotta et al., 1992; Lee et al., 1993) including the proinflammatory mediators, bacterial lipopolysaccharide (LPS), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF) and the interleukins 1 and 6. These agents all promote neutrophil survival by delaying neutrophil apoptosis, whereas tumour necrosis factor-α (TNFα) has been shown to accelerate neutrophil apoptosis at early time points (Takeda et al., 1992; Murray et al., 1997). Several of these agents also act to prime the functional responsiveness of neutrophils. Thus, it appears that the balance of inflammatory mediators present at an inflamed site may potentially regulate both neutrophil function and longevity. We became interested in the possibility that other changes in the neutrophil's environment may also hold the potential to regulate the functional life-span of these cells and, since under many physiological and pathological conditions the site of inflammation is deprived of oxygen, we proceeded to investigate the effect of hypoxia on neutrophil apoptosis.

3.2 Results

3.2.1 Effect of hypoxia on neutrophil apoptosis

To evaluate whether hypoxia could influence the rate of neutrophil apoptosis the effects of incubating purified human peripheral blood neutrophils in an atmosphere with a reduced oxygen content (2.5% opposed to 21% oxygen) were examined at 6 and 20 hour time points. These oxygen concentrations were achieved by placing cultured neutrophils in sealed plastic boxes, flushed through with a gas mixture containing either 2.5% (hypoxic conditions) or 21% oxygen (normoxic conditions). This method has previously been validated and used to examine the effect of hypoxia on macrophage TNFα release (Scannell et al., 1993). Figure 3.2.1.1B shows the mean results from 5 different experiments. After 6 hours in culture the percentage of neutrophils exhibiting morphological features of apoptosis in 21% and 2.5% oxygen environments was not significantly different ($p<0.05$). After 20 hours incubation the mean percentage apoptosis of neutrophils incubated in 2.5% oxygen was less than that of neutrophils incubated in 21% oxygen (46.2 ± 5.3 versus 56.3 ± 5.3), however, this difference was again not significant. When the pO_2 of the medium under these two conditions was quantified using a blood-gas analyser (figure 3.2.1.1A) it was found that the pO_2 of the medium only decreased to approximately 9 kPa under the 'hypoxic' condition. Although this value was significantly lower than control, it is only slightly lower than the pO_2 of normal arterial blood and very similar to the oxygen tension in normal tissues, even at rest. In addition, these levels were not maintained for the duration of the experiments, suggesting that the containers used were partially oxygen permeable.

Thus, a second set of experiments was performed in which different containers were used. These containers maintained the required atmosphere (and medium pO_2) for the 20 hour duration of the experiment (figure 3.2.1.2A). In addition, a third condition was introduced where neutrophils were incubated in an MK3 anaerobic incubator. Under this condition the pO_2 of the medium was reduced to less than 3.5 kPa (figure 3.2.1.2A). Figure 3.2.1.2B shows the effect of these levels of hypoxia on the rate of neutrophil apoptosis. After 6 hours, when the index of apoptosis is low, there was no noticeable effect of hypoxia. However, after 20 hours incubation

A

	15 minutes	6 hours	20 hours
21% Oxygen pO_2	26.3 ± 0.1	22.1 ± 2.2	19.9 ± 2.3
21% Oxygen pCO_2	7.5 ± 0.8	4.3 ± 0.6	4.2 ± 0.6
2.5% Oxygen pO_2	8.3 ± 1.9	15.1 ± 1.6	20.2 ± 0.5
2.5% Oxygen pCO_2	7.6 ± 1.0	4.4 ± 0.2	4.3 ± 0.4

B

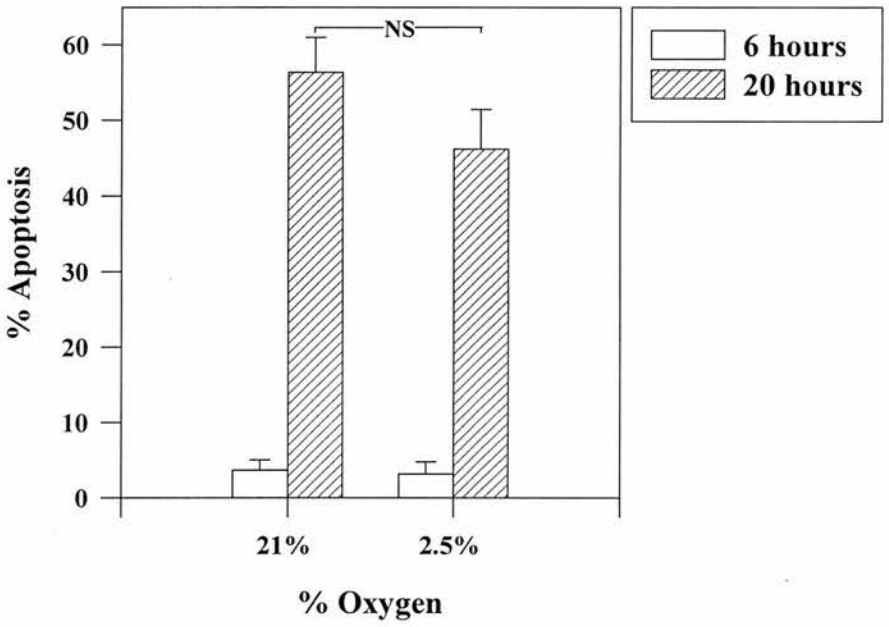


Figure 3.2.1.1 The effect of hypoxia on the rate of neutrophil apoptosis using existing methology. (A) Changes in the pO_2 and the pCO_2 of the culture medium over the time course of the experiments shown in B. The results are expressed in kPa as the mean ± SD from 2 separate experiments. **(B)** Neutrophils (5×10^6) were incubated in atmospheres containing either 21% or 2.5% oxygen. After 6 and 20 hours cytocentrifuge preparations were made in triplicate and the percentage of apoptosis assessed by examining cell morphology under oil immersion light microscopy. When the pO_2 of the culture medium was monitored (A) the initial pO_2 of the culture medium was found not to remain constant for the duration of the experiment. Data are expressed as the mean ± SEM of 5 separate experiments. The difference in the percentage apoptosis between the conditions was not significant at 6 or 20 hours ($p<0.05$).

A

	15 minutes	6 hours	20 hours
21% Oxygen pO_2	26.9 ± 0.6	22.8 ± 0.8	22.4 ± 0.2
21% Oxygen pCO_2	8.8 ± 0.8	4.7 ± 0.2	4.1 ± 0.5
2.5% Oxygen pO_2	8.7 ± 0.7	8.6 ± 0.4	9.5 ± 1.7
2.5% Oxygen pCO_2	8.9 ± 0.2	4.7 ± 0.4	3.9 ± 0.2
0 % Oxygen pO_2	12.7 ± 0.8	3.3 ± 0.3	3.3 ± 0.3
0% Oxygen pCO_2	7.2 ± 0.2	7.6 ± 0.4	8.3 ± 0.1

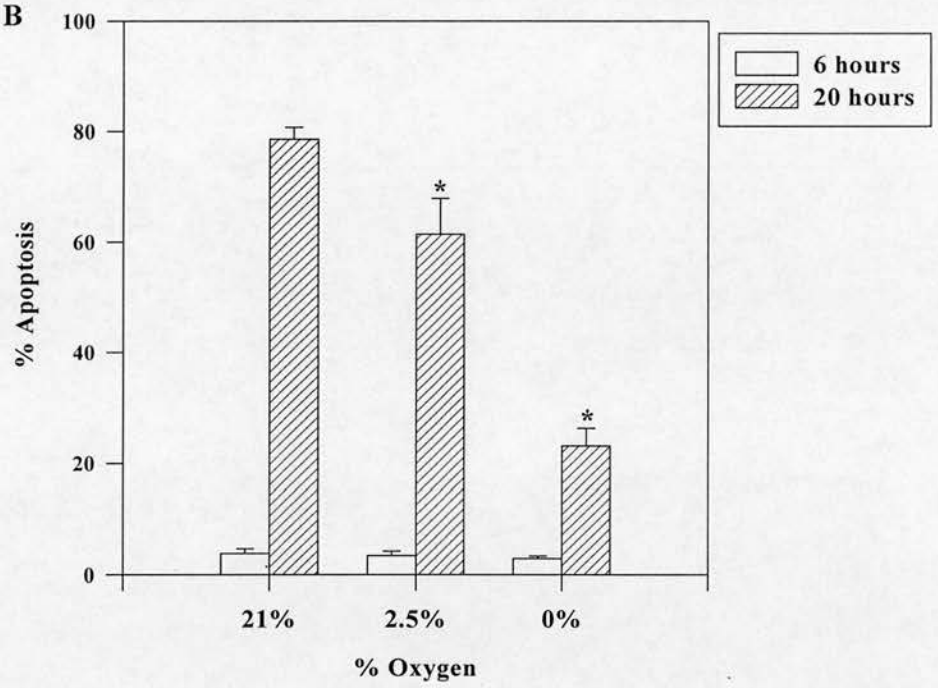


Figure 3.2.1.2 Hypoxia inhibits neutrophil apoptosis in a concentration dependent manner. **(A)** Changes in the pO_2 and the pCO_2 of the culture medium over the time course of the experiments shown in B. The results are expressed in kPa as the mean ± SD from 2 separate experiments. **(B)** Neutrophils were incubated and evaluated as described in figure 3.2.1 except the pO_2 of the culture medium was maintained for the duration of the experiment (A). Cells were also incubated in an anaerobic environment (0% oxygen) to further reduce the pO_2 of the medium. Data are expressed as the mean ± SEM from n = 5 separate experiments each performed in triplicate. The difference in the percentage of apoptosis between all three conditions is significant at 20 hours but not at 6 hours (*$p < 0.05$ compared to values obtained in 21% O_2).

hypoxia caused a significant and concentration-dependent inhibition of neutrophil apoptosis.

3.2.2 Time-course for the effect of hypoxia on neutrophil apoptosis

Due to the dramatic inhibition of neutrophil apoptosis found under hypoxic conditions, an extended time-course experiment was undertaken in order to determine at which point between 6 and 20 hours hypoxia began to inhibit neutrophil apoptosis and to evaluate the duration of this protective effect. Neutrophils were incubated in atmospheres containing either 21% or 0% oxygen and the percentage of apoptotic cells was assessed using morphological criteria at time points up to and including 44 hours (figure 3.2.2.1). At 44 hours neutrophils incubated in 21% oxygen showed a large increase in the percentage of necrotic cells (i.e. trypan blue positive). Therefore, at this time point the resulting cytospins showed considerable cell debris and it was not possible to assess the percentage of apoptotic neutrophils. In contrast, neutrophils incubated in 0% oxygen were all viable (trypan blue negative) and the percentage of neutrophil apoptosis was still only 21.5 ± 4.4% (figure 3.2.2.2). The difference in the percentage of apoptotic neutrophils under hypoxic and normoxic conditions was significant at 10, 20, 30 and 44 hours but not at 6 hours ($p < 0.05$).

3.2.3 Confirmation of the hypoxic inhibition of neutrophil apoptosis by annexin V binding

One of the earliest events in programmed cell death is the externalisation of phosphatidylserine, a membrane phospholipid normally restricted to the inner leaflet of the lipid bilayer. Annexin V is an endogenous human protein with a high affinity for membrane bound phosphatidylserine. Hence, this protein can be used as a sensitive probe for phosphatidylserine exposure on the cell membrane and as an index of apoptosis (Pepper et al., 1998; Vermes et al., 1995). Neutrophils were incubated for 6 and 20 hours in atmospheres containing 21% and 0% oxygen and FITC-labelled annexin V binding was assessed by flow cytometry. Figure 3.2.3A shows representative histograms demonstrating less annexin V binding in hypoxic

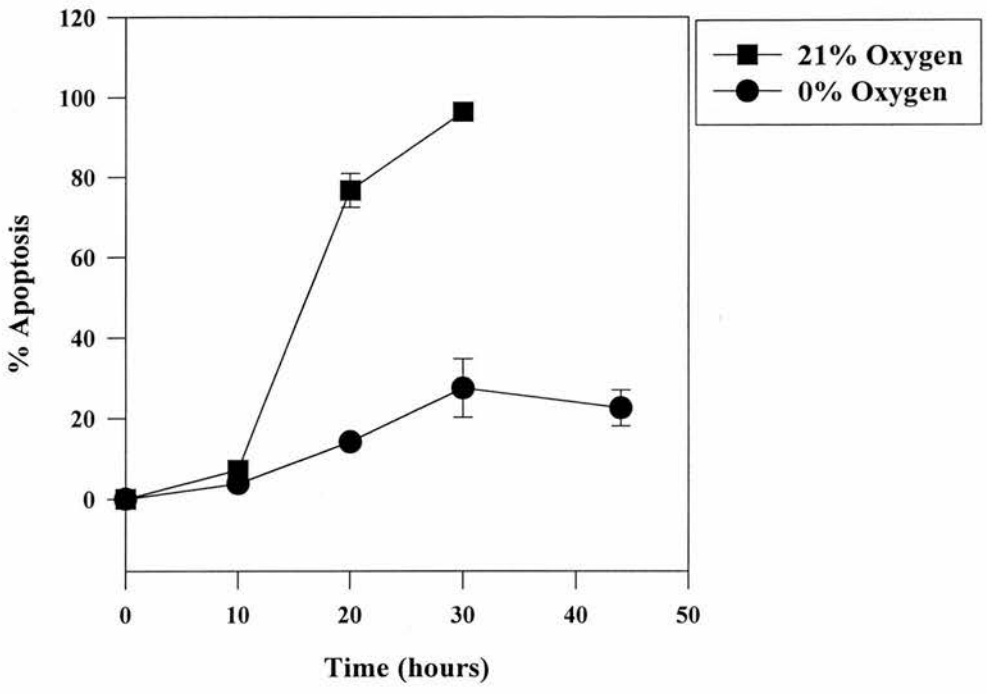
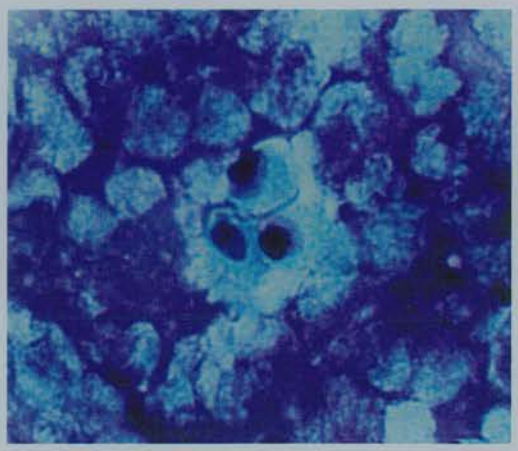


Figure 3.2.2.1 Time course for the inhibition of neutrophil apoptosis by hypoxia. Neutrophils (5 x 10^6/ml) were incubated in atmospheres containing either 21% or 0% oxygen. At time points up to and including 44 hours the percentage of apoptosis was evaluated as described in figure 3.2.1. At 44 hours neutrophils incubated in 21% oxygen showed an increase in necrotic cells to 26% (as assessed by trypan blue exclusion), thus it was not possible to assess the percentage of apoptotic cells at this time point. Results are from a representative experiment of 2 and data are expressed as mean ± SEM of triplicate determinations.

A



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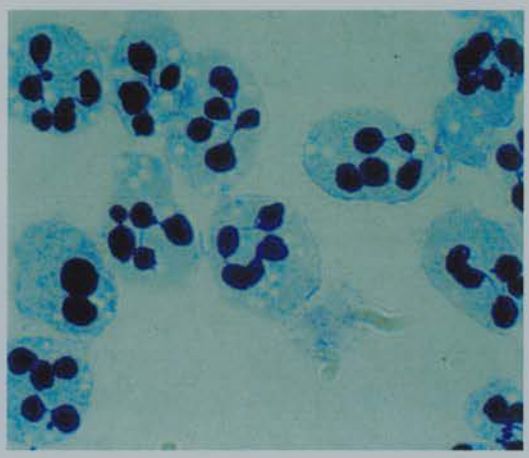
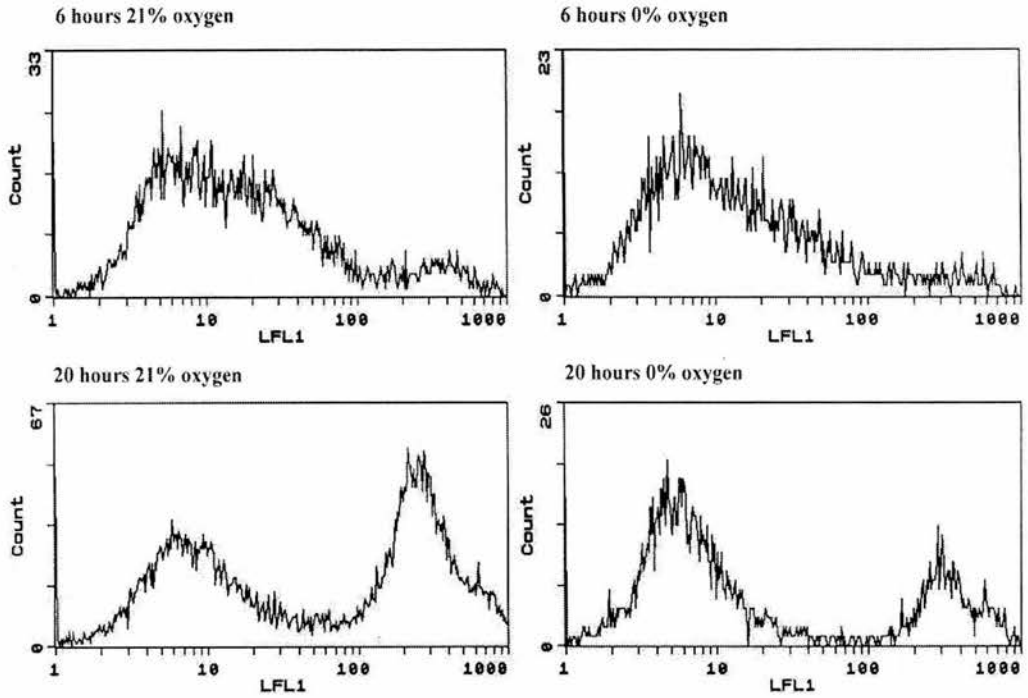


Figure 3.2.2.2 Cytological appearance of human neutrophils aged in culture for 44 hours in 21% oxygen (A) or 0% oxygen (B). (magnification x1000). Neutrophils were isolated by methods that cause minimal cell activation and cultured at 5 x 10^6 /ml in Iscove's MDM. Cytocentrifuge preparations were fixed and stained with May Grunwald/Geimsa. The cells cultured in 21% oxygen showed a significant increase in the number of necrotic cells (assessed by trypan blue exclusion prior to cell fixation). There was no such increase observed in the neutrophils incubated in 0% oxygen. **(A)** Due to the increase in cell necrosis it was not possible to assess the percentage of apoptotic neutrophils. **(B)** In 0% oxygen the proportion of cells exhibiting the morphological features of apoptosis was still only 21.4%.

A



B

Method	6hr 21%	6hr 0%	20hr 21%	20hr 0%
Annexin V	7.6 ± 1.4	5.9 ± 1.4	59.6 ± 4.4	32.1 ± 2.7*
Morphology	3.5 ± 1.4	2.9 ± 1.3	67.3± 3.5	38.4 ± 2.2*

Figure 3.2.3 Effect of hypoxia on annexin V binding in human neutrophils. Neutrophils (5 x 10^6/ml) were incubated in atmospheres containing 21 or 0% oxygen. At 6 and 20 hours neutrophils were harvested and incubated with FITC-labelled annexin V and fixed in 3% paraformaldehyde prior to analysis using an Epics Profile II. **(A)** Shows representative flow-cytometry histogram (cell count vs log fluorescence) profiles. **(B)** Cytospins were also prepared in parallel and % morphological apoptosis compared with % apoptosis as assessed by annexin V labelling. Data is expressed as mean ± SEM from 4 separate experiments (* $p < 0.05$ compared to cells cultured for 20 hours in 21% O_2).

cells as compared to control cells. This difference is most striking at 20 hours. The mean percentage apoptosis, as assessed by annexin V binding, correlated well with values obtained using morphological criteria (figure 3.2.3B). Apoptosis values at 6 hours were slightly higher for both conditions using annexin V binding compared to values obtained by morphology, but as the translocation of phosphatidylserine proceeds morphological changes, this was not unexpected. Although the externalisation of phosphatidylserine also occurs during necrosis, this possibility was eliminated in these experiments using trypan blue exclusion (data not shown).

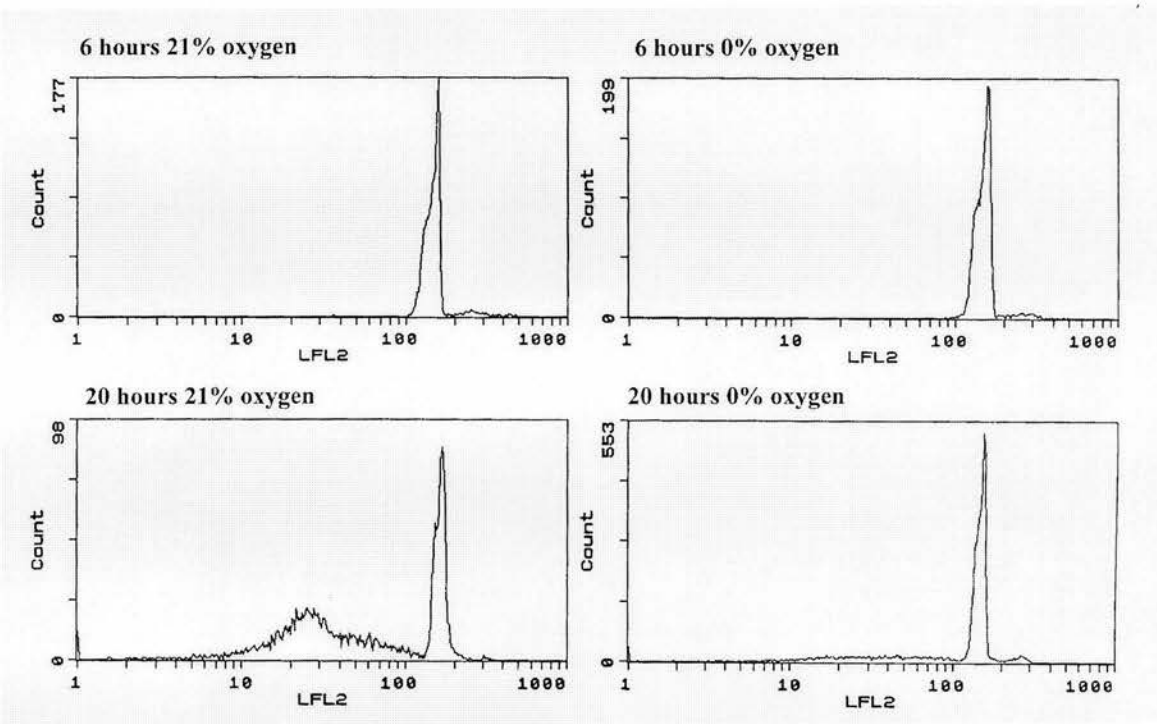
3.2.4 Effect of hypoxia on propidium iodide staining in neutrophils

The inhibitory effect of hypoxia on neutrophil apoptosis was further confirmed using propidium iodide DNA staining and flow cytometry. The DNA fragmentation that occurs during apoptosis results in reduced binding of propidium iodide and, hence, as a cell undergoes apoptosis there is decreased binding of the fluorescent dye propidium iodide to DNA, which can be analysed by flow cytometry (Nicoletti et al., 1991). Figure 3.2.4A shows the decrease in the percentage of cells with hypodiploid DNA content following hypoxic (0% O_2) incubation compared to cells incubated under normoxic conditions (21% O_2) at 20 hours. The percentage apoptosis obtained using propidium iodide and flow cytometry closely match the extent of apoptosis assessed by morphological criteria in parallel incubations (figure 3.2.4B). Hence, this data confirms that the above morphological and annexin V data reflect genuine apoptosis.

3.2.5 Kinetics of the inhibition of neutrophil apoptosis by hypoxia

In order to characterise the hypoxic inhibition of neutrophil apoptosis in greater detail we investigated whether hypoxia could inhibit apoptosis at any stage of the apoptotic program, or whether neutrophils must be hypoxic from time 0. This was achieved by incubating neutrophils for progressively longer times (3, 6, 9, 12, 15, 18 and 21 hours) in 21% oxygen before transferring them to a hypoxic environment for the rest of the 24 hour experiment. The effect of the length of the normoxic incubation time on the rate of apoptosis was assessed and compared to control values (figure 3.2.5A, B and C). These data demonstrate that hypoxia can delay the rate of

A



B

Method	6 hrs 21%	6 hrs 0%	20 hrs 21%	20 hrs 0%
Propidium Iodide Staining	1.1 ± 0.1	0.9 ± 0.1	65.2 ± 2.0	33.9 ± 4.3*
Morphology	1.4 ± 0.3	0.9 ± 0.2	69.3 ± 1.3	33.0 ± 1.3*

Figure 3.2.4 Effect of hypoxia on propidium iodide staining in human neutrophils. Neutrophils (5 x 10^6/ml) were incubated in atmospheres containing 21% or 0% oxygen. Following a 6 or 20 hour incubation period cells were resuspended in ice-cold 70% ethanol, washed in PBS and incubated with propidium iodide in the presence of RNase prior to analysis using an EPICS Profile II. **(A)** Shows representative flow-cytometry histogram (cell count vs log fluorescence) profiles. **(B)** Cytospins were prepared in parallel and % morphological apoptosis compared with % apoptosis assessed with propidium iodide staining. Data is expressed as mean ± SEM from 3 separate experiments (* $p < 0.05$ compared to cells cultured in 21% oxygen).

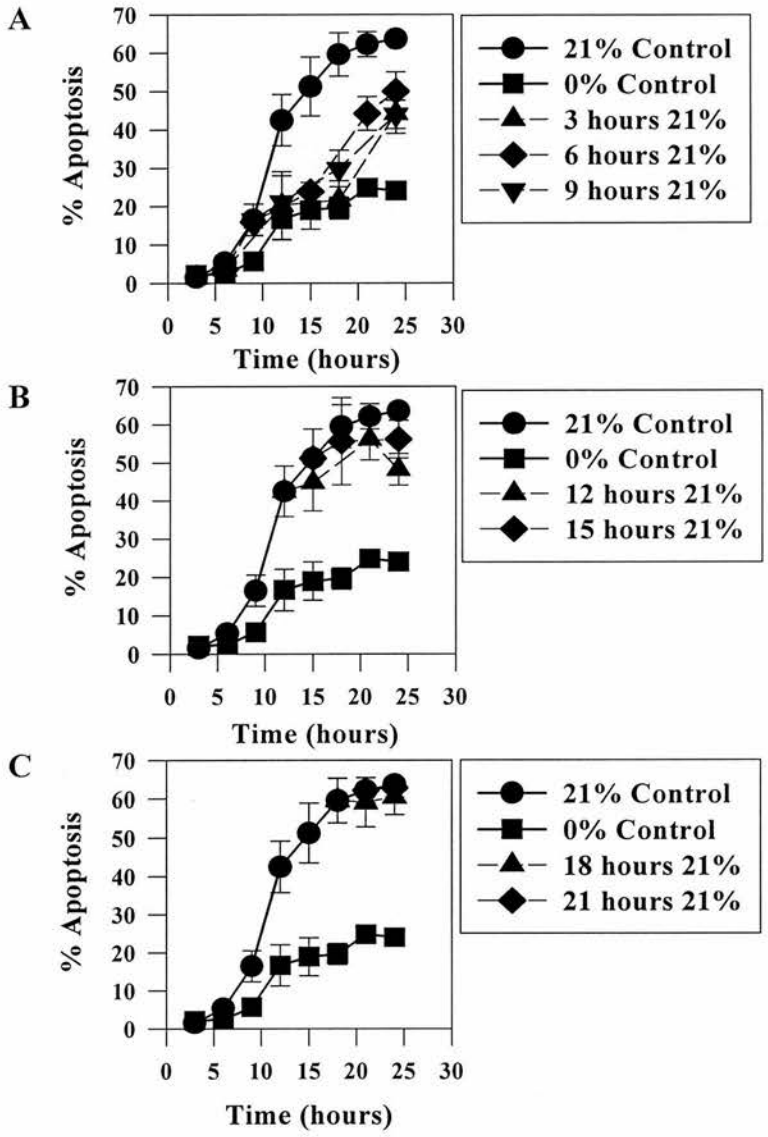


Figure 3.2.5 The kinetics of the hypoxic inhibition of neutrophil apoptosis. Neutrophils (5 x 10^6/ml) were incubated in 21% oxygen for increasing amounts of time before being transferred to an anoxic incubator and the rate of apoptosis assessed over a 24 hour period. Control cells were incubated in either 21% or 0% oxygen for the entire duration of the experiment. Data are expressed as the mean ± SEM from n=4 separate experiments each performed in triplicate and is shown on 3 graphs for reasons of clarity.

constitutive cell death even when introduced relatively late (e.g. After 3-15 hours of normoxic incubation) and at a time when an exponential increase in apoptosis is occurring. By 18 hours, however, the rate of constitutive apoptosis begins to plateau in the 21% O_2 control cells and, thus, it was not possible to see any effect on the rate of apoptosis in cells that were made hypoxic at 18 hours or more into the experiment. These results suggest that hypoxia can provide some protection against apoptosis in neutrophils even after prolonged incubation in 21% O_2.

3.2.6 Effect of re-oxygenation on neutrophil apoptosis

We also examined the effect of returning hypoxia-treated neutrophils to a normoxic environment. As an initial experiment neutrophils were incubated under anoxic conditions for 20 hours, at which point they were transferred to an atmosphere containing 21% oxygen. The rate of apoptosis of these cells was then assessed for the following 13 hours in parallel with control cells that remained under normoxic and hypoxic conditions throughout the experiment. Over this subsequent time period, the rate of apoptosis of the 0% → 21% (re-oxygenated) neutrophils was very similar to that seen in the cells maintained at 0% O_2 throughout the experiment (figure 3.2.6.1). These data indicate that after a prolonged hypoxic incubation period (20 hours) neutrophils remain resistant to spontaneous apoptosis for a significant period of time. To clarify further this observation and define the minimum initial hypoxic period required to delay or inhibit neutrophil apoptosis, we investigated whether shorter periods of hypoxia could generate a similar effect. These experiments are essentially the reverse of those described in section 3.2.5. Neutrophils were incubated for 3, 6, 9, 12, 15, 18 or 21 hours in 0% oxygen prior to transfer to normoxic conditions for the remainder of the 24 hour experiment and the corresponding rates of apoptosis assessed (figure 3.2.6.2). These data demonstrated that cells returned to a normoxic environment after an initial hypoxic incubation of 3-15 hours entered a normal rapid sigmoid increase in apoptosis, identical to that observed at earlier times in normoxic treated cells. Hence cells incubated for up to 15 hours under anoxic conditions did not lose their apoptotic potential or undergo apoptosis at an increased rate when returned to a normoxic environment. Thus, it

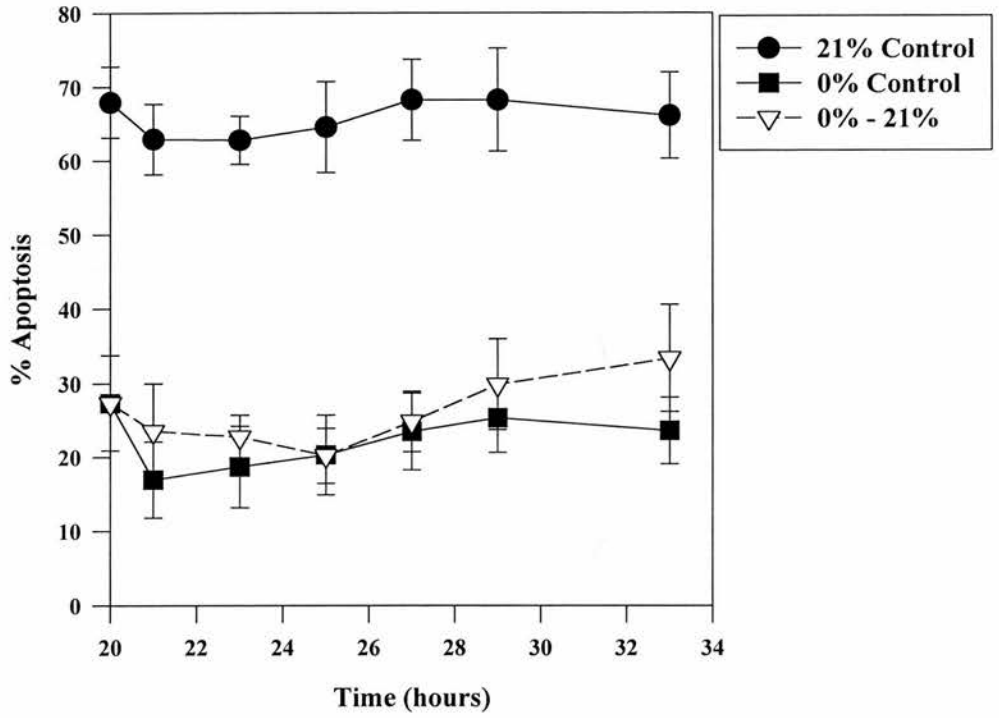


Figure 3.2.6.1 The effect of re-oxygenation on neutrophil apoptosis.
Neutrophils (5 x 10^6/ml) were incubated in 0% oxygen for 20 hours and then transferred to an atmosphere containing 21% oxygen. The rate of apoptosis of these cells was assessed over a subsequent 13 hour time period using the method described in figure 3.2.1. Control cells were kept in 21% or 0% oxygen for the duration of the experiment. Data is expressed as mean ± SEM from n=5 separate experiments each performed in triplicate.

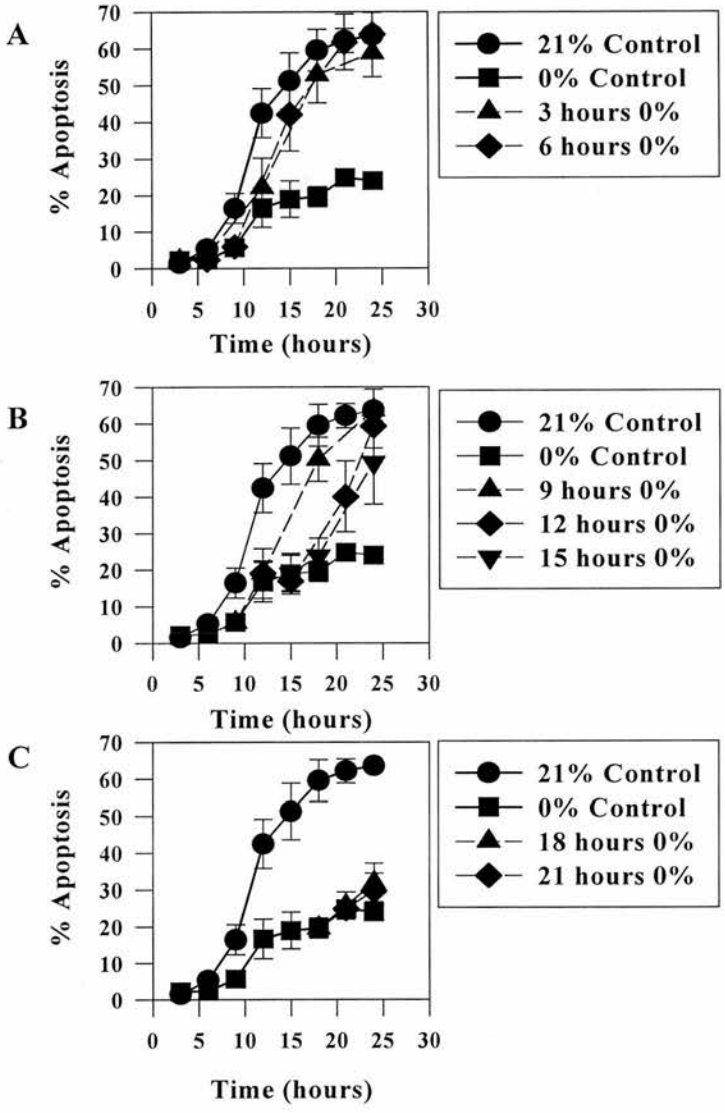


Figure 3.2.6.2 The effect of re-oxygenation on neutrophil apoptosis.
This experiment is the reverse of that shown in figure 3.2.5. Neutrophils (5 x 10^6/ml) were incubated in 0% oxygen for increasing amounts of time before being transferred to a normoxic environment and the rate of apoptosis assessed over a 24 hour period. Control cells were incubated in 21% and 0% oxygen for the entire duration of the experiment. Data are expressed as the mean ± SEM from n=4 separate experiments each performed in triplicate and is shown on 3 graphs for reasons of clarity.

appears that sustained protection (re-programming) only occurs after prolonged hypoxia.

3.3 Discussion

In the above experiments we have shown by several well established methods that hypoxia profoundly inhibits the rate of constitutive neutrophil apoptosis *in vitro*. These findings are in complete contrast to those of Jacobson and Raff (1995) and Shimizu et al (1995) who have demonstrated that hypoxia induces apoptosis in SV-40 transformed human fibroblasts (that lacked mitochondria) and a rat pheochromocytoma cell line, respectively. A similar induction of apoptosis by hypoxia has now been reported in WEHI 7.1 T-lymphoma cells (Muschel et al., 1995), thymocytes (Steffanelli et al., 1995), neonatal rat cardiomyocytes (Tanaka et al., 1994), the human breast cancer cell line MCF-7 (Amellem et al., 1997), human adenocarcinoma HT29 cells (Yao et al., 1995), PC-12 cells (Yoshimura et al., 1998), sympathetic neurons (Rosenbaum et al., 1994) and cultured hepatocytes (Shimizu et al., 1996).

Despite such observations, there are clearly a number of instances *in vivo* where a hypoxic environment appears to play a role in protecting cells against PCD. For example, hypoxic regions are a common feature of solid tumours (Moulder and Rowckwell, 1984; Vaupel et al., 1991); this is due to certain features of tumour physiology including limited arteriolar supply causing arteriolar deoxygenation (Dewhirst et al., 1996), relatively low vascular density and disorderly vascular architecture (Secomb et al., 1993), oxygen consumption rates that are mis-matched with oxygen supply (Secomb et al., 1995) and an unstable blood supply (Kimura et al., 1996). Although hypoxia has been shown to induce apoptosis in a number of tumour cells when studied in monolayer culture (Yao et al., 1995; Shimizu et al., 1995), cells in hypoxic regions of tumours appear to be more resistant than their normoxic counterparts to the effects of radiotherapy and conventional chemotherapeutic agents (for review see Teicher, 1994) and this factor is thought to contribute importantly to disease relapse.

Hypoxia has also been shown to protect against dexamethasone and calcium ionophore (A23187) induced apoptosis in thymocytes, although over longer periods hypoxia itself induces apoptosis in this cell type (Stefanelli et al., 1995). These

experiments are in agreement with the findings of Matthews et al. (1987) and Shen et al. (1987) who found hypoxia inhibited both TNFα cytolysis of L929 cells and doxorubicin mediated cytotoxicity in Chinese hamster ovary cells respectively. Thus, two seemingly opposing effects of hypoxia exist, one protective and the other inducing cell death.

An interesting anomaly of the experiments shown above was that the pO_2 of the medium failed to reflect the atmospheric oxygen content. When an atmosphere containing 2.5% oxygen was used the corresponding pO_2 of the medium was approximately 9 kPa, while an anaerobic atmosphere only reduced the medium pO_2 to 3.3 kPa. The anaerobic incubator used (MK3, Don Whitley Scientific Ltd., UK) incorporates a nickel catalyst to ensure a constant oxygen content of 0-0.1 kPa and this is monitored constantly by assessing the growth of organisms within the incubator that are strict facultative anaerobes. It is possible that the blood-gas analyser used to measure the medium pO_2 was inaccurate or poorly calibrated at such low pO_2 values, however, similar results were obtained using an independent oxygen electrode (World Precision Instruments Inc., USA; data not shown) making these unlikely explanations. Thus it would appear that even under protracted anaerobic conditions the medium does not equilibrate fully with the atmosphere.

In order to assess the relevance of these findings to *in vivo* situations it is necessary to compare these pO_2 values with both physiological and pathophysiological values. Cells are routinely cultured in atmospheric oxygen (the pO_2 of dry air at sea level is 21.2 kPa), however, this level of oxygen is clearly 'supra' physiological with reference to tissue levels. Maximum arterial pO_2 levels found in the body are approximately 13 kPa. These values are found in the lung and large arteries. However, as the majority of inflammatory sites are extrapulmonary, it is of more relevance to look at tissue pO_2. As discussed in section 1.3.3, there are several ways of measuring tissue pO_2, however, these are all open to criticism, mainly that tissue pO_2 is not uniform. In general, tissue pO_2 lies in the range of 1.3-6.7 kPa, although in certain physiological and pathological states tissue pO_2 will be further reduced. For example, a significant proportion of any injured tissue exists in conditions of

very low O_2 tension as the injury has, by definition, destroyed or interrupted the local circulation. Attempts at measuring wound deadspace pO_2 have given values of 0.8 and 1.5 kPa (Niikinski et al., 1972; Hunt et al., 1967). Other pathologic conditions that produce localised regions of hypoxia include myocardial infarction and rheumatoid arthritis. In a dog model of myocardial infarction, occlusion of the left anterior descending artery caused a rapid drop of myocardial tissue pO_2 to a mean level of 0.3 ± 0.1 kPa (Feola et al., 1979). The pO_2 of synovial fluid taken from rheumatoid arthritis patients has also been quantified and shown to be significantly lower than that of control (non-rheumatoid) synovial fluid values (3.8 kPa versus 6.7 kPa) (Ellis et al., 1994). Diseases which are associated with more generalised hypoxaemia and tissue hypoxia include lung disease such as ARDS and endotoxaemia. Indeed multiple organ failure (MOF), which often occurs concomitantly with ARDS and sepsis, and is at least partially responsible for the poor prognosis associated with these diseases, has been proposed to be caused in part by tissue hypoxia.

Thus, the pO_2 levels used in our experiments can be considered to fall in the range relevant to *in vivo* oxygen tensions. The pO_2 of the medium incubated in 0% oxygen (3.3 kPa) lies in the lower region of some tissue pO_2 values but corresponds to that of hypoxia in other tissues and the medium incubated in 2.5% oxygen (9 kPa) is close to the pO_2 of arterial blood. Our results, showing that these levels of pO_2 significantly inhibit neutrophil apoptosis compared to cells cultured in 21% oxygen, imply that examination of apoptosis under routine (21% O_2) culture conditions represents a relative hyperoxic challenge and may alter the susceptibility of cells to apoptosis and does not accurately reflect the O_2 environment *in vivo*. In addition, pathological studies have suggested that neutrophils may remain viable for considerably longer periods when recruited to an inflammatory focus (e.g. an emphysema cavity) where there is local and often systemic hypoxia. The effect of extreme tissue hypoxia on neutrophil survival that would delay their removal, could potentially exacerbate the disease state and result in additional neutrophil-mediated tissue destruction. Although neutrophils have been shown to phagocytose normally under anaerobic conditions (Sbarra and Karnovsky, 1959) it might be expected that

the respiratory burst, which is necessary for the effective killing of certain microorganisms (Mandell, 1974), would also be inhibited under these conditions. However, Gabig and co-workers (1979) have demonstrated that neutrophils have the capacity to mount a nearly normal respiratory burst at oxygen tensions down to 1 kPa, suggesting that not only do neutrophils live longer under hypoxic conditions but that they also remain fully functional.

We have also described the kinetics of hypoxia-mediated survival of neutrophils. The ability of hypoxia to protect neutrophils from apoptosis after they had been cultured in 21% O_2 for time points up to, and including, 15 hours implies that hypoxia can inhibit neutrophil apoptosis even in aged cells and, hence, is similar to the apoptosis protection effects of other agents such as GM-CSF (S. Hannah, personal communications). More importantly, we also found that when neutrophils were returned to a normoxic environment after a lengthy hypoxic incubation they lost their apoptotic potential for a considerable time period thereafter. While, following shorter hypoxic incubations, neutrophils underwent apoptosis at rates similar to those observed in control cells. In many other cell types re-oxygenation after a hypoxic period induces cell death both by necrosis and apoptosis (Shimizu et al., 1996; Van Der Laarse et al., 1979; Halsey et al., 1991; Iwata et al., 1994; Vogt et al., 1998; Bossenmeyer et al., 1998), however, the resistance of neutrophils to cell death following re-oxygenation may be of relevance to the pathophysiology of ischaemia-reperfusion injury. Ischaemia is a common clinical event in a number of organs with potentially serious consequences. Evidence suggests that a major part of tissue injury occurs upon reperfusion and the neutrophil has been heavily implicated as contributing significantly to this damage (Welbourn et al., 1991). A great deal of the evidence for the role of neutrophils in reperfusion injury has come from cardiac studies, however, this phenomenon has also been observed in other organs. For example, in a rat model of hepatic ischaemia and reperfusion, pre-treatment with a mAb to neutrophils resulted in an 86% decrease in the accumulation of these leukocytes in the affected lobe. This was accompanied by a significant attenuation in tissue necrosis, measured after 24 hours of reperfusion, from 80% in controls to 28% in the treated groups (Jaeschke et al., 1990). Similarly, in a model of renal ischaemia

and reperfusion in rats, administration of an anti-neutrophil serum resulted in a reduction in both creatinine levels and tubular necrosis (Klausner et al., 1989). Thus, the relative resistance of neutrophils themselves to re-oxygenation-mediated cell death may underlie their destructive potential in reperfused tissues.

Hypoxia has also been reported to modulate neutrophil receptor expression and, thus, potentially alter cellular functions and responses to inflammatory cytokines. Knowles and co-workers (1997) have reported that hypoxia increases whole blood neutrophil phagocytosis in the presence of IL-8, TNFα and IL-1β compared to normoxic, control cells and, furthermore, that this enhanced phagocytosis was due to increased expression of certain complement and/or FcγR receptors. Similarly, Scannell and colleagues (1995) have reported increased expression of the leukocyte adhesion molecules CD11b/CD18 on hypoxic whole blood neutrophils and, in addition, demonstrated an augmented LPS-induced upregulation of these molecules following hypoxia. Contrary to these findings in whole blood, Simms and D'Amico (1993) reported that hypoxia reduced isolated neutrophil FcγR III (CD16) and FcγR II (CD32) expression, while having no effect on complement receptor type 1 (CD35) and CD11b/CD18 expression. However, adherence of neutrophils in the presence of the Arg-Gly-Asp-Ser (RGDS)-binding epitope of fibronectin was found to significantly restore neutrophil FcγR II and FcγR III expression in the presence of hypoxia. Neutrophils have also been reported to upregulate IL-1β type 1 R, TNFαR (p75) and IL-8R expression in response to hypoxia (Simms and D' Amico, 1996). Interestingly, this response was abrogated when the neutrophils were bound to the matrix proteins, fibronectin and laminin. The same study also found that, although no change in receptor expression on unbound neutrophils was observed following hypoxia/reoxygenation, this treatment caused cells adhered to laminin or fibronectin to upregulate TNFα (p55 and p75) and IL-8 expression. It would thus appear that hypoxia not only has the potential to affect expression of neutrophil cytokine receptors and cell surface receptors that mediate cell adherence, bactericidal activity and activation state but, in addition, that the effect of matrix proteins on these cell surface molecules is also potentially regulated by oxygen tension.

It is important to note that hypoxia and/or hypoxia-re-oxygenation have several other potential pro-inflammatory effects. Albina and co-workers (1995) demonstrated that macrophages grown in an anoxic environment have increased total L-arginine metabolism, arginase activity and increased release of TNFα and IL-6, suggesting that anoxia may also act as an inducer of macrophage activation. This proposal is substantiated by other studies, which have demonstrated an increase in the production and release of other inflammatory agents from these cells in response to changes in pO_2. VanOtteren et al. (1995) showed that anoxia followed by re-oxygenation resulted in the increased expression of both TNFα and macrophage inflammatory protein-1 alpha (MIP-1α) from LPS stimulated alveolar macrophages. Naldini and co-workers (1997) showed that hypoxia increased IL-2, IL-4 and interferon (IFN)-γ production in human peripheral mononuclear cells, and Metinko et al. (1992) demonstrated that anoxic pre-treatment of monocytes primed them for augmented production of IL-8. Finally, hypoxia has been reported to increase LPS-induced release of IL-1 and TNFα from alveolar macrophages (Hempel et al., 1996). Many of these substances are chemotactic for and/or directly activate neutrophils. For example, TNFα mediates inflammation by co-ordinating leukocyte migration via the upregulation of endothelial cell and leukocyte adhesion molecules (Strieter et al., 1989; Ulich et al., 1991; Pober and Cotran, 1991; Nawroth et al., 1986). In addition, TNFα has been shown to activate multiple cell types including neutrophils, lymphocytes and monocytes (Wewers et al., 1990; Shalaby et al., 1985; Klebanoff et al., 1986). MIP-1α was initially characterised as having significant neutrophil activating and chemokinetic properties. While IL-8 interacts with its own specific neutrophil receptor to induce motility (chemokinesis), directional migration (chemotaxis), exocytosis of storage enzymes, NADPH oxidase priming and activation and expression of the leukocyte integrin CD11b/CD18 complex. Its biological activity is maintained in the presence of significant changes in pH, and is relatively resistant to proteolysis and denaturation as compared to other known chemotactic factors (Matsushima and Oppenheim, 1989; Baggiolini et al., 1989; Westwick et al., 1989). The early and sustained generation and stability of IL-8 suggests that its production at sites of acute inflammation may result in prolonged

biological activity, making it a pivotal cytokine in acute neutrophil-mediated disease states.

Macrophages are not the only cells that have been shown to release pro-inflammatory agents in response to changes in pO_2 tension. Endothelial cells have been reported to generate PAF (Milhoan et al., 1992) and IL-8 (Pober and Cotran, 1991) following a hypoxic challenge. H_2O_2, a product of the endothelial xanthine oxidase enzyme system (Welbourn et al., 1991), is also upregulated following hypoxia and re-oxygenation and this molecule is also capable of causing neutrophil activation. Indeed, a stimulation of neutrophil phagocytosis and chemotaxis has been observed when these cells were cultured with serum from tissue exposed to severe ischaemia (Freischlag and Hanna, 1992). Hypoxia-treated endothelial cells have also been shown to stimulate human neutrophils to produce superoxide anions and leukotriene B_4 (LTB_4) (Arnould et al., 1994). However, this activation by hypoxic endothelial cells required close contact between both cell types, as conditioned medium from hypoxic cells alone was not able to induce neutrophil activation.

A further and important pathophysiological effect of ischaemia and reperfusion is a rapid and sustained elevation of neutrophil adhesion to vascular endothelium. Rainger et al. (1995) found that adhesion of neutrophils to endothelial cells was time-dependent with 30, 60 and 100 min of exposure causing graded increments in neutrophil recruitment. *In vitro* studies of the effects of endothelial cell hypoxia and reoxygenation have shown that the enhanced neutrophil adhesion is susceptible to blockade by antibodies against CD18, ICAM-1 and P-selectin (Arnould et al., 1993; Yoshida et al., 1992). The report that hypoxia increases the expression of neutrophil adhesion molecules CD11b/CD18 (Scandell et al., 1995) is in accordance with this, however, production of PAF by endothelial cells has also been implicated in this mechanism (Milhoan et al., 1992).

Thus it appears that hypoxia is capable of causing a plethora of cellular responses, which favour neutrophil margination, recruitment and activation within tissues. The ability of hypoxia to dramatically enhance the neutrophil's life span is a further

factor that may enable us to explain the often severe tissue destruction associated with inflammatory foci that are known to possess a low pO_2 and redox potential.

CHAPTER 4

INTRACELLULAR SIGNALLING PATHWAYS IN THE INHIBITION OF NEUTROPHIL APOPTOSIS BY HYPOXIA

4.1 INTRODUCTION

In the previous chapter we established and characterised the ability of hypoxia to profoundly inhibit neutrophil apoptosis. This inhibitory effect of hypoxia on neutrophil apoptosis is opposed to the pro-apoptotic effect of hypoxia in many other cell types. For example, hypoxia induces apoptosis in cardiomyocytes (Tanaka et al., 1994), the T-lymphoma cell line, WEH7.1 (Muschel et al., 1995), cultured neurons (Rosenbaum et al., 1994), adenocarcinoma cells (Yao et al., 1995), macrophage-like RAW 264.7 cells (Yun et al., 1997) and transformed rat fibroblasts (Graeber et al., 1996). However, hypoxia has also been shown to inhibit several forms of stimulus-induced cell death, namely TNFα-mediated cytolysis of L929 cells (Matthews et al., 1987), dexamethasone-induced apoptosis in rat thymocytes (though prolonged hypoxia still induces spontaneous apoptosis in this cell type) (Stefanelli et al., 1995) and doxorubicin (an anthracycline cytotoxic antibiotic) cytotoxicity in Chinese hamster ovary cells (Shen et al., 1987). The mechanisms underlying these effects are not fully elucidated. However, in some cells TNFα acting at the TNFα-receptor results in a rapid rise in the levels of intracellular reactive oxygen species (ROS) (Larrick and Wright, 1990; Matthews et al., 1987) and in various cell types TNFα-mediated apoptosis can be inhibited by antioxidants (Matsuda et al., 1991; Chang et al., 1992). In addition, cellular sensitivity or resistance to TNFα is correlated with decreased or increased levels of superoxide dismutase respectively (Hirose et al., 1993). These findings led to the proposal that hypoxia may be inhibiting TNFα-induced cytotoxicity via its negative effect on intracellular ROS levels. The protective effects of antioxidants are not limited to TNFα cytotoxicity; N-acetyl cysteine (NAC) blocks the induction of apoptosis in thymocytes by T-cell receptor cross linking, and ionising radiation (McLaughlin et al., 1996); NAC, vitamin C and trolox protect neurons against apoptosis induced by growth factor deprivation (Mayer and Noble, 1994); and various antioxidants protect HL-60 cells from UV and

drug induced apoptosis (Verhaegen et al., 1995). These results may imply that ROS can activate or trigger apoptosis.

The inhibition of dexamethasone-induced apoptosis in rat thymocytes by hypoxia can also be mimicked by antioxidants (McLaughlin et al., 1996). However, more recent experiments have shown that ATP depletion can also inhibit apoptosis in this system and that ATP is required for glucocorticoid binding (Stefanelli et al., 1997). Thus, this group proposed that the inhibition of dexamethasone-induced apoptosis by hypoxia is due to the adverse effect of hypoxia on intracellular ATP pools and not ROS levels. A recent review has proposed the cellular ATP level as an important determinant for cell death (Richter et al., 1996). It has been shown that, in growth factor-dependant neural and haematopoetic cells, ATP levels decreased after growth factor withdrawal (Mills et al., 1995; Whetton and Dexter, 1983). However, viability and proliferation potential were maintained in the presence of an ATP-regenerating system (Whetton and Dexter, 1983). In contrast, Chou and co-workers (1995) demonstrated that reduction of intracellular ATP blocks actinomycin D-induced apoptotic cell death. Likewise, Eguchi et al. (1997) showed that ATP depletion, induced with an inhibitor of mitochondrial ATPase, completely blocks Fas/APO-1 stimulated apoptosis.

The increased resistance of Chinese hamster ovary cells to doxorubicin by hypoxia has been associated with the induction of the glucose-regulated protein (GRP) system (Shen et al., 1987). Glucose deprivation is the most common stimulus used to induce the GRP response, however, in addition to glucose deprivation this system can also be induced by 2-deoxyglucose, the calcium ionophore A23187 and chronic hypoxia. Shen et al. (1987) showed that all these stimuli concomitantly stimulated the GRP response and led to cellular resistance to doxorubicin. It is notable that, in this system, hypoxia proved the strongest inducer of GRPs and provided the greatest protection against doxorubicin. The GRP system represents a subset of a group of stress proteins that includes the major heat shock proteins (HSPs). In the case of the HSPs, it has been shown that the application of HSP-inducing stress results in the expression of a heat-resistance state referred to as thermotolerance. However, as

well as playing a role in protecting cells from elevated temperatures, the HSPs, particularly Hsp 70 and Hsp 27, have also been demonstrated to protect against apoptosis induced by various stimuli, including serum withdrawal (Mailhos et al., 1993), Fas/APO-1, staurosporine (Mehlen et al., 1996), doxorubicin (Oesterreich et al., 1993) and LPS (Wong et al., 1996). In addition, Hsp 70 has been postulated as a factor that may limit cell damage during myocardial and cerebral ischaemia (Dillman et al., 1986; Kawagoe et al., 1992). Hypoxia has been shown to induce Hsp 70 expression in cultured cells (Benjamin et al., 1990), although in macrophage like cell lines the same stimulus caused a decrease in constitutive Hsp 70 (Hsc 70) levels, an effect that was linked to hypoxia-induced apoptosis (Yun et al., 1997). These data suggest that hypoxia may have different effects on HSP expression depending on the cell type.

Another pathway that has a potential role in apoptosis and may be induced by hypoxia is the p38 mitogen-activated protein (MAP) kinase pathway. The family of p38 MAP kinases is a recently identified group of signalling molecules that mediate environmental stress responses in various cell types. The p38 MAP kinase activity is activated by dual phosphorylation on a Thr-Gly-Tyr motif in response to endotoxin, cytokines, physical stress (such as hyperosmolarity), chemical stress (such as hydrogen peroxide) (Han et al., 1993; Han et al., 1994; Raingeaud et al., 1995; Rouse et al., 1994; Freshney et al., 1994; Lee et al., 1994) and, although less well characterised, hypoxia (Seko et al., 1997). In neutrophils, p38 activity has been demonstrated upon stimulation with certain inflammatory mediators (Nahas et al., 1996) and appears to play a role in neutrophil activation (Schnyder et al., 1998; Zu et al., 1998). In other cells, p38 MAP kinases have been implicated in gene regulation (Tan et al., 1996; Wang and Ron, 1996), morphological alterations (Lin et al., 1997; Lavoie et al., 1993) and have been suggested to play a role in the regulation of apoptosis (Batistatou et al., 1993; Pittman et al., 1993; Kummer et al., 1997). For example, p38 inhibitors inhibit glutamate-induced apoptosis in cerebellar granular cells (Kawasaki et al., 1997), while activation of p38 was shown to protect against anisomycin-induced cell death in cardiac myocytes (Zechner et al., 1998), suggesting the effect of p38 MAP kinase on apoptosis may be cell type-dependent.

Although the molecular and biochemical events underlying apoptosis remain to be fully elucidated, there are several factors that appear to be common to most models of apoptosis. Firstly, the bcl-2 oncogene (the mammalian homologue of the ced-9 gene in C. elegans) and other members of the bcl-2 family have been shown to inhibit stimulus-induced cell death in a variety of cell types. Transgene-enforced overexpression of Bcl-2 and Bcl-XL have demonstrated the capacity of these proteins to confer apoptotic resistance to a number of different cells and tissues including lymphocytes (McDonnell et al., 1989; Strasser et al., 1991), neurons (Martinou et al., 1994), hepatocytes (Lacronique et al., 1996), prostrate endothelium (Zhang et al., 1997) and ovary follicles (Hsu et al., 1996). Cell culture studies have shown Bcl-2 and Bcl-XL to protect cells from a range of stimuli, including hypoxia (Jacobson and Raff, 1995), ultraviolet irradiation (Zhai et al., 1996), p53 (Schott et al., 1995), growth factor withdrawal (Farlie et al., 1995), nitric oxide (Messmer et al., 1996), hydrogen peroxide (Satoh et al., 1996) and stimulation with Fas-L (Zhang et al., 1996). Secondly, dissipation of the mitochondrial transmembrane potential ($\Delta\psi_m$), a characteristic of mitochondrial dysfunction, appears to be a general feature of apoptosis, irrespective of the cell type or apoptotic stimulus. In addition, pharmacological or genetic manipulations that prevent apoptosis also abolish the $\Delta\psi_m$ disruption that usually precedes cell death (for review see Kroemer et al., 1998). Mitochondrial dysfunction and dissipation of the $\Delta\psi_m$ are also associated with an inhibition of ATP generation and mitochondrial calcium handling, an increased level of mitochondrial ROS generation and the release of apoptosis inducing factor-1 (AIF-1) and cytochrome c. Thus, although there is strong evidence that mitochondria play a key role in regulating the apoptotic pathway, the precise involvement of each of these factors is unclear.

Hypoxia is not the only factor capable of inhibiting neutrophil apoptosis. A wide range of inflammatory mediators including IL-1β, TNFα, IL-6, G-CSF and GM-CSF, the bacterial product LPS and the chemoattractant C5a have all been shown to increase the life span of the neutrophil by attenuating apoptosis (Colotta et al., 1992a; Lee et al., 1993). While the intracellular mechanisms underlying these effects are poorly understood, there are data to support a role for tyrosine phosphorylation and

intracellular acidification in GM-CSF/LPS and G-CSF-mediated inhibition of cell death respectively (Yousefi et al., 1994; Sweeny et al., 1998, Gottlieb et al., 1995). Prevention of neutrophil apoptosis by GM-CSF has also been associated with induction of RNA and protein synthesis and the RNA and protein synthesis inhibitors, actinomycin D and cycloheximide have been shown to impede GM-CSF mediated protection (Brach et al., 1992).

In this study we have investigated the signalling pathway(s) underlying the protective effect of hypoxia on neutrophil apoptosis by examining:

(1) whether hypoxia acts through a similar mechanism to other agents known to promote neutrophil survival,
(2) whether factors common to other models of apoptosis could be involved, and
(3) the role of other pathways, which have been reported in the literature to be regulated by hypoxia and are capable of modulating apoptosis in other cell types.

4.2 Results

4.2.1. Modulation of the GM-CSF survival effect in neutrophils by hypoxia

It is now well documented that several inflammatory cytokines are capable of modulating the life span of the neutrophil. One such cytokine is GM-CSF, which has been shown to inhibit neutrophil apoptosis (Lee et al., 1993). In view of this, the possibility that the inhibitory effects of hypoxia and GM-CSF were mediated by a common mechanism or that hypoxia stimulated GM-CSF production from either the neutrophils themselves (Lindemann et al., 1989) or the small numbers of 'contaminating' monocytes (< 0.1%) present in the neutrophil preparations was investigated. Neutrophils were incubated with GM-CSF (50 U/ml) for 20 hours in atmospheres containing 21%, 2.5% or 0% oxygen. These experiments demonstrated that the GM-CSF- and hypoxia-mediated survival of neutrophils were clearly additive (figure 4.2.1), suggesting that the hypoxia and GM-CSF effects are discrete and act via independent mechanisms.

4.2.2 Effect of hypoxic culture on Bcl-2 expression in neutrophils

Studies on the nematode, *C. elegans*, have shown that individual cell survival is signalled by the gene, ced-9 (Hengartner et al., 1992). The mammalian homologue of the Ced-9 protein is Bcl-2 (Hengartner et al., 1994), which is capable of causing survival of a number of cell lines and B-lymphocytes (Reed et al., 1987; Nunez et al., 1990). The possibility that the protective effect of hypoxia was mediated by an increase in Bcl-2 gene expression was examined by taking fixed, permeabilized cells that had been previously incubated in atmospheres containing 21% or 0% oxygen and measuring Bcl-2 levels in these cells using an anti-Bcl-2 mAb and flow cytometry. MAC 387, which recognises an intracellular antigen in neutrophils, was used to confirm efficient permeabilization of the neutrophils (relative mean fluorescence > 50). Neither control nor hypoxia-treated neutrophils expressed Bcl-2 (table 4.2.2). Thus, it is unlikely that the enhanced survival of neutrophils under hypoxic conditions was Bcl-2 related. The Bcl-2 protein was also not present in neutrophils treated with other agents known to effect neutrophil apoptosis, namely TNFα and GM-CSF, both alone or in conjunction with hypoxia (table 4.2.2). These observations support experiments showing that neutrophils do not express Bcl-2

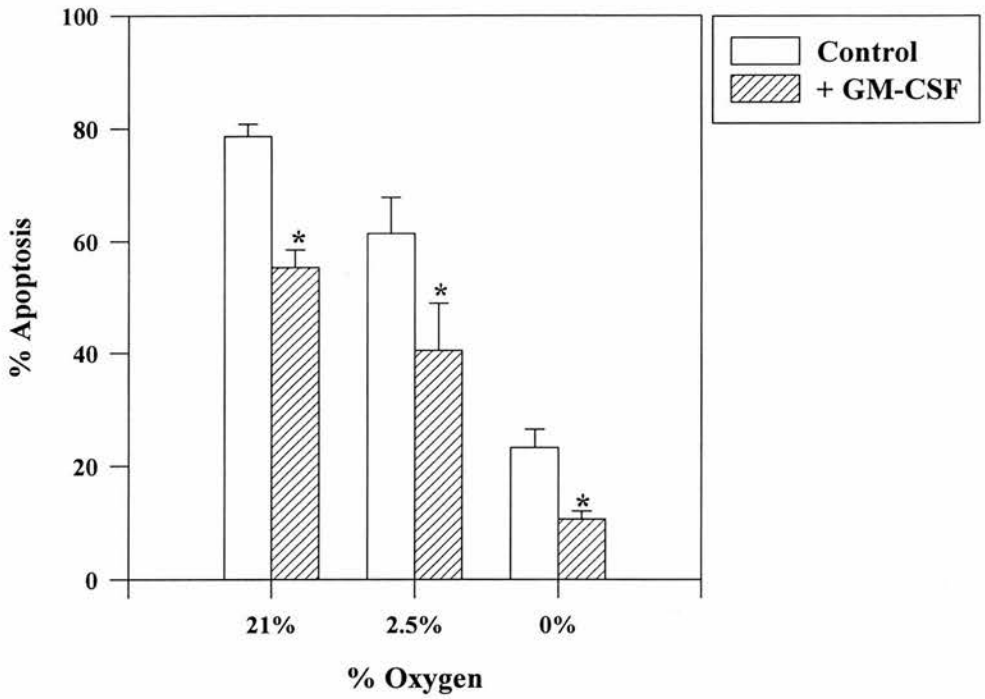


Figure 4.2.1 Modulation of the GM-CSF survival effect in neutrophils by hypoxia. Neutrophils were incubated with GM-CSF (50 U/ml) for 20 hours in atmospheres containing 21%, 2.5% or 0% oxygen. Control cells were incubated under the same conditions but in the absence of cytokine. Apoptosis was assessed morphologically on cytospins. The data are expressed as the mean ± SEM of n = 5 separate experiments, each performed in triplicate. Under each oxygen condition tested, the addition of GM-CSF caused a significant inhibition of apoptosis ($P<0.05$).

	6 hrs control	6 hrs + TNFα	20 hrs control	20 hrs + GM-CSF
21% Oxygen	1.08 ± 0.12	1.11 ± 0.12	1.09 ± 0.12	1.01 ± 0.01
0% Oxygen	1.17 ± 0.17	1.08 ± 0.08	1.14 ± 0.14	1.01 ± 0.24

Table 4.2.2 Bcl-2 production by neutrophils in response to 6 or 20 hours and/or TNFα or GM-CSF treatment. Neutrophils were incubated in the presence or absence of TNFα (12.5 ng/ml) for 6 hours or GM-CSF (50 U/ml) for 20 hours in atmospheres containing 0% or 21% oxygen. The amount of Bcl-2 in the cells, once permeabilized, was measured by flow cytometry as detailed in the methods section and expressed as mean fluorescence relative to the negative control (MOPC21 antibody). In all cases, Bcl-2 could not be detected in the neutrophils. HL-60 cells, which are known to express Bcl-2, were used as a positive control (relative mean fluorescence = 4.43). MAC 387, which recognises an intracellular antigen in neutrophils, was used to confirm the efficient permeabilization of the neutrophils (relative mean fluorescence > 50).

(Hockenbery et al., 1991). HL-60 cells which are known to produce Bcl-2 were used as a positive control (relative mean fluorescence = 4.43 ± 0.91) as opposed to untreated neutrophils at time 0 (relative mean fluorescence = 1.07 ± 0.08).

4.2.3 Effect of antioxidants on neutrophil apoptosis

To examine whether the effect of hypoxia was mediated solely by the inhibition of ROS generation, the effect of exogenous addition of the anti-oxidant enzymes, SOD and catalase, and methionine, an essential thiol containing amino acid, on neutrophil apoptosis was investigated. Under normoxic (21% oxygen) conditions methionine (5 mM) and SOD (200 μg/ml) did not influence the rate of constitutive neutrophil apoptosis at 20 hours (figure 4.2.3A), suggesting that hypochlorous and superoxide radicals do not play a significant role in regulating neutrophil apoptosis. In contrast, catalase (250 μg/ml), both alone and in combination with SOD, caused a significant inhibition of neutrophil apoptosis at 20 hours (figure 4.2.3A). It is probable, however, that this effect of catalase is non-specific and independent of its effect on hydrogen peroxide levels since when neutrophils were incubated in the presence or absence of 250 μg/ml catalase in atmospheres containing 21% or 0% oxygen for 20 hours the anti-apoptotic effect of catalase was still present in 0% oxygen (figure 4.2.3B).

4.2.4 Role of p38 MAP kinase in hypoxic-mediated inhibition of neutrophil apoptosis

The potential role of the MAP kinase cascade in mediating the anti-apoptotic effect of hypoxia on neutrophils was examined by incubating cells with SB 203580 (20 μM), a specific inhibitor of p38 kinase (Lee et al., 1994), under both hypoxic and normoxic conditions. SB 203580 was originally discovered as an inhibitor of LPS-induced cytokine (IL-1 and TNFα) synthesis in THP-1 monocytes (IC_{50} 1 μM) (Lee et al., 1994) and was subsequently shown to inhibit LPS-induced activation of MAPKAP kinase-2, the physiological substrate for p38 kinase (IC_{50} 0.6 μM). SB203580 has also been shown to prevent the phosphorylation of Hsp 27 in response to IL-1, cellular stress and LPS in KB and PC-12 cells (Cuenda et al., 1995) and block glutamate-stimulated apoptosis in cerebellar granule cells (Kawasaki et al.,

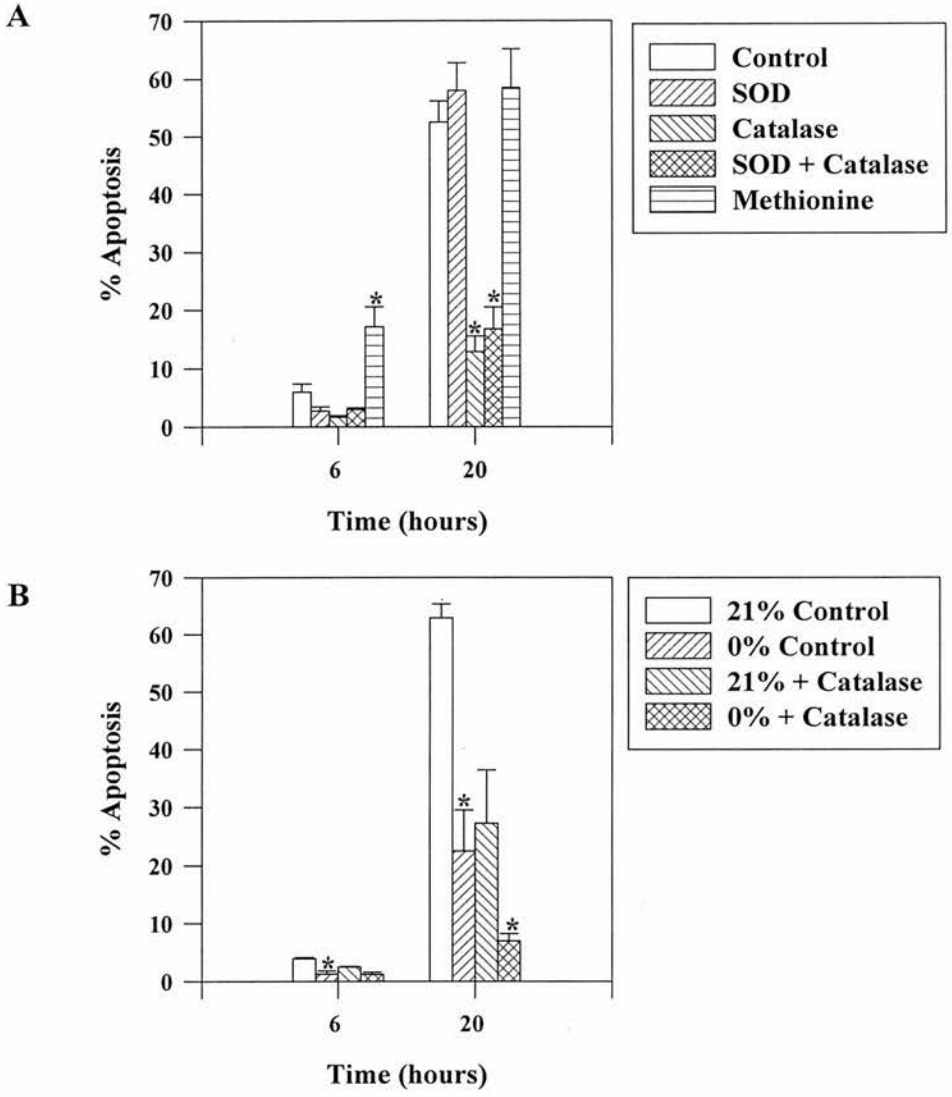


Figure 4.2.3 Effect of antioxidants on neutrophil apoptosis. (A) Neutrophils ($5x10^6$/ml) were cultured under normoxic (21% oxygen) conditions for 6 or 20 hours in the presence of 200 μg/ml SOD, 250 μg/ml catalase, 200 μg/ml SOD and 250 μg/ml catalase or 5 mM methionine. Control untreated neutrophils were also prepared. **(B)** Neutrophils ($5x10^6$/ml) were cultured for 6 or 20 hours in either normoxic or hypoxic conditions in the presence or absence of 250 μg/ml catalase. Apoptosis was assessed morphologically. Data represent mean ± SEM of n = 3 separate experiments, each performed in triplicate (*$p<0.05$ compared with control values).

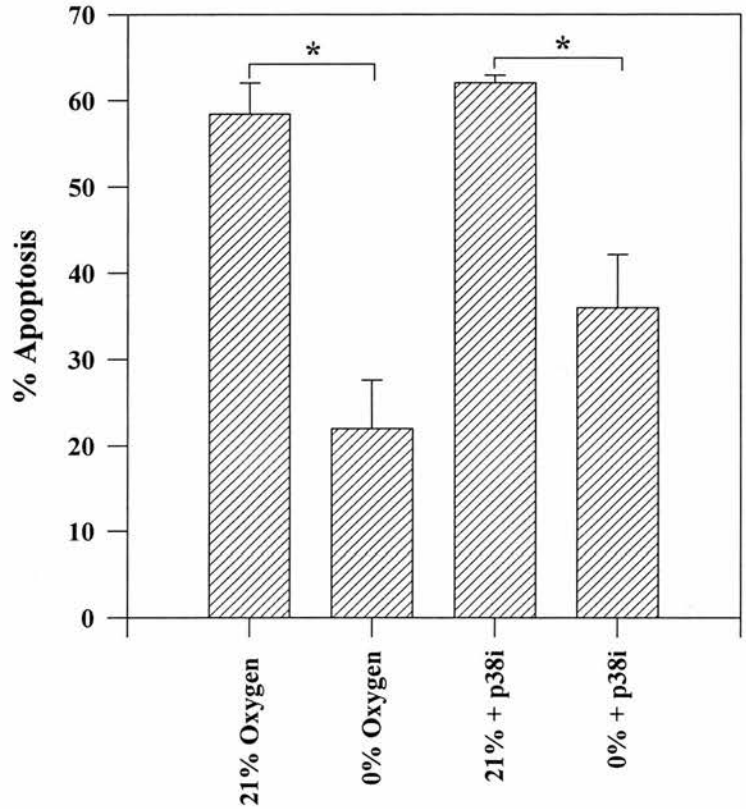


Figure 4.2.4 The role of p38 MAP kinase in hypoxia-mediated inhibition of neutrophil apoptosis. Human neutrophils ($5x10^6$/ml) were incubated in the presence and absence of 20μM of the p38 MAP kinase inhibitor (SB 203580) in atmospheres containing 21% or 0% oxygen. Data represent mean ± SEM of n = 3 separate experiments, each performed in triplicate (*$p<0.05$).

1997). The presence of p38 MAP kinase in neutrophils, and the ability of SB 203580 to inhibit this activity, was confirmed by assessing MAPKAP kinase-2 activity in neutrophil lysates (Stokoe et al., 1993). These experiments, performed by Dr. A Paul (University of Strathclyde), used neutrophils prepared in the Rayne lab and demonstrated both basal and TNFα and sorbitol-stimulated MAPKAP kinase-2 activity (a specific and immediate target of p38 MAP kinase) (data not shown). Incubation of neutrophils with SB 203580 (20 μM), while having no effect on the extent of constitutive apoptosis at 20 hours, partially inhibited the anti-apoptotic effect of hypoxia, although this effect was not significant ($p<0.05$) (figure 4.2.4). These data suggest that p38 MAP kinase activation does not play a major role in hypoxic-mediated inhibition of neutrophil apoptosis.

4.2.5 Effect of glucose deprivation on neutrophil apoptosis

To address whether the protective effect of hypoxia in neutrophils involves depletion of intracellular ATP pools, the ability of protracted glucose deprivation to mimic this response was examined. Neutrophils were cultured in a glucose and sodium pyruvate free medium, conditions which have been shown to rapidly decrease intracellular ATP levels in MCF-7/ADR cells (Lee et al., 1997). Glucose has also been implicated in inducing cell death through a free radical-mediated mechanism (Donnini et al., 1996). Thus, to investigate whether glucose levels affect neutrophil apoptosis, an additional high glucose condition (10 g/L, 50 mM glucose) was also included in the experiment. Control cells were incubated in medium containing 4.5 g/L (25 mM) glucose. In these experiments all media (high glucose, control and glucose-free) was supplemented with 10% dialysed (glucose-free) fetal calf serum (FCS) in place of autologous serum. This substitution caused a reduction in the control basal rate of apoptosis (autologous serum 42.4 ± 8.2 vs FCS 28.3 ± 8.55), however the hypoxic inhibitory effect was uninfluenced. Neutrophils were then incubated under normoxic or hypoxic conditions for 20 hours. To verify that the glucose contents of these media did vary significantly, their glucose contents were analysed using a Randox glucose test. This assay was performed by Clinical Biochemistry (Edinburgh Royal Infirmary). The control medium was found to contain 20.2 mM glucose, the glucose free medium <0.6 mM glucose and the high

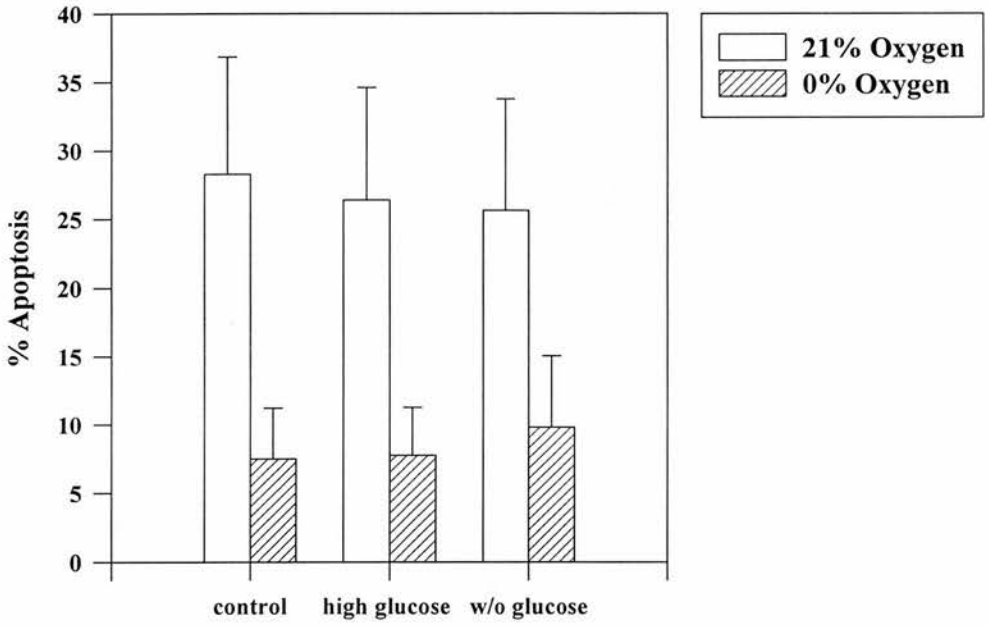


Figure 4.2.5 Effect of glucose deprivation on neutrophil apoptosis. Neutrophils ($5x10^6$/ml) were cultured under normoxic or hypoxic conditions for 20 hours in either normal Iscove's MDM (4.5 g/L glucose) (control), Iscove's DMEM without glucose or sodium pyruvate (w/o glucose) or Iscove's containing 10 g/L glucose (high glucose). In all the conditions the Iscove's was supplemented with 10% dialysed (glucose free) fetal calf serum. Apoptosis was assessed morphologically. The data are expressed as the mean ± SEM of n = 4 separate experiments each performed in triplicate.

glucose medium >41 mM glucose. These measurements also confirm the absence of detectable glucose in the dialysed FCS. The concentration of glucose in the culture medium had no effect on the rate of neutrophil apoptosis under either 21% or 0% oxygen (figure 4.2.5). Although not measured directly, these data suggest that intracellular ATP levels do not affect neutrophil apoptosis and that the hypoxic protective effect is not caused by depletion of intracellular ATP pools. In addition, as the rate of neutrophil apoptosis did not increase under high glucose conditions, these findings imply that the generation of free radicals by auto-oxidation of glucose, likewise, is not a contributing factor to constitutive neutrophil apoptosis.

4.2.6 Effect of heat shock on neutrophil apoptosis

In a number of studies upregulation of the heat shock proteins (HSPs), especially Hsp 70 and Hsp 27, have been associated with an increased resistance to apoptotic stimuli. For example, WEHI-s cells overexpressing Hsps 27 and 70 showed an increased resistance to apoptosis inducing agents, such as actinomycin D and etoposide (Samali and Cotter, 1996), and constitutive expression of Hsp 27 in murine L929 cells blocked Fas/APO-1 mediated programmed cell death (Mehlen et al., 1996). In view of these reports, and the fact that hypoxia has been reported to cause activation of the heat shock transcription factor in mammalian cells (Benjamin et al., 1990), the possible role of HSPs in hypoxic inhibition of apoptosis was investigated. Our initial protocol for induction of HSPs involved incubating cells at either 42°C or 4°C for one hour, after which time they were returned to 37°C. Both these treatments have previously been shown to induce Hsp 70 expression in human neutrophils (Eid et al. 1987 and Cox et al., 1993). Control cells were kept at 37°C throughout, while hypoxia-treated cells were incubated at 37°C in 0% oxygen. After 5 and 20 hours neutrophils were harvested and the rate of apoptosis assessed morphologically. Neither the heat shock or cold shock treatments influenced the rate of neutrophil apoptosis (figure 4.2.6A), although hypoxia did exert its normal inhibitory effect in the same experiment.

In order to ensure that Hsp proteins were being induced, lysates from identically treated cells were analysed for Hsp 70 expression using Western blotting. A number

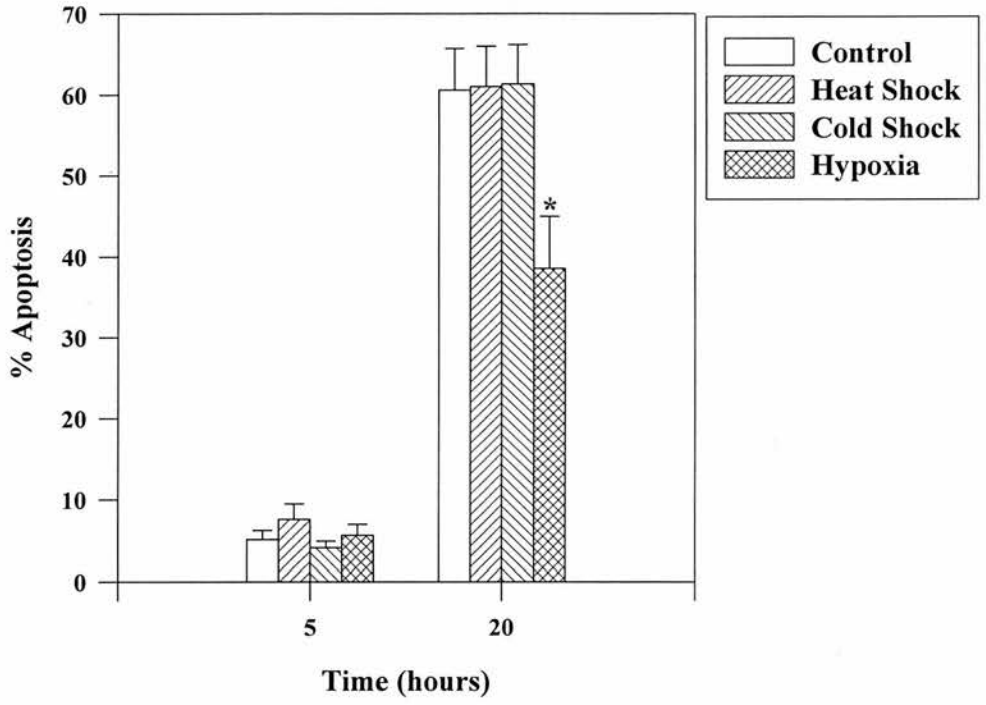


Figure 4.2.6A Effect of heat shock on neutrophil apoptosis. Neutrophils ($5x10^6$/ml) were heat shocked or cold shocked by being placed at 42°C or 4°C respectively for one hour before being returned to 37°C. Control cells were incubated at 37°C in 21% oxygen, while hypoxic cells were incubated at 37°C in 0% oxygen. After 5 and 20 hours cells were harvested, cytospins were prepared and apoptosis assessed morphologically. Data represent mean ± SEM of n = 7 separate experiments, each performed in triplicate (*$p<0.05$ compared with control values).

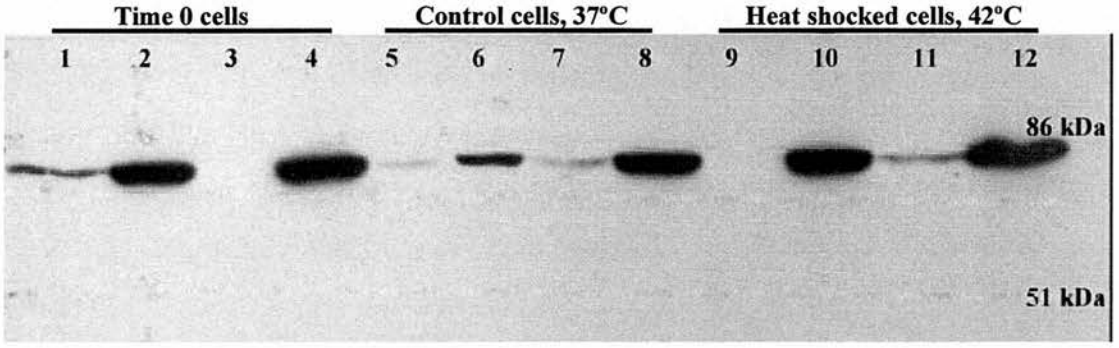


Figure 4.2.6B Western blot showing Hsp 70 levels in human neutrophils. Neutrophil lysates were prepared by either direct lysis in boiling sample buffer (odd numbered lanes) or a TCA based lysis method (even numbered lanes) and separated by 10% SDS-PAGE. Western blot was developed with an antibody to Hsp 70. After isolation neutrophils were either lysed immediately (time 0, lanes 1 and 2) or incubated in platelet poor plasma, PPP, (37°C, 45 min) before lysing (time 0, PPP, lanes 3 and 4). Neutrophils with (lanes 7 and 8) or without (lanes 5 and 6) the PPP incubation were then cultured for 4 hours at 37°C (control). Parallel cells were heat shocked for 1 hour at 42°C before being returned to 37°C for 3 hours prior to lysing (lanes 9 and 10 no PPP incubation; lanes 11 and 12 with PPP incubation). Identical data was obtained in 2 additional experiments.

C

Time	Control	Heat shock	Hypoxia
0	3.44		
4 hours	2.91	2.63	2.4
20 hours	3.27	2.2	2.92

D

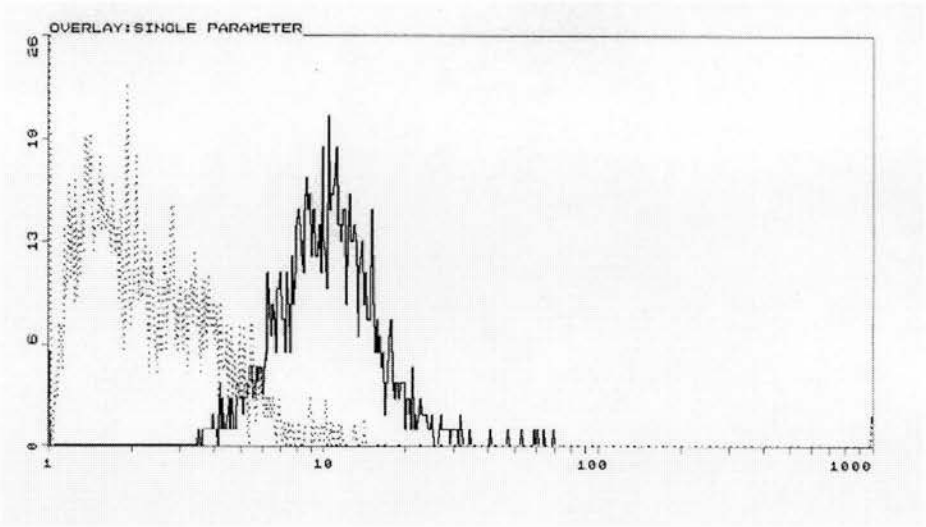


Figure 4.2.6 C and D Flow immunocytometric analysis of Hsp 70 in human neutrophils. (C) Neutrophils ($5x10^6$/ml) were heat shocked by being placed at 42°C for one hour before being returned to 37°C. Control cells were incubated at 37°C in 21% oxygen while hypoxic cells were incubated at 37°C in 0% oxygen. At the time points stated the amount of Hsp 70 was measured as detailed in chapter 2 and is expressed as mean fluorescence relative to the negative antibody (MOPC) control. Equal amounts of Hsp 70 were detected under all conditions tested. MAC 387, which recognises an intracellular antigen in neutrophils, was used to confirm the efficient permeabilization of the neutrophils (relative mean fluorescence = 25 ± 8). **(D)** Representative flow-cytometry (EPICS Profile II) histogram (cell count vs log fluorescence) showing profile of neutrophils after labelling with MOPC antibody (negative control, dashed outline) and Hsp 70 antibody (black outline).

of lysis methods were employed as parallel Commassie blue stained gels demonstrated inconsistent extraction, and in particular loss of high molecular weight protein forms, when using standard lysis techniques. These problems occurred despite inclusion of a broad spectrum of protease inhibitors and may reflect the major protease content of these cells. Figure 4.2.6B shows a comparison of samples prepared either by direct lysis into boiling laemmli sample buffer or by a TCA based method (see chapter 2 for details). Samples were otherwise treated and analysed identically. Of the various protocols tried, the TCA method produced the best results, both on Western analysis and on Commassie blue stained gels. These data show the presence of large amounts of Hsp 70 in neutrophils, even when lysed immediately after they had been prepared from whole blood (figure 4.2.6B, lane 2), suggesting that the isolation method itself (even when pre-warmed solutions were used and dextran sedimentation performed at 37°C) may cause upregulation of this protein. In an attempt to downregulate HSP expression before commencing the experiment, neutrophils were incubated in pre-warmed platelet poor plasma (37°C, 45 min) (figure 4.2.6B, lane 4). However, even employing such a 'recovery period', subsequent heat shocking failed to further upregulate Hsp 70 compared to control cells at 4 hrs (figure 4.2.6B lanes 6, 8, 10 and 12). This finding is contrary to that reported by Eid et al. (1987) who found upregulation of Hsp 70 in neutrophils at this time point after an identical heat shock treatment. Incubating cells under hypoxic conditions also failed to modulate Hsp 70 expression (data not shown). These findings were substantiated by flow immunocytometric analysis (figures 4.2.6C and D), where Hsp 70 was found to be still present at 20 hours in all conditions tested. The presence of equal amounts of Hsp 70 in neutrophils independent of treatment, suggests that this protein does not play a role in the modulation of either basal neutrophil apoptosis or in the inhibition of apoptosis by hypoxia.

4.2.7 Effect of mitochondrial inhibitors on the rate of neutrophil apoptosis

To examine whether the inhibition of neutrophil apoptosis by hypoxia was due to an effect of hypoxia on oxidative phosphorylation, the effect of mitochondrial inhibitors on the rate of neutrophil apoptosis was investigated. Freshly isolated neutrophils were treated with 0.1 μg/ml rotenone, 1 mM sodium azide or 1 mM potassium

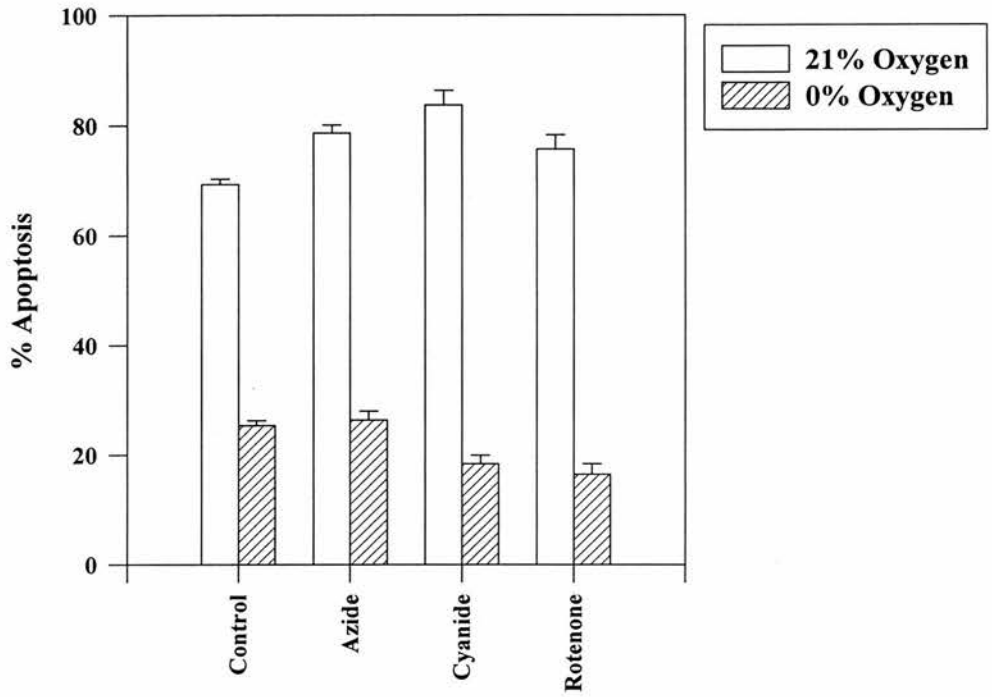


Figure 4.2.7 Effect of mitochondrial inhibitors on the rate of neutrophil apoptosis. Neutrophils ($5x10^6$/ml) were cultured under normoxic or hypoxic conditions for 20 hours in the presence of 1 mM sodium azide, 1 mM potassium cyanide and 0.1 μg/ml rotenone. Control, untreated neutrophils were also prepared. Data represent mean ± SEM of triplicate incubations from a single representative experiment of 2.

cyanide and incubated in 21% or 0% oxygen for 20 hours. None of the mitochondrial inhibitors mimicked or abrogated the action of hypoxia on neutrophil apoptosis (figure 4.2.7). This lack of effect of mitochondrial inhibitors implies that the hypoxic inhibition of neutrophil apoptosis is specific for hypoxia and cannot be induced by other cellular stresses associated with making the cells hypoxic, namely the compromise of oxidative metabolism.

4.2.8 The protective effect of hypoxia on neutrophil apoptosis is cycloheximide-dependent

To investigate whether the inhibition of neutrophil apoptosis by hypoxia was protein synthesis dependent, the effect of cycloheximide on this response was studied. Neutrophils were treated with the protein synthesis inhibitor, cycloheximide (50 μM, 14 μg/ml), and incubated in atmospheres containing either 21% or 0% oxygen for 6 and 20 hours. A more detailed time course over a 24 hour period was also performed. In agreement with previously published data (Whyte et al., 1997), incubation of neutrophils with 50 μM cycloheximide significantly increased basal apoptosis at all time points studied (figures 4.2.8A and B). These data imply that continuous synthesis of some regulatory protein(s) may be necessary to prevent neutrophils from undergoing apoptosis. Cycloheximide was also able to override the protective effect of hypoxia, increasing the levels of apoptosis in hypoxic neutrophils to those of normoxic, cycloheximide treated cells. Thus, it appears that the protective effect of hypoxia on neutrophil apoptosis requires *de novo* protein synthesis. Recently, it has been reported that the protective effect of dexamethasone on neutrophil apoptosis can be overcome by a far lower concentration of cycloheximide that has no effect on basal apoptosis (Cox and Austin, 1996). In view of this, we investigated whether this was also the case with the hypoxic protective effect. At doses of 1 μg/ml, a concentration that has been shown to interfere with, but not abolish, protein synthesis in neutrophils (Cox et al., 1994), cycloheximide did not alter basal levels of survival over 20 hours (figure 4.2.8C). However, the increased rate of neutrophil survival observed under hypoxic conditions was inhibited in a concentration-dependent fashion by co-treatment with cycloheximide (0.1-10 μg/ml). Induction of apoptosis under normoxic conditions was only

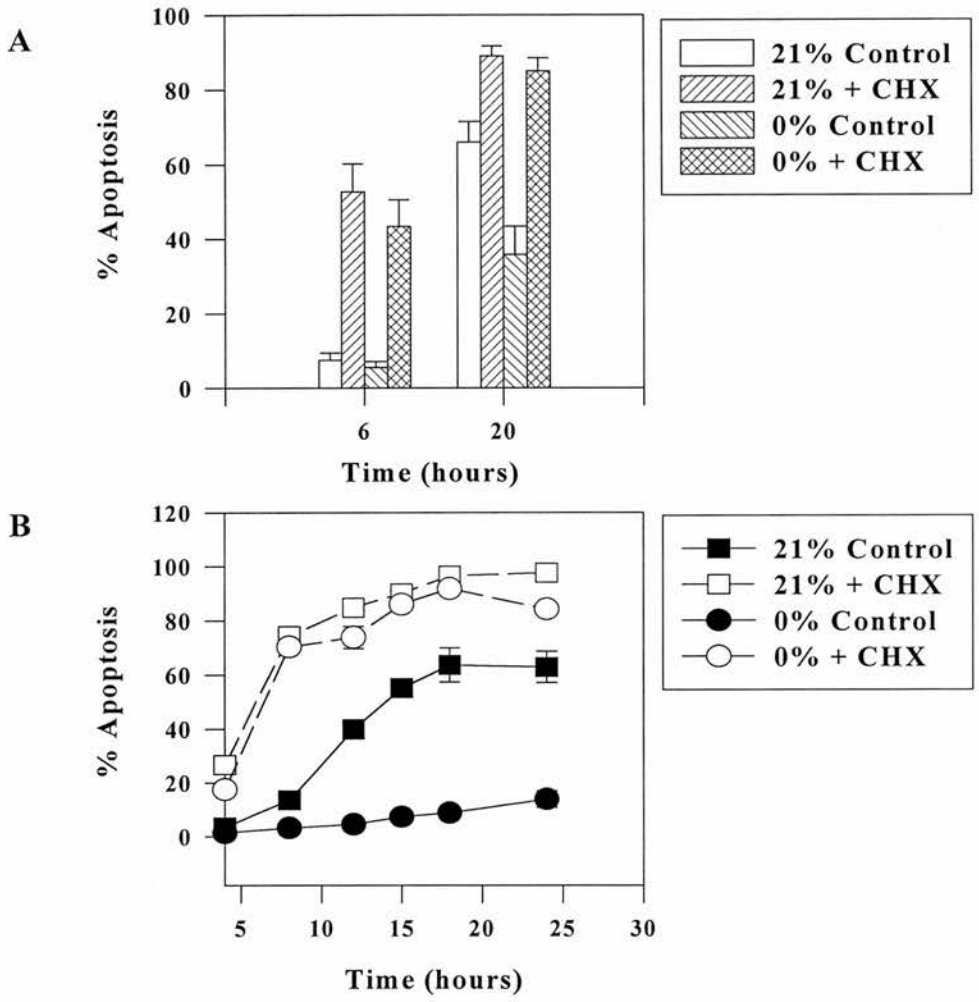


Figure 4.2.8 The protective effect of hypoxia on neutrophil apoptosis is cycloheximide-dependent. (A) Neutrophils ($5x10^6$/ml) were cultured in the presence or absence of cycloheximide (50 μM) in atmospheres containing either 21% or 0% oxygen for 6 or 20 hrs. Apoptosis was assessed morphologically. Data represent mean ± SEM of n = 6 separate experiments, each performed in triplicate **(B)** Neutrophils were treated as in (A) and the rate of apoptosis assessed at intervals over a 24 hour period. Data represent mean ± SEM of triplicate incubations from a single representative experiment of 2.

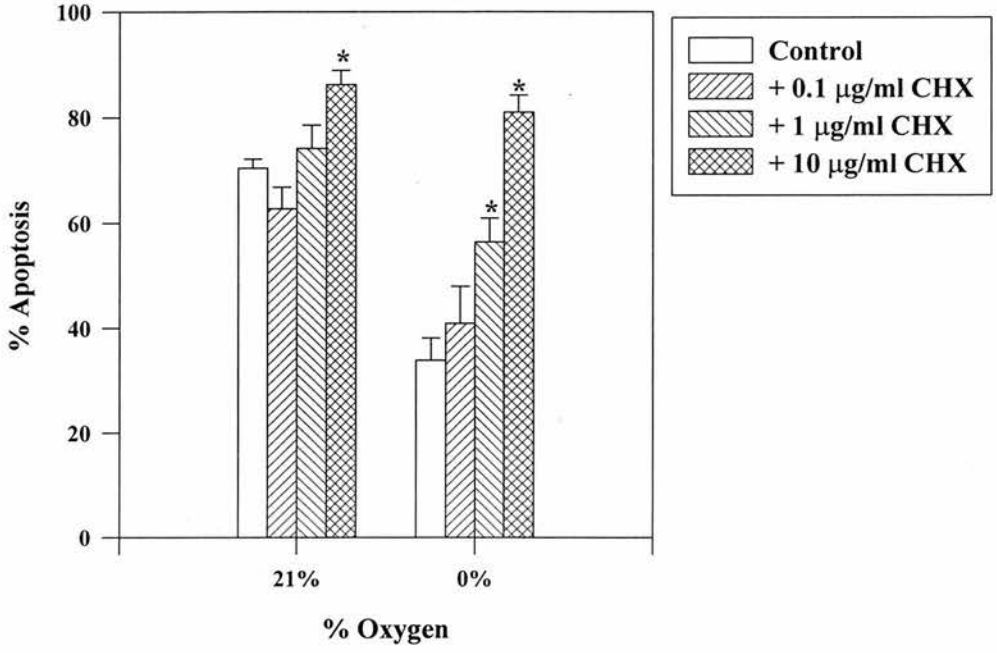


Figure 4.2.8C The hypoxic protective effect is more sensitive to CHX than basal apoptosis. Neutrophils ($5x10^6$/ml) were cultured in the presence or absence of increasing concentrations of cycloheximide in atmospheres containing either 21% or 0% oxygen for 20 hrs. Apoptosis was assessed morphologically. Data represent mean ± SEM of n = 6 experiments using 3 separate donors (*$p<0.05$ compared with control values).

observed at a cycloheximide concentration of 10 μg/ml. These findings provide strong evidence that protein synthesis is required for hypoxic inhibition of neutrophil apoptosis, and suggest that hypoxia may be inducing upregulation of a survival protein(s).

4.3 Discussion

In these studies we have sought to identify the intracellular mechanism(s) underlying the inhibition of neutrophil apoptosis by hypoxia.

Granulocyte macrophage-colony stimulating factor (GM-CSF) is an inflammatory cytokine produced by activated lymphocytes, fibroblasts, endothelial cells (Sallerfors, 1994; Munker et al., 1986) monocytes (Yamaoka et al., 1998), macrophages (Cavaillon, 1994), and mesenchymal cells (Vandermolen et al., 1996) following stimulation by cytokines (IL-1 and TNFα) or bacterial products. Neutrophils themselves are also capable of synthesising this cytokine (Lindemann et al., 1989). As well as GM-CSF's ability to 'prime' the neutrophil for subsequent enhanced responses to secretagogues such as fMLP (Guthrie et al., 1984; Haslett et al., 1985), this cytokine is also able to inhibit neutrophil apoptosis in a concentration-dependent manner (Colotta et al., 1992a; Lee et al., 1993).

Although enhanced survival of neutrophils by GM-CSF has shown to be RNA and protein synthesis dependent (Brach et al., 1992), the signal transduction mechanisms underlying this effect are poorly understood. However, a role for tyrosine phosphorylation has been proposed based on the observations that treatment of neutrophils with GM-CSF was associated with increased tyrosine phosphorylation of proteins and the inhibition of neutrophil apoptosis by GM-CSF could be overcome by the tyrosine kinase inhibitor, genistein (Yousefi et al., 1994).

We postulated that GM-CSF and hypoxia could be promoting survival of neutrophils through a common mechanism or that hypoxia might induce GM-CSF release from the neutrophils and thereby inhibit apoptosis indirectly. To test this hypothesis, we examined the combined effects of hypoxia and GM-CSF on neutrophil apoptosis. Under all oxygen tensions tested, including 0%, we found GM-CSF further inhibited neutrophil apoptosis. The fact that the hypoxic and GM-CSF effects were additive, suggests that these anti-apoptotic stimuli exert their effects through independent mechanisms.

The best defined genetic pathway of cell death exists in the nematode, *Caenorhabditis elegans*. Systemic genetic analyses have elucidated three genes, ced-3, ced-4 and ced-9, that are important in the regulation of nematode cell death. Mutations of ced-3 and ced-4 abolish all somatic cell deaths, which normally occur during the development of *C. elegans*, suggesting that these genes encode effector components of the pathway (Ellis and Horvitz, 1986). In contrast, ced-9 encodes a negative regulator that functions to suppress inappropriate cellular suicide (Hengartner et al., 1992). Mammalian homologues of Ced-9 have been identified and include proteins belonging to the Bcl-2 family (Hengartner and Horvitz, 1994). Bcl-2 can substitute functionally for Ced-9 in preventing nematode cell death, further emphasising the highly conserved nature of the cell death pathway (Hengartner and Horvitz, 1994). In mammalian cells Bcl-2 can also block apoptotic cell death following a variety of stimuli. Bcl-2 conferred a death-sparing effect in a number of haemopoietic cell lines following growth factor withdrawal (Vaux et al., 1988; Hockenbery et al., 1990; Nunez et al., 1990). Similarly, Bcl-2 has also been shown to protect primary neuronal cell cultures (Garcia et al., 1992) and rat adrenal derived pheochromocytoma (PC-12) cells (Batistatou et al., 1993) from nerve growth factor withdrawal-induced cell death. Finally, transgenic mice that redirected Bcl-2 to cortical thymocytes markedly extended the role for Bcl-2 as an antidote to apoptosis; thymocytes overexpressing Bcl-2 were resistant to glucocorticoid and γ irradiation-induced apoptosis as well as anti-T cell receptor-induced death (Sentman et al., 1991; Strasser et al., 1991).

Initially a mechanism where Bcl-2 protects against programmed cell death by inhibiting the generation or action of ROS was proposed (Hockenbery et al., 1993; Kane et al., 1993; Veis et al., 1993; Buttke and Sandstrom, 1994). This theory was based on the observations that Bcl-2 is predominantly localised to the mitochondria, endoplasmic reticulum and nuclear membrane (Hockenbery et. al., 1990; Monaghan et al., 1992; Jacobson et al., 1993), all important sites for ROS generation. In addition, Bcl-2 was able to block γ radiation-induced cell death (ionising radiation produces hydroxyl radicals in aqueous solutions) (Sentman et al., 1991; Strasser et al., 1991). Finally, overexpression of Bcl-2 has been reported to inhibit ROS

generation directly (Kane et al., 1993) and block apoptosis caused by glutathione depletion (Ellerby et al., 1996). However, evidence against this theory came from experiments where Bcl-2 protected against apoptosis induced under anoxic conditions where ROS generation is ablated (Shimizu et al., 1995; Jacobson and Raff, 1995) and it has now been proposed that Bcl-2 acts at a mitochondrial level by preventing the dissipation of the mitochondrial transmembrane potential ($\Delta\Psi_m$) and consequential release of protease activators (Kroemer et al., 1995; Yang et al., 1997).

In view of the ability of Bcl-2 to suppress apoptosis in a variety of cell types and circumstances (Reed, 1994), we investigated the possibility that upregulation of Bcl-2 was mediating the hypoxic inhibition of neutrophil apoptosis. However, we were unable to detect Bcl-2 protein expression in either normoxic or hypoxic treated cells. Treatment of cells with other agents known to modulate the rate of neutrophil apoptosis, namely GM-CSF and TNFα, were also unable to induce Bcl-2 expression. These results are in accord with the observations that endogenous expression of Bcl-2 is restricted to early myeloid cells in the bone marrow and is absent in mature neutrophils (Hockenbery et al., 1991; Delia et al., 1992). It is notable, however, that studies using transgenic mice that expressed Bcl-2 in mature neutrophils have shown that Bcl-2, if present, does have the capacity to block apoptosis in this cell type (Lagasse and Weissman, 1994).

Although these experiments rule out a role for Bcl-2 in the inhibition of neutrophil apoptosis by hypoxia, it remains possible that other members of the Bcl-2 family which have also been found to be death antagonists (e.g. Bcl-X_L, Bcl-w Bfl-1, Brag-1, Mcl-1 and A1) could be involved.

Oxidative mechanisms have been widely implicated in playing a role in the induction of apoptosis. Early evidence for this theory came from observations that many of the chemical and physical treatments capable of inducing apoptosis are also known to evoke oxidative stress. For example, both ionising and ultra-violet radiation are capable of inducing apoptosis and both generate ROS such as H_2O_2 and $OH^{\bullet}$ (Halliwell and Guttereridge, 1990). Exposure to low concentrations of H_2O_2 induces

apoptosis in a variety of cell types (Lennon et al., 1991), thereby establishing oxidative stress as a direct mediator of apoptosis. Some agents that induce apoptosis are not free radicals themselves but may elicit ROS formation. Doxorubicin, cisplatin and a number of ether-linked lipids are anti-neoplastic agents which induce both apoptosis and oxidative damage in sensitive cells (Benchekroun et al., 1993; Stamler et al., 1992; Wagner et al., 1993; Takagi et al., 1974). In addition, anti-oxidants have been reported to provide protection against many different types of apoptosis. N-acetyl-cysteine and N-t-butyl-phenylnitrone abolished Fas-induced programmed cell death in T-Cells (Gulbins et al., 1996). Furthermore, the cellular sensitivity or resistance to the cytotoxic effects of TNFα has been shown to correlate with decreased or increased levels of superoxide dismutase respectively (Hirose et al., 1993).

Neutrophils are phagocytotic cells and, when activated, are capable of generating and releasing large amounts of ROS via the NADPH oxidase system. Since the generation of ROS is entirely dependent on the availability of molecular oxygen (Chance et al., 1979), an obvious explanation for the inhibition of neutrophil apoptosis by hypoxia is that it is caused by a direct effect of hypoxia on the levels of intracellular ROS. We investigated this hypothesis by examining whether exogenous addition of SOD and catalase, which selectively metabolise O_2^- and H_2O_2 respectively, and methionine, an essential thiol-containing amino acid, would mimic the hypoxic inhibition of neutrophil apoptosis. Methionine and SOD did not influence the rate of neutrophil apoptosis, suggesting that hypocholorous and superoxide radicals do not play a significant role in regulating neutrophil apoptosis. This supports a previous study, which demonstrated that SOD was ineffective in preventing cell death in human lung fetal fibroblasts (Kurita and Namiki, 1994). In contrast, catalase, both alone and in combination with SOD, caused a significant inhibition of neutrophil apoptosis at 20 hrs. It is probable, however, that this effect of catalase is independent of its effect on hydrogen peroxide levels since a similar non-specific anti-apoptotic effect of catalase has been demonstrated in WEHI 231 cells (Fang et al., 1995). In addition, catalase has now been identified as the anti-apoptotic factor present in the conditioned medium of CCRF-CEM T-cell leukaemia

cells (Sandstrom and Buttke, 1993). Further evidence to support this comes from the experiment in which neutrophils were incubated in the presence or absence of catalase in normoxic and anoxic atmospheres. Catalase was still able to inhibit neutrophil apoptosis in 0% oxygen, again suggesting that its anti-apoptotic abilities are independent of its effect on hydrogen peroxide levels. These data have now been confirmed by a more recent study in which catalase, but not exogenous SOD or $HO^{\bullet}$ scavengers, reduced spontaneous apoptosis in ageing neutrophils. In this study depletion of cellular glutathione levels likewise did not induce apoptosis (Rollet-Labelle et al., 1997). Despite this, one group (Kettritz et al., 1996) has reported that exogenous SOD can inhibit neutrophil apoptosis. This discrepancy may be explained by the fact that the neutrophil isolation technique used in these experiments is known to cause cell activation.

In contrast to the above data, two studies have reported the ability of antioxidants to prevent phagocytosis-stimulated apoptosis in neutrophils. The thiols, GSH and NAC, have been shown to be able to inhibit an increase in apoptosis associated with neutrophil-mediated *E. Coli* ingestion, although not affecting basal apoptosis (Watson et al., 1996). It is also notable that, in this system, catalase had no effect on neutrophil apoptosis. In accord with this study, Coxon and co-workers (1996) reported that phagocytosis of opsonized particles by neutrophils rapidly induced apoptosis. Treatment of neutrophils with diphenylene iodonium (an NADPH oxidase inhibitor), which effectively inhibited phagocytosis-induced superoxide generation, while having no effect on phagocytosis itself, blocked phagocytosis-induced apoptosis. Similarly, apoptosis following phagocytosis did not occur in neutrophils from patients suffering from chronic granulomatous disease, which lack NADPH oxidase.

Thus, it would appear that ROS do not play a pivotal role in spontaneous apoptosis in cultured neutrophils, although ROS may be implicated in the induction of apoptosis by certain stimuli e.g. *E. Coli* and opsonized particle ingestion (Watson et al., 1996; Coxon et al., 1996) or Fas-L (Watson et al., 1997). These findings suggest that the inhibition of neutrophil apoptosis by hypoxia is not caused by a direct effect of

hypoxia on intracellular ROS levels and also argue against generation of ROS being a universal mechanism underlying apoptosis in all cell types. Evidence supporting this conclusion comes from experiments in which anoxic incubation failed to attenuate stimulus-induced apoptosis. Hence, Jacobson and Raff (1995) found that hypoxia did not attenuate staurosporine-induced apoptosis in a fibroblast cell line and Muschel et al. (1995) found that hypoxia did not inhibit the induction of apoptosis in T-lymphoma cell lines S49.1 and WEH7.1 by dexamethasone. In fact, hypoxia itself induced apoptosis in the WEH7.1 cells, a response now observed in a number of cell types including cultured neurons (Rosenbaum et al., 1994) and human adenocarcinoma cells (Yao et al., 1995).

There has been a recent surge of interest in mitogen-activated protein kinase (MAPK)-mediated signal transduction cascades, which are conserved eukaryotic signalling pathways that convert receptor derived signals into a variety of cellular functions (for reviews see Davis, 1993; Marshall, 1995; Hunter, 1995). MAPK pathways have at their 'core' a three-component protein kinase cascade consisting of a serine/threonine protein kinase (MAPKKK), which phosphorylates and activates a dual-specificity protein kinase that, in turn, activates another serine/threonine protein kinase (MAPK) (see figure 4.3.1). Currently three distinct mammalian MAPKs, each with apparently unique signalling functions, have been identified. These pathways serve to link signals from the cell surface to cytoplasmic and nuclear events. The Ras/Raf/MEK/ERK pathway is one of these. In this pathway Raf correlates to MAPKKK, MEK correlates to MAPKK and ERK-1 and ERK-2 correspond to MAPK. The second MAPK pathway in mammalian cells involves Ras and mediates some of the signals, environmental stresses and pro-inflammatory cytokines (Sanchez et al., 1994), which result in the N-terminal phosphorylation of Jun. It involves Ras, MEKK-1, a dual-specificity kinase (SEK1), and the MAPK Jun Kinase (JNKs/SAPKs) (Hibi et al., 1993; Minden et al., 1994; Yan et al., 1994).

The third group of MAPKs are mammalian homologues of HOG1 found in yeast and have been designated p38 MAP kinases on account of their molecular weight in murine cells (Han et al., 1994). $p21^{racl}$ is one of the small GTP-binding proteins, and

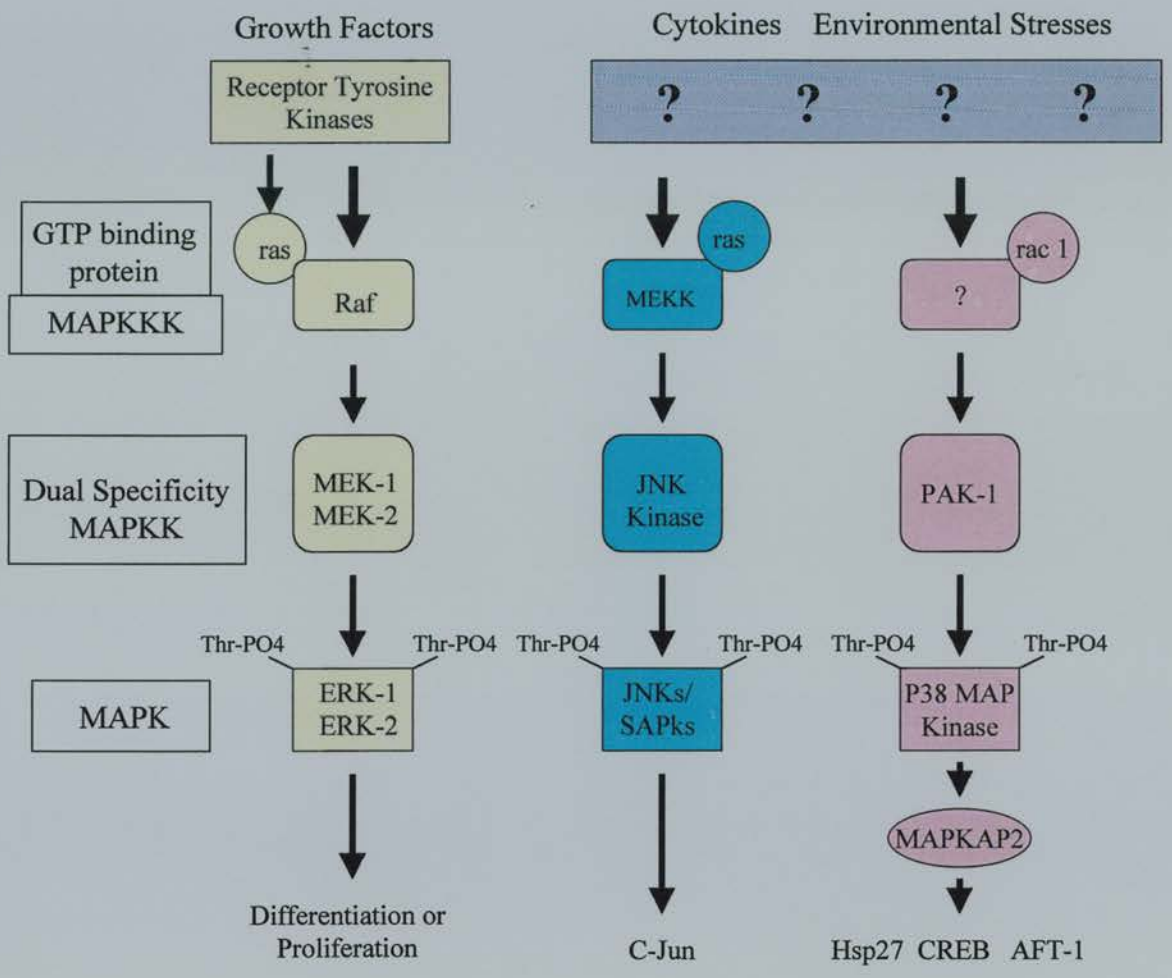


Figure 4.3.1 MAPK pathways in mammalian cells. (Modified from Marshall et al., 1995). Both receptor tyrosine kinase and stress response pathways contain a central core of a serine/threonine kinase, MAPKKK; a dual specificity kinase, MAPKK; and a serine/threonine kinase, MAPK. Receptor tyrosine kinase signalling occurs through Ras, and there is evidence that some stress responses, e.g. UV radiation may also activate Ras (Hibi et al., 1993). The upstream components of the p38 pathway are still uncertain. PAK-1, Rac and Cdc42 have been shown to activate p38 MAPK (Zhang et al., 1995) although, they also activate JNK in the same system.

belongs to the rho subfamily of ras-related proteins. A serine/threonine protein kinase $p65^{PAK}$ (also called p21-activated kinase 1 [PAK1]) was identified as a target for $p21^{racl}$ (Manser et al., 1994). $p21^{racl}$ has been shown to regulate p38 MAPK through the downstream mediator PAK 1 (Zhang et al., 1995). In response to environmental stresses, mitogens and pro-inflammatory cytokines, p38 MAPK is activated, and in turn stimulates MAPK-activated protein (MAPKAP) kinase-2 (Rouse et al., 1994; Freshney et al., 1994), which has been shown to be activated in neutrophils following stimulation with PMA or fMLP (Zu et al., 1996). The *in vivo* substrates of MAPKAP kinase-2 include heat shock protein (Hsp) 27 and the transcription factors CREB, AFT1 and 2 (Cuenda et al., 1995, Tan et al., 1996; Raingeaud et al., 1995). The phosphorylation of Hsp 27 is thought to stimulate the phosphorylation of actin and thus help repair the actin microfilament network that becomes disrupted during cellular stress, thereby aiding cell survival (Lavioe et al., 1995).

Studies of signalling mechanisms have revealed that the MAPKs may play important roles in the pathways that regulate both cell growth and apoptosis. For example, the removal of nerve growth factor from PC-12 cells leads to apoptosis (Batistatou et al., 1993; Pittman et al., 1993), as well as the activation of JNK and p38 MAPK and the inhibition of ERK (Xia et al., 1995; Park et al., 1996). Kawasaki et al. (1997) recently proposed the involvement of p38 MAPK in glutamate induced neurotoxicity: addition of glutamate, NMDA, Ca^{2+} or the Ca^{2+} ionophore A23187 to cultures of rat cerebellar granule cells, all of which induce apoptosis in this system, markedly increased p38 MAP kinase activity. The p38 MAPK inhibitor SB 203580 also inhibited glutamate-induced apoptosis by about 70%. In addition, inhibition of p38 by insulin or the pyridinyl imidazole inhibitor, PD 169316, blocked apoptosis in Rat-1 fibroblasts and PC-12 cells induced by trophic factor withdrawal (Kummer et al., 1997).

Although studies looking at p38 MAPK activation in response to reduced oxygen tensions are at present limited, both hypoxia and hypoxia/reoxygenation have been shown to cause rapid activation of PAK 1 and p38 MAPK, as well as

phosphorylation of activating transcription factor (ATF)-2 in cardiac myocytes (Seko et al., 1997). In addition, phosphorylated p38 MAPK levels increased in microglia after global ischaemia (Walton et al., 1998) providing evidence that hypoxia can cause activation of p38 MAPK, at least in some cell types. In view of such data and the evidence that p38 MAPK may play a protective role in apoptosis, we investigated the effect of SB 203580, a specific inhibitor of p38 kinase (Cuenda et al., 1995; Lee et al., 1994), on the anti-apoptotic effect of hypoxia in human neutrophils.

As illustrated, 20μM SB 203580 caused a small, although not significant, reversal of the hypoxia-mediated survival of neutrophils at 20 hrs, while having no effect on the extent of apoptosis in control cells. A similar effect of SB 203580 has been observed with respect to ameliorating the ability of LPS to delay neutrophil apoptosis (J. Murray, ER Chilvers, personal communications). Hence, if hypoxia does induce p38 MAPK activation in neutrophils (and this was not assessed directly in these experiments), this signal does not appear to be the major mediator of the anti-apoptotic effect of hypoxia in this system. The activation of p38 MAPK under conditions where apoptosis is stimulated (Kummer et al., 1997; Bastistatou et al., 1993; Pittman et al., 1993; Xia et al., 1995; Park et al.; 1996 Kawasaki et al., 1997) may represent an autoregulatory response by the cell to prevent cell death ensuing. This hypothesis would be supported by the observations that TNFα stimulates p38 MAPK activity in neutrophils and that SB 203580 can potentiate TNFα-mediated induction of apoptosis at 6 hours (J. Murray, unpublished data). Certainly, in cardiac myocytes, activation of p38 MAPK results in protection of this cell type from apoptosis induced by antisomyocin or overexpression of constitutively active MEKK1 (Zechner et al., 1998) but such an effect would not seem to be as marked in neutrophils under the conditions studied. It would be of interest to see whether hypoxia can modulate other MAP kinase pathways in neutrophils and whether such effects underlie hypoxia-mediated survival of human neutrophils.

The intracellular energy status of a cell is controlled by the balance between the rates of ATP utilisation and formation, which depends upon the availability of substrates such as oxygen, glucose and glutamine for ATP formation. Thus, incubation of cells

under reduced oxygen conditions is likely to have a detrimental effect on intracellular ATP levels. Several studies have found ATP to be necessary for cells to undergo apoptosis. For example, it has been shown that ATP generation is required for the execution of the final phase of apoptosis in human T-cells (Leist et al., 1997) and a study using isolated thymocyte nuclei found the presence of ATP to be necessary for both the movement of chromatin to the nuclear periphery and apoptotic body formation (Kass et al., 1996). However, it should be noted that this is not true of all cell types, for example, in MCF-7/ADR cells depletion of intracellular ATP was associated with the induction of apoptotic cell death (Lee et al., 1997). It has also been proposed that intracellular ATP levels are a determinant of the mode of cell death, i.e. if cells are depleted of ATP they undergo necrotic cell death whereas if ATP is available the cells die by apoptosis (Leist et al., 1997; Eguchi et al., 1997). Thus, the possibility that hypoxia is inhibiting neutrophil apoptosis by depletion of intracellular ATP stores was investigated. Culturing neutrophils in medium without glucose or sodium pyruvate, conditions which have been shown to cause a rapid decrease in intracellular ATP levels in MCF-7/ADR cells (Lee et al., 1997), had no effect on the rate of apoptosis compared to control levels. These data imply either that this protocol (despite being widely used to induce this effect) does not significantly impact on ATP levels in neutrophils or that intracellular ATP levels are not a crucial factor regulating constitutive neutrophil apoptosis. Furthermore, ATP depletion does not appear to play a role in mediating the inhibition of neutrophil apoptosis by hypoxia.

It is also important to note that glucose deprivation results in the induction of a set of proteins referred to as glucose-regulated proteins (GRPs). The induction of the GRP response has been linked to cellular resistance to chemotherapeutic agents (Shen et al., 1987). In three breast cancer cell lines and two lymphoma lines the induction of Grp 78 expression correlated with resistance to thapsigargin (an inhibitor of ER Ca^{2+} uptake)-induced apoptosis (McCormick et al., 1997). In addition to glucose deprivation, this system can also be induced by 2-deoxyglucose, the calcium ionophore A23184 and, most interestingly, anoxia. In fact, one proposal for why cells within the hypoxic core of tumours are resistant to chemotherapeutic drugs is

the ability of hypoxia to upregulate GRPs. Thus, the inability of glucose deprivation to affect neutrophil apoptosis is of further interest as it also suggests that the GRP response is not involved in the protective effect of hypoxia.

There is now convincing evidence that glucose, under physiological conditions, may auto-oxidise generating free radicals (Wolff et al., 1987; Hunt et al., 1988). High glucose conditions have been shown to induce apoptosis in a FRTL5 cell line, an effect amplified by co-incubation with buthionine-sulfoximine (BSO), a transition state inhibitor that blocks glutathione synthesis (Donnini et al., 1996). In endothelial cells, high glucose causes both increased apoptosis and necrosis, effects blocked by the addition of the antioxidant SOD (Baumgartner-Parzer et al., 1995). To investigate whether the extent of neutrophil apoptosis seen under 'basal' control conditions could be due, at least in part, to glucose-dependent oxidative stress (a process that would be blocked by hypoxia), neutrophils were incubated in a high glucose medium. Again, this condition had no effect on the rate of neutrophil apoptosis, suggesting that free radical production by auto-oxidisation of glucose is not a contributory factor in this system.

Cultured cells or whole organisms react to increased temperatures by synthesising a small number of highly conserved proteins, termed heat shock proteins or HSPs. This heat shock response has been found in every cell and organism examined to date. Also universally, several of the proteins induced by heat are induced by a variety of other stresses. Although the particular constellation of effective inducers varies somewhat from organism to organism, in nearly all cells anoxia, ethanol and certain heavy metal ions induce protein expression (for review see Lindquist and Craig, 1988). The first proposed function for HSPs was thermotolerance, the ability of cells pre-exposed to non-lethal temperatures to survive subsequent exposure to temperatures lethal under normal conditions, and although there has long been controversy about their specific role in thermotolerance, recent experiments indicate that HSPs and in particular Hsp 70, indeed play an important role in this phenomenon (Johnston and Kucey, 1988; Riabowol et al., 1988).

The upregulation of HSPs has been associated with increased resistance of cells, not just to lethal temperatures, but also to a broad range of cytotoxic stimuli and this cytoprotective effect extends to protection against apoptosis as well as necrosis. For example, heat shock treatment of tumour cell lines protected cells from apoptosis induced by actinomycin-D, camptothecin and etoposide and this protection was mimicked in cells transfected with Hsp 27 or Hsp 70 (Samali and Cotter, 1996). Similarly, constitutive expression of Hsp 27 blocked Fas/APO-1-mediated cell death in a murine fibrosarcoma cell line and expression of either human Hsp 27, Drosophila Hsp 27 or the small stress protein human αB-crystallin protected these cells from staurosporine (a protein kinase C inhibitor) induced apoptosis (Mehlen et al., 1996). Wong et al. (1996) found that induction of the heat shock response in endothelial cells attenuated the pro-apoptotic effect of LPS, an effect that could also be achieved by transient overexpression of Hsp 70. Hsps 70 and 27 have also been implicated in cellular resistance to chemotherapeutic drugs (Oesterreich et al., 1993; Ciocca et al., 1992). This, and other evidence (Mosser et al., 1997; Mailhos et al., 1993; Mosser and Martin, 1992; Yun et al., 1997), point to a role for the HSPs and especially Hsps 27 and 70 in the protection of cells from apoptosis, however, their mode of action remains unclear.

As previously noted, the HSPs can be induced by a range of stimuli including hypoxia. The effect of hypoxia has been most widely studied on the regulation of Hsp 70. This protein has been demonstrated to be induced by transient ischaemia in rodent brain (Gonzalez et al., 1991; Kirino et al., 1991; Vass et al., 1988) and differential expression of Hsp 70 and Hsc70 has been linked to the different susceptibilities among brain cell populations to the transient ischaemia (Kawagoe et al., 1992). Hsp 70 has also been proposed as a factor that may limit cell damage during myocardial ischaemia (Dillman et al., 1986; Currie et al., 1988), a hypothesis that prompted Benjamin et al. (1990) to investigate the biochemical events resulting in activation of Hsp 70 gene transcription in cultured myogenic cells. This group found that exposure of these cells to hypoxia stimulated DNA binding of heat shock transcription factor (HSTF) through mechanisms independent of new protein synthesis and suggested that hypoxia and heat shock induce expression by a similar,

if not identical mechanism (for review of transcriptional control of HSPs see Linquist, 1986). It should be noted, however, that the kinetics of HSTF binding and Hsp 70 gene expression differed depending on which of the two inducers were used; heat shock induced these responses rapidly (< 20 min) while hypoxia took considerably longer (2 hrs).

We proceeded to investigate the possibility that upregulation of HSPs could be mediating the hypoxic-induced survival of neutrophils by examining the effect of heat shock on neutrophil apoptosis. Heat shocking or cold shocking neutrophils, treatments that have previously been shown to induce Hsp 70 expression in these cells (Eid et al., 1987; Cox et al., 1993), had no effect on the rate of neutrophil apoptosis, although hypoxia exerted its usual protective effect in the same experiment. When we examined identically treated cells for expression of Hsp 70 using both Western blot analysis and flow immunocytochemistry we found that Hsp 70 was present in time 0 cells and also present in equal amounts in the control, heat shock and hypoxically treated cells. These findings suggest that either neutrophils constitutively express Hsp 70 or (more likely) that the isolation method used to obtain purified neutrophils causes maximal activation of the heat shock response. Other reports show little or no expression of Hsp 70 at 24 hr in control neutrophils (Cox et al., 1994) and an upregulation of HSPs in freshly prepared neutrophils in response to heat shock (Eid et al., 1987). These findings are contrary to our own and suggest that neutrophils do not constitutively express Hsp 70. In addition Cox et al. (1993) showed that certain neutrophil isolation procedures cause upregulation of Hsp 70, thus it seems most likely that our results are due to the isolation method used. However, we were unable to prevent the upregulation of Hsp 70 even when we adapted the neutrophil isolation technique to that shown by Cox et al (1993) not to activate the heat shock response. The reason for this discrepancy is unclear. Incubation of neutrophils in platelet poor plasma (37°C, 45 min) immediately after isolation also failed to reduce basal Hsp 70 expression. Although we failed to manipulate Hsp 70 expression, the consistent presence of Hsp 70 in control, heat shocked and hypoxic cells suggests this protein does not modulate neutrophil apoptosis and is not responsible for the hypoxic-mediated survival effect in these

cells. These data agree with those of Cox et al. (1994) who found that, although human bronchial epithelial cell conditioned medium (HBEC-CM) induced Hsp 70 expression in neutrophils and had an inhibitory effect on neutrophil apoptosis, the active constituents of HBEC-CM, G-CSF and GM-CSF, did not mimic this upregulation, thus showing Hsp 70 expression was associated with, but not necessary for, suppression of apoptosis.

These findings in neutrophils appear to be contrary to the many examples in the literature reporting that Hsp 70 expression is associated with improved cell survival. Perhaps this difference can be explained by the fact that all the other studies look at apoptosis induced by various stimuli, whereas in the current study, we have examined effects on spontaneous or constitutive apoptosis.

Mitochondria are considered a key site for the initial induction of the nuclear events in apoptosis. The reasons for this are manifold. Firstly, alterations in mitochondria structure and function have been characterised in several cell types presented with apoptotic stimuli (Petit et al., 1995; Schulze-Osthoff et al., 1992). The protective Bcl-2 protein is mainly located in the inner mitochondrial membrane (Korsmeyer et al., 1995). Also, in a cell free system of apoptosis, isolated mitochondria can contribute to the apoptotic disintegration of isolated nuclei (Newmeyer et al., 1994). However the precise mechanisms by which mitochondria are involved and which mitochondrial features, such as mitochondrial membrane potential, the electron transport chain, ROS production, mitochondrial calcium handling, the cellular ATP level and cytochrome c or AIF release, are essential for apoptosis are still not fully elucidated. It is, therefore, perhaps not surprising that mitochondrial inhibitors (or 'chemical' hypoxia) have been shown to modulate apoptosis in multiple systems. In many cultured cells mitochondrial toxins have been shown to induce apoptosis (Dipasquale et al., 1991; Hartley et al., 1994; Mochizuki et al., 1994; Wolvetang et al., 1994). However, certain mitochondrial inhibitors have also been shown to inhibit apoptosis induced by specific stimuli, for example, complex I inhibitors have been shown to inhibit apoptosis induced by ceramide in human myeloid leukaemia cells (Quillet-Mary et al., 1997) and TNFα induced apoptosis in murine fibrosarcoma

cell lines (Schulze-Osthoff et al., 1992). Notably, dexamethasone-induced apoptosis in thymocytes can be inhibited both by hypoxia (Steffanelli et al., 1995) and by mitochondrial inhibitors (Steffanelli et al 1997).

In order to see if hypoxia could be exerting its protective effect on neutrophil apoptosis by acting on the mitochondria, we investigated the effect of a series of mitochondrial inhibitors on neutrophil apoptosis. The complex I inhibitor, rotenone, and the complex IV inhibitors, potassium cyanide and sodium azide, at concentrations similar to those shown to modulate TNFα-induced apoptosis in murine fibrosarcoma cell lines (Schulze-Osthoff et al., 1992), had no effect on apoptosis in neutrophils incubated under normoxic or hypoxic conditions. Thus, it appears that 'chemical' hypoxia does not mimic the survival effect of anoxic hypoxia in human neutrophils, a finding that suggests the inhibition of neutrophil apoptosis may be quite specific for hypoxia and does not reflect the broader metabolic consequences of making cells hypoxic. Neutrophils are not alone in their resistance to mitochondrial inhibitors; rotenone has also been shown to have no effect on the human lung carcinoma (A549) cell line (Hartley et al., 1994).

A fundamental question to answer when trying to elucidate any effector pathway is to determine whether or not *de novo* protein synthesis is a requirement. Traditionally, neutrophils have been considered to be pre-programmed end, or terminally differentiated, cells capable of functioning with little or no protein synthesis throughout their short life span (Bainton et al., 1971). This belief was derived from observations that much of the nuclear chromatin of mature neutrophils is coarsely clumped, suggesting that a considerable portion of the genome is inactive (Murphy, 1976). In addition, neutrophils lack evidence of a nucleolus and contain very little endoplasmic reticulum or ribosomes. However, more recently neutrophils have been shown to both synthesise mRNA transcripts and selectively synthesise a large number of proteins (Jack and Fearon, 1988), allowing these cells to modulate their responses to environmental factors. Additional evidence shows that new RNA synthesis is required for the production of leukocyte pyrogen (Nordlund et al., 1970), elastase (Hart, 1984) and plasminogen activator (Granelli-Piperno et al., 1977).

Indeed, neutrophils are now known to produce a range of cytokines, including G- and GM-CSF (Lindemann et al., 1989), demonstrating that neutrophils do indeed possess significant biosynthetic activity.

To investigate whether the survival effect of hypoxia on neutrophils requires *de novo* protein synthesis, we examined the action of the protein synthesis inhibitor, cycloheximide, on this effect. Using a concentration of 50 μM (14 μg/ml) cycloheximide, a concentration previously shown by Whyte et al. (1997) to almost completely inhibit protein synthesis in neutrophils, both control and hypoxically treated neutrophils showed accelerated rates of apoptosis at all time points examined. The observation that cycloheximide accelerates constitutive neutrophil apoptosis agrees with previous data (Brach et al, 1992; Payne et al.; 1994; Whyte et al., 1997) and has led to the proposal that continuous synthesis of some regulatory (anti-apoptotic) protein(s) may be necessary to prevent neutrophils undergoing spontaneous apoptosis. Acceleration of apoptosis by protein synthesis inhibitors has also been demonstrated in monocytic myeloid cells (Waring, 1990), the myeloid leukaemic cell line (HL-60) (Martin et al., 1990) and in the U-937 leukaemic cell line (Kochi and Collier, 1993). However, this is certainly not true of all cell types. In thymocytes, death via apoptosis, induced by multiple stimuli, can be delayed or abrogated by RNA or protein synthesis inhibitors (Wyllie et al., 1984; Cohen et al., 1984; Sellins et al., 1987; Kizaki et al., 1989), as can apoptosis in hepatocytes (Ledda-Columbano et al., 1992) and neurons (Martin et al., 1992). Thus, it would appear that, in these cell types, molecules necessary for the execution of the death program are synthesised *de novo* in response to the inducing stimulus, a finding more in agreement with apoptosis being a cellular form of active rather than passive suicide. It has been proposed that in cell types, which retain the capacity to proliferate, RNA and protein synthesis inhibitors delay or prevent the development of apoptosis, while cells such as neutrophils, which have lost the potential to proliferate, do not depend on new protein synthesis to permit apoptosis and treatment with these agents will typically accelerate basal rates of cell death (Cohen, 1991).

The ability of cycloheximide to overcome completely the protective effect of hypoxia in neutrophils, increasing levels of apoptosis in hypoxically treated neutrophils to nearly those of cycloheximide treated control cells, is compatible with the hypothesis that the hypoxic protective effect is protein synthesis dependent. However, as cycloheximide at this concentration (50 μM; 14 μg/ml) itself exerts such a powerful effect on basal apoptosis, it was also possible that the protective effect of hypoxia was simply not strong enough to combat the pro-apoptotic effect of cycloheximide. Thus, in order to further investigate the role of *de novo* protein synthesis in the protection of neutrophil apoptosis by hypoxia the effect of lower concentrations of cycloheximide on this phenomenon was examined. When cells were treated with concentrations of 0.1, 1, and 10 μg/ml cycloheximide the enhanced survival of neutrophils by hypoxia was overcome in a concentration-dependent manner. Cycloheximide at 1μg/ml, a dose that did not induce apoptosis in control cells, effectively overcame the protective effect of hypoxia. Previous studies (Cox et al., 1994) have shown this dose of cycloheximide is sufficient to markedly interfere with, but not abolish, protein synthesis in neutrophils. These data provide strong evidence that the prolonged survival of neutrophils by hypoxia is associated with newly synthesised proteins that prevent programmed cell death. Clearly much more work is required in order to elucidate the identity of these proteins.

Cycloheximide has also been shown to overcome dexamethasone (Cox et al., 1996), GM-CSF (Brach et al., 1992), sodium butyrate (Stringer et al., 1996) and LPS (Hachiya et al., 1995) induced survival of neutrophils, implicating a role for *de novo* protein synthesis in all of these inhibitory effects. However, the survival proteins involved have not been identified in any of these systems. Cox et al. (1994), found increased expression of MnSOD and Hsp 70, two proteins that have been associated with enhanced survival in certain *in vitro* cell culture systems (Warner et al., 1991; Peng et al., 1986; Angelidis et al., 1991; Kantanagwa et al., 1991), in response to treatment of neutrophils with human bronchial epithelial cell conditioned medium (HBEC-CM). However the major anti-apoptotic factors present in HBEC-CM, namely G-CSF and GM-CSF, did not mimic the upregulation, implying that the induction of these two stress proteins is not necessary for suppression of apoptosis.

Our data on Hsp 70 expression in neutrophils shown earlier in this chapter certainly does not support a role for this protein in the protective effect of hypoxia in neutrophils. Our previous data, and that of others (Hockenbery et al., 1991) showing a lack of expression of the oncoprotein Bcl-2 in neutrophils and the inability of glucose deprivation to modulate neutrophil apoptosis also makes Bcl-2 and the GRPs unlikely candidates for the role. However, there remain numerous putative proteins that could be responsible for inhibiting neutrophil apoptosis, for example other members of the Bcl-2 family, and the possibility that an, as yet, unknown protein is involved is also feasible. Also it would be dangerous to assume that all these inhibitory effects involve the same protein(s). Although it is not impossible that the same anti-apoptotic proteins are involved, our data showing that the GM-CSF and hypoxic protective effects are additive suggests, at least between these two protective effects, that there are likely to be differences between the mechanisms involved.

CHAPTER 5

INVOLVEMENT OF TRANSCRIPTION FACTORS IN THE HYPOXIC INHIBITION OF NEUTROPHIL APOPTOSIS

5.1 Introduction

In the previous chapter we investigated the intracellular signalling pathways underlying the hypoxic inhibition of neutrophil apoptosis. We found that this pathway(s) was not sensitive to other cellular stresses, including glucose deprivation, heat shock or chemical hypoxia. Antioxidants were also ineffective in mimicking the inhibitory effect of hypoxia. However, this effect was sensitive to a protein synthesis inhibitor, cycloheximide, indicating that *de novo* protein synthesis is required, or involved, in mediating the anti-apoptotic effect of hypoxia in human neutrophils.

The archetypal model for gene regulation in response to an extracellular stimulus involves ligand-receptor interaction followed by a series of downstream 'second-messenger' events that target cytosolic transcriptional regulators and, hence, the expression of appropriate genes. This commonly occurs via the induction or repression of transcription, although additional mechanisms have been described, including changes in mRNA stability or in the rate of translation. Most extracellular ligands influence cell behaviour purely by interacting with a specific cellular receptor and, hence, act solely as discrete and 'confined' messenger molecules. Such ligands include polypeptide hormones, growth factors and a variety of smaller molecules (such as catchecholamines, steroids and thyroid hormones). In contrast, oxygen plays a central function in aerobic metabolism and, under normal conditions, this is likely to preside over and dominate any additional role this molecule has in signalling via a sensor-receptor mechanism. Thus, it seems likely that the mechanism(s) available for oxygen sensing and signalling will deviate from these well established systems.

One of the most studied mechanisms underlying oxygen sensing in mammalian cells is that underlying hypoxia-induced upregulation of erythropoietin. Erythropoietin

(EPO) is a glycoprotein hormone required for the proliferation and differentiation of erythroid cells (reviewed by Jelkmann, 1992). Upon hypoxic exposure, the kidney, and to a lesser extent the liver, increase EPO synthesis by enhancing both transcription of the EPO gene and EPO mRNA stability. Studies into the mechanisms involved in EPO regulation were greatly enhanced by the discovery of several human hepatoma cell lines, which are capable of producing EPO in a hypoxia-dependent manner. We noted that the inhibition of neutrophil apoptosis by hypoxia shared several characteristics with EPO production. For instance, the induction of EPO mRNA and protein has also been reported to be insensitive to mitochondrial inhibitors, certain antioxidants, heat shock and glucose deprivation, but induction can be inhibited by the protein synthesis inhibitor, cycloheximide. EPO production can also be stimulated by cobalt, nickel and manganese ions in a concentration-dependent manner. These observations lead Goldberg and co-workers (1988) to propose that the oxygen sensor involved in hypoxic regulation of EPO production was a haemoprotein, which was active in its deoxy state. While such a protein system has yet to be identified, it is known that cobalt, nickel and manganese can all substitute for the ferrous ion in the porphyrin of haem but have a much lower affinity for oxygen. Goldberg et al., (1988) hypothesised that these metal ions would lock the haem sensor in its (active) deoxy-state. Observations that carbon monoxide (a haem inhibitor) and 4, 6-dioxoheptanoic acid (a haem synthesis inhibitor) were able to inhibit hypoxia-induced EPO production further substantiated this theory. However, later observations that iron chelators were also potent inducers of EPO mRNA expression were hard to accommodate using this model as haem iron is not chelatable. Other proposals for how the cell may sense oxygen levels include the involvement of ROS as signalling intermediates (Fandrey et al., 1994) and the involvement of a flavoprotein oxidoreductase. This latter suggestion was based on observations that iodonium compounds are powerful inhibitors of the hypoxic induction of EPO gene expression (Gleadle et al., 1995b).

Although the molecular processes underlying the oxygen-sensing mechanism remain unclear, the elements involved in the transcriptional control of EPO have been more fully characterised. Through experiments involving the introduction of human EPO

gene constructs into transgenic mice (Semenza et al., 1990; Semenza et al., 1991a) and Hep3B cells (Semenza et al., 1991b; Semenza and Wang, 1992) an important transcriptional regulatory element in the 3'-flanking region of the gene was identified. A 50-nucleotide (nt) sequence from this region was shown to function as a hypoxia-inducible enhancer of transcription in Hep3B cells and a trans-acting factor that binds to this enhancer was found in nuclear extracts from Hep3B cells exposed to hypoxia but not in control cells (Semenza and Wang, 1992). This DNA-binding activity, termed hypoxia-inducible factor-1 (HIF-1), fulfilled the criteria for a physiological regulator of EPO transcription. Other inducers of EPO expression (e.g. $CoCl_2$ and desferrioxamine) also induced HIF-1 binding, while inhibitors of EPO expression, including cycloheximide, blocked induction of HIF-1 activity. Finally, mutations in the EPO 3' flanking region that eliminated HIF-1 binding also eliminated enhancer function (Semenza, 1994). It is important to note that, while EPO expression is cell type specific, induction of HIF-1 activity by hypoxia, cobalt or desferrioxamine has been detected in many other mammalian cell lines (Wang and Semenza, 1993b). Indeed, HIF-1 has been shown to mediate the hypoxic regulation of vascular endothelial growth factor (VEGF), the primary regulator of angiogenesis (Forsythe et al., 1996). HIF-1 has also been implicated in transcriptional activation of the genes encoding iNOS and haem oxygenase 1 (Lee et al., 1997; Melillo et al., 1995) and several glycolytic enzymes (Firth et al., 1994; Semenza et al., 1994), suggesting that HIF-1 plays a very general role in mediating transcriptional responses to hypoxia.

Glycerol gradient sedimentation, UV crosslinking, and methylation interference studies have indicated that HIF-1 is a heterodimer consisting of a 120 kDa HIF-1α subunit and a 91-94 kDa HIF-1β subunit and that both subunits contact DNA in the major groove (Wang and Semenza, 1993a; Wang and Semenza, 1993c; Wang and Semenza, 1995). These findings were confirmed by the analysis of purified HIF-1, and subsequent protein microsequence analysis and cDNA cloning have now revealed the molecular identity of these two subunits (Wang et al., 1995). Both HIF-1 subunits are basic-helix-loop-helix proteins containing a PAS domain (bHLH-PAS). PAS is an acronym derived from the first three proteins observed to contain

this motif. These include the product of the *period* gene of *Drosophilia melanogaster* (Jackson et al., 1986), the aryl hydrocarbon receptor nuclear transporter (ARNT) gene of mammals (Burbach et al., 1992) and the product of the fruit fly *single-minded* gene (Nambu et al., 1991). HIF-1α was identified as a novel bHLH-PAS protein while HIF-1β is identical to ARNT, which appears to be a common subunit for a family of bHLH-PAS proteins. Although there is still some controversy about hypoxic regulation of HIF-1 mRNA levels (Wang et al., 1995; Huang et al., 1996), the general consensus is that HIF-1 function is regulated primarily by HIF-1α protein levels (see Semenza, 1998 and Ratcliffe et al., 1998 for recent reviews). HIF-1β can be found in both the nucleus and the cytoplasm of cells under normoxic conditions, although translocation of the protein to the nucleus occurs only upon hypoxic stimulation, while HIF-1α can only be detected at very low levels in unstimulated cells. Western analysis of whole cell extracts has demonstrated that hypoxic stimulation results in a rapid accumulation of HIF-1α, with levels decaying rapidly ($t_{1/2} < 5$ min) when cells are returned to a normoxic environment. The rapid increase in HIF-1α levels in stimulated cells could involve enhanced translation or enhanced stabilisation. Evidence for the latter mechanism comes from treatment of hypoxic cells with cycloheximide, which has indicated that, under hypoxic conditions, HIF-1α is relatively stable and there is evidence that HIF-1α is rapidly degraded under normoxic conditions by the ubiquitin-protease system (Salceda and Caro, 1997). Stabilisation of HIF-1α is also induced by cobalt, nickel, manganese and iron chelators (Salceda and Caro, 1997), as would be predicted from the ability of these compounds to induce HIF-1 activity. Jiang and co-workers (1996) indicated that the C-terminal end of HIF-1α contained the putative degradation domain. Further work by Pugh et al. (1997) has provided evidence suggesting that amino acids 530-634 and within that fragment, amino acids 549-582, are involved in the oxygen-regulated degradation of the protein. In addition to this, an iron-binding site in fragment 529-658 has been reported and suggested as a plausible mechanism for this oxygen-regulated degradation, excluding the need for a separate oxygen sensor (Srinivas et al., 1998). Consistent with the theory that HIF-1 activity is controlled by HIF-1α protein levels, forced overexpression of the α

subunit in normoxic cells is itself sufficient to drive HIF-1-dependent reporter gene expression. However, hypoxic stimulation of these cells further enhanced activity, suggesting that hypoxia can also modify HIF-1 DNA-binding (Jiang et al., 1996). In agreement with this, further analysis of C-terminal sequences from HIF-1α showed that amino acids lying immediately N-terminal to position 786 suppressed total activity and conferred inducibility, an effect which appeared to be independent of the level of expressed protein (Ratcliffe et al., 1998). Thus, the evidence to date suggests that HIF-1 activity is regulated primarily by oxygen-dependent modulation of the specific activity of HIF-1α activation domains in addition to an effect on HIF-1α protein levels.

Importantly, HIF-1 induction is observed in response to physiologically relevant O_2 concentrations. A report by Jiang and co-workers (1996) demonstrated that HIF-1 DNA-binding activity and HIF-1α protein expression increased exponentially as cells were subjected to decreasing O_2 concentrations, with a half maximal response between 1.5 and 2% O_2 and a maximal response at 0.5% O_2. Such levels of oxygen are comparable with those present in many tissues under conditions of normal or compromised oxygen delivery (see Chapter 1, sections 1.3.2 - 4). Interestingly, below 0.5% O_2, rather than reaching a plateau, HIF-1 binding and protein levels actually declined.

Although HIF-1 is probably unique in its specificity for hypoxia, at least in mammalian cells, it is by no means the only transcription factor that has been reported to be activated in response to hypoxia. Two other such transcription factors are NF-κB (nuclear factor-κB) and AP-1 (activator protein-1). Unlike HIF-1, it has been suggested that these transcription factors are induced by either (near-) anoxic stress conditions (where the O_2 concentration is below approximately 0.02%) or even reoxygenation dependent processes (Wenger and Gassman, 1997). However, there are other reports suggesting AP-1 may be induced by less strenuous hypoxia (5-10% oxygen) (Millhorn et al., 1997; Norris and Millhorn, 1995).

NF-κB exists in the cytoplasm of the majority of cells as a homo- or heterodimer family of structurally related proteins (for reviews see Baldwin, 1996 and Ghosh et al., 1998). To date, five proteins belonging to the NF-κB family have been identified in mammalian cells: p65, c-Rel, RelB, p50 and p52. NF-κB activity is regulated by a class of inhibitory proteins called IκB; seven of these molecules have been identified so far, the best characterised being IκBα and IκBβ. The IκB proteins bind to NF-κB dimers keeping them sequestered in the cytosol. In response to an activating stimulus, the IκB protein is phosphorylated on specific serine residues prior to ubiquitination and subsequent proteosomal degradation, leaving NF-κB free to translocate to the nucleus where it binds specific κB DNA sequences. This mechanism allows for extremely rapid activation of NF-κB making it ideally suited for its pivotal role in many cellular responses to environmental stress. Indeed, NF-κB can be activated by a manifold of stimuli known to cause cellular stress including various cytokines, bacterial and viral products, ROS, UV light and γ-irradiation. More recently, hypoxia has been proposed as a further inducer of NF-κB, however, there is still some controversy associated with this. Notably, many of the inducers of NF-κB activation are also known for their apoptotic effects and thus, perhaps unsurprisingly, NF-κB has been implicated as playing a pivotal role in controlling apoptosis. There is evidence that inhibition of the constitutive NF-κB activity in WEHI 231 cells prevents apoptosis (Wu et al., 1996). In contrast, $p65^{-/-}$ mice die during embryonic development through massive apoptosis of hepatocytes (Wang et al., 1996) suggesting an anti-apoptotic role for NF-κB. This role has been further supported by data showing that cell lines, previously resistant to the cytotoxic effects of TNFα and other pro-apoptotic agents, became extremely sensitive when lacking p65 or when expressing a dominant negative form of IκBα (Beg and Baltimore, 1996; Wang et al 1996). Finally, a recent report has proposed an important regulatory role for NF-κB in neutrophil apoptosis (Ward et al., 1999). Treatment of neutrophils with gliotoxin, a potent inhibitor of NF-κB, caused a marked enhancement of both constitutive and TNFα-induced apoptosis, suggesting an anti-apoptotic role for NF-κB in this cell type.

Like NF-κB, the AP-1 transcription factor is a dimeric DNA-binding protein consisting of homo- and heterodimer subunits (for recent reviews see Foletta et al., 1998; Liebermann et al., 1998). These subunits are encoded by the proto-oncogenes c-fos and c-jun and their related gene family members. The fos family consists of c-fos, fra-1, fra-2, fosB and fosB2, while, to date, three jun family members have been described (c-jun, junB and junD). Dimerization between Fos and Jun proteins occurs via a structure referred to as a leucine zipper. The most stable dimers are those formed between jun and fos family members, although Jun (but not Fos) proteins can also homodimerize. AP-1 activity is regulated primarily by changes in DNA-binding activity and abundance of the AP-1 protein. The levels of AP-1 protein are primarily regulated by controlling transcription of their genes; however, more recent evidence suggests that the abundance of both c-Jun and c-Fos can also be regulated by modulation of their stability. In the case of c-Jun the phosphorylation of serines and threonines at its amino-terminal activation domain by MAPK reduces the ubiquitination of this protein and hence its degradation. In addition, AP-1 activity is regulated by phosphorylation and is also sensitive to redox potential. In a variety of cells, AP-1 activity can be enhanced by the administration of both organic antioxidants as well as by thioredoxin, a protein with reducing capability, whereas hydrogen peroxide and sulfhydryl-blocking reagents inhibit activation. Site-directed mutagenesis has localised the critical redox sensitive sites to a single conserved cysteine residue in the DNA binding domains of Fos and Jun. Replacement of these cysteines by serine resulted in enhanced activity that is resistant to oxidants. It is also noteworthy that this Cys→Ser replacement is also present in the oncogenic v-Jun. There is also much evidence indicating that the c-fos and c-jun family of genes are inducible by hypoxia. Exposure of cardiac myocytes to severe hypoxia induced mRNA expression of c-fos, c-jun, junD and junB. Likewise, when the brains of new born rats were rendered acutely ischaemic, expression of c-fos and c-jun was induced and in hypoxic Hep 3B cells expression of c-jun and junB was upregulated. Consistent with these observations hypoxia has also been shown to induce AP-1 binding to its DNA sequence motif in several cell types including Hela, Hep-3B and endothelial cells (Bandyopadhyay et al., 1995; Rupec and Baeuerle, 1995).

Experiments showing that c-jun and c-fos expression was upregulated in cells treated with pro-apoptotic stimuli prompted suggestions that AP-1 may play a role in promoting apoptosis (Colotta et al., 1992b). Further evidence supporting this theory came from a study using *fos-lacZ* transgenic mice, which found that there is continuous expression of c-fos hours or days before cell death in numerous cell populations which were undergoing terminal differentiation (Smeyne et al., 1993). More recently JNK and c-Jun have been implicated in ceramide-induced apoptosis of human myeloid and lymphoid tumour cell lines and growth factor withdrawal-induced apoptosis in cultured neurons (Ham et al., 1995; Verheij et al., 1996). Although the evidence that indicates that AP-1 and JNK are involved in some forms of apoptosis is increasing, there are also a number of reports that suggest AP-1 proteins and JNK are not essential for cells to undergo apoptosis. For example, programmed cell death can occur in embryos lacking either c-Fos or c-Jun or both c-Fos and c-Jun (Roffler-Tarlov et al., 1996). In addition, splenic and thymic primary cultures taken from c-fos deficient mice underwent apoptosis after treatment with etoposide in identical manner to wild-type cultures (Gajate et al., 1996).

The aim of the work presented in this chapter was, firstly, to investigate the oxygen-sensing mechanism underlying the anti-apoptotic effect of hypoxia and, secondly, to examine the potential involvement of redox-sensitive transcription factors.

5.2 Results

5.2.1 Effect of desferrioxamine and cobaltous chloride on neutrophil apoptosis

One of the most studied mammalian oxygen sensing mechanisms is that which controls the regulation of the transcription factor hypoxia inducible factor (HIF)-1. This transcription factor was originally discovered due to its involvement in the hypoxic mediation of erythropoietin synthesis but has subsequently been proposed to be a part of a universal oxygen sensing mechanism. One of the characteristics of this system is the ability of iron chelators and certain divalent metal ions, as well as hypoxia, to cause activation of HIF-1. Thus, to examine the hypothesis that HIF-1 may be involved in the inhibition of neutrophil apoptosis by hypoxia, we investigated the effect of the iron chelator, desferrioxamine (DFO), and cobaltous chloride, two agents that have been shown to cause activation of HIF-1 in other cell types, on the rate of neutrophil apoptosis. Neutrophils were incubated in the presence of 100 μM DFO or 100 μM cobaltous chloride in atmospheres of either 21% or 0% oxygen for 20 hours. While neither of these agents had any effect on the rate of apoptosis compared to untreated cells in 0% oxygen, in 21% oxygen DFO significantly inhibited neutrophil apoptosis ($p<0.05$), although cobaltous chloride still had no effect (figure 5.2.1). To ensure that the lack of effect of cobaltous chloride was not because we were using an inappropriate concentration, a range of concentrations from 1 μM to 10 mM was also tested. Concentrations of 1 mM and above proved toxic to neutrophils and at no concentration did cobalt ions inhibit neutrophil apoptosis (data not shown).

5.2.2 Iron chelators inhibit neutrophil apoptosis in a concentration-dependent manner

To further investigate the observation that DFO inhibits neutrophil apoptosis (i.e. mimics the effect of hypoxia), the influences of a range of concentrations (1 μM - 10 mM) of DFO and a second iron chelator, CP94 (1 μM - 1 mM), was examined. Both these iron chelators inhibited neutrophil apoptosis in a concentration-dependent manner (figure 5.2.2A) and, at the maximally effective concentrations, were able to inhibit apoptosis to a similar extent as hypoxia. In addition, the anti-apoptotic effect of the iron chelators was only evident under hypoxic conditions at very high

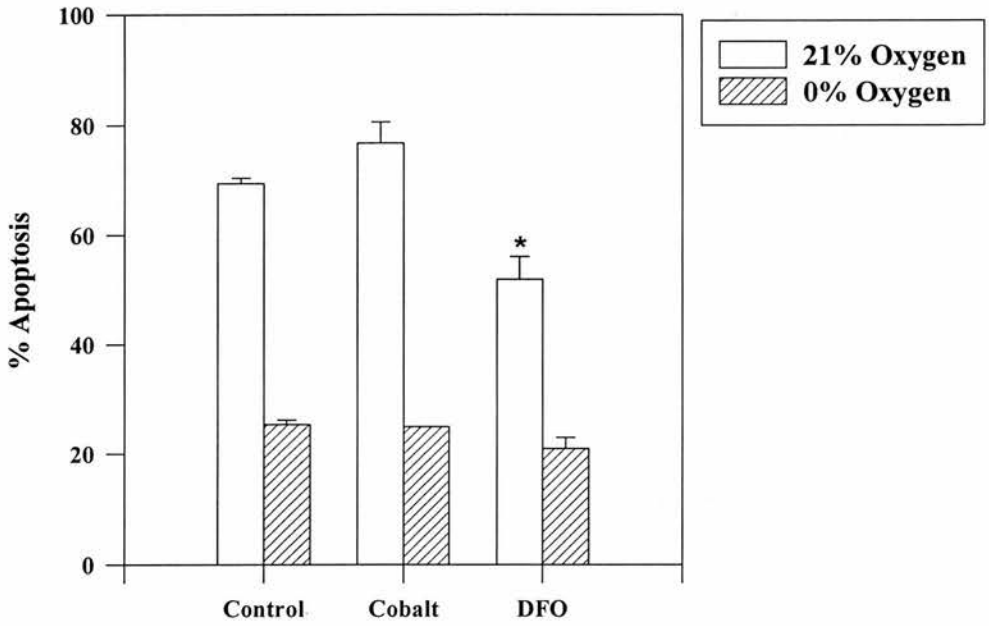


Figure 5.2.1 Effect of desferrioxamine and cobaltous chloride on neutrophil apoptosis. Neutrophils (5 x 10^6/ml) were incubated with cobaltous chloride (100 μM) or the iron chelator, desferrioxamine (100 μM), for 20 hours in atmospheres containing 21% or 0% oxygen. Control, untreated cells were also prepared. Apoptosis was assessed morphologically and data represent mean ± SD of triplicate incubations from a single representative experiment of 2 (*$p<0.05$ compared with control values).

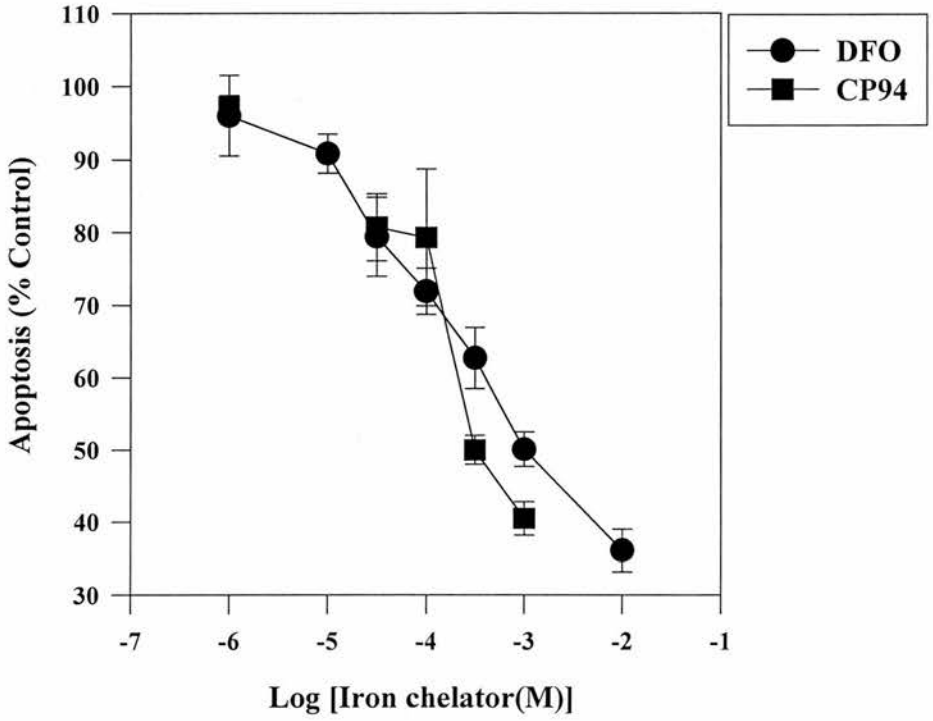


Figure 5.2.2A Iron chelators inhibit neutrophil apoptosis in a concentration-dependent manner. Neutrophils (5 x 10^6/ml) were cultured under normoxic conditions for 20 hours in the presence of increasing concentrations of desferrioxamine (DFO, 1 μM-10 mM) or CP94 (1 μM-1 mM). Control, untreated neutrophils were also prepared. Apoptosis was assessed morphologically and apoptosis expressed as percentage of the control values. Data represent mean ± SEM of n = 3 (DFO) and n = 4 (CP94) separate experiments each performed in triplicate.

concentrations (1 mM and above; data not shown), suggesting that hypoxia and the iron chelators were acting through a common mechanism. It is notable that CP94 and DFO are structurally different, suggesting that this effect was due to their iron chelating properties and not some common secondary action. This was demonstrated experimentally by showing that a molar excess of iron (Fe^{2+}) could overcome the protective (anti-apoptotic) effects of these iron chelators. Neutrophils were incubated with 300 μM DFO or CP94 in the presence and absence of 3 mM ferrous chloride ($FeCl_2$). The protective effects of CP94 and DFO were completely abrogated when $FeCl_2$ was present (figure 5.2.2B). These results suggest a role for chelatable iron in the oxygen-sensing mechanism and may indicate the presence and involvement of a ferro-protein sensor. In the context of other published work, these data also support a putative role for the transcription factor HIF-1 in the inhibition of neutrophil apoptosis by hypoxia.

5.2.3 Expression of hypoxia inducible factor (HIF)-1 in human neutrophils

In view of the ability of agents known to induce HIF-1 activation in other cells to mimic the effect of hypoxia on neutrophil apoptosis, we examined expression of HIF-1 in human neutrophils. HIF-1 is a heterodimer consisting of the HIF-1α and HIF-1β subunits. Both these subunits have been classified as basic helix-loop-helix (bHLH)/PAS domain proteins and, while HIF-1α is a novel protein, HIF-1 β was found to be identical to the previously recognised aryl hydrocarbon nuclear translocator, ARNT (Wang et al., 1995). In many studies (Huang et al., 1996; Wenger et al., 1996a; Wood et al., 1996) HIF-1α and HIF-1β mRNAs have been found to be expressed in normoxic cells, indicating that HIF-1 activation may involve both translational and post-translational mechanisms. As an initial approach, we verified that neutrophils have the potential to express the HIF-1 protein by demonstrating that neutrophils express HIF-1α mRNA. This was achieved by isolating RNA from freshly isolated, unstimulated neutrophils and performing an RNA protection assay. This approach was also used to identify endothelial PAS domain protein 1 (EPAS1), another PAS domain protein, which shares 48% sequence homology with HIF-1α (Tian et al., 1997). Although HIF-1α mRNA was shown to be present in both freshly isolated neutrophils and monocytes, EPAS1

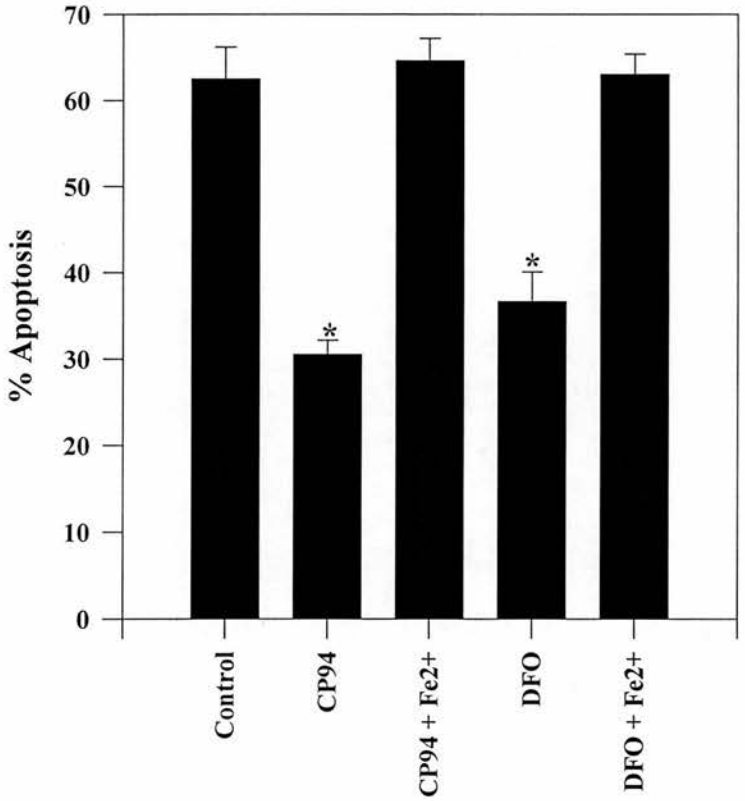


Figure 5.2.2B The protective effects of desferrioxamine and CP94 on neutrophil apoptosis can be overcome by the addition of excess Fe^{2+} ions. Neutrophils were incubated under normoxic conditions for 20 hours in the presence of: 300 μM CP94; 300 μM CP94 and 3 mM $FeCl_2$; 300 μM DFO; and 300 μM DFO and 3 mM $FeCl_2$. Control untreated cells were also prepared and apoptosis was assessed morphologically. Data represent mean ± SEM of n = 3 separate experiments, each performed in triplicate (*$p<0.05$ compared with control values).

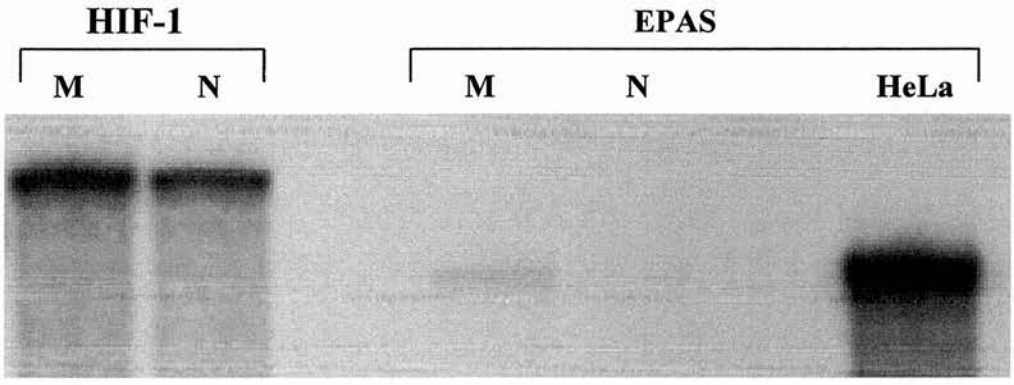


Figure 5.2.3A RNase protection assay showing monocytes and neutrophils constitutively express HIF-1α mRNA but not EPAS1 mRNA. Human neutrophils and monocytes were isolated from whole blood and total cellular RNA was prepared. Ribonuclease protection of total RNA from monocytes (M) and neutrophils (N) was performed for EPAS-1 and HIF-1α (40 μg each) with HeLa RNA utilised as a positive control. RNA analysis was performed by Dr Michael Wiesener, Wellcome Trust Centre for Human Genetics, Oxford. Data is a representative blot of n = 2.

mRNA was not found in either (figure 5.2.3A), indicating that both cell types have the potential to upregulate HIF-1, but not EPAS1, in response to hypoxia.

The presence of HIF-1β protein has been reported in both cytoplasm and nuclear extracts from normoxic cells, while HIF-1α is only expressed at very low levels in unstimulated cells (Wang et al., 1995), suggesting that HIF-1 activity is regulated, at least partially, by hypoxic-modulation of HIF-1α protein levels. In view of this, we examined whether neutrophils would upregulate HIF-1α protein expression in response to hypoxic-stimulation and iron chelator treatment. Human neutrophils were incubated under normoxic conditions in the presence and absence of DFO, while parallel cells were made hypoxic by resuspension in medium that had been pre-incubated in 0% oxygen conditions overnight. After 3 hours in these culture conditions the cells were harvested and whole cell lysates prepared using a TRIZOL® based lysis method (see section 2.2.6.2a for details). The lysates were then analysed for HIF-1α using western blotting. HIF-1α was not present in the control cells but was found in the hypoxic and DFO treated neutrophils (figure 5.2.3B), demonstrating that neutrophils upregulate HIF-1α protein in response to these stimuli.

Finally, in order to show that not only HIF-1α protein can be induced in response to hypoxic-stimulation, but also that HIF-1-DNA binding can occur under these conditions, a DNA binding assay for HIF-1 was performed using neutrophil nuclear extracts. Neutrophils were incubated under hypoxic and normoxic conditions and after 3 hours cells were harvested and nuclear extracts prepared. These extracts were incubated with a ^{32}P-labelled double stranded oligonucleotide containing the HIF-1 binding site (see section 2.2.7.3 for details) and an electrophoretic mobility-shift assay (EMSA) performed. Identically treated HeLa cells were used as a positive control. Figure 5.2.3C shows a representative EMSA with protein complexes binding the oligonucleotide and retarding the mobility of the probe. The HeLa nuclear extracts demonstrate the normal binding pattern for HIF-1 (J. Gleadle, personal communications) with both constitutive and inducible binding complexes present. However, in comparison, the neutrophil extracts exhibit a very different binding pattern with no obvious inducible band. The reason for these differences is,

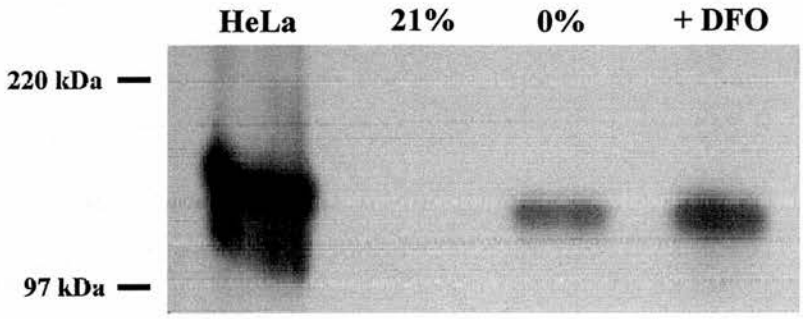


Figure 5.2.3B Western blot showing HIF-1α protein expression in human neutrophils. Neutrophil lysates were prepared by a Trizol® based lysis method and separated by 10% SDS-PAGE. Western blot was developed with an antibody to HIF-1α. After isolation neutrophils were incubated in either a normoxic atmosphere (21% oxygen) or a hypoxic environment (0% oxygen) or incubated in the presence of desferrioxamine (DFO, 1 mM) for 3 hours before cell lysis. Whole cell lysate from hypoxia treated HeLa cells was used as a positive control. Western blot analysis was performed by Dr Michael Wiesener, Wellcome Trust Centre for Human Genetics, Oxford. Data is a representative blot from n = 2.

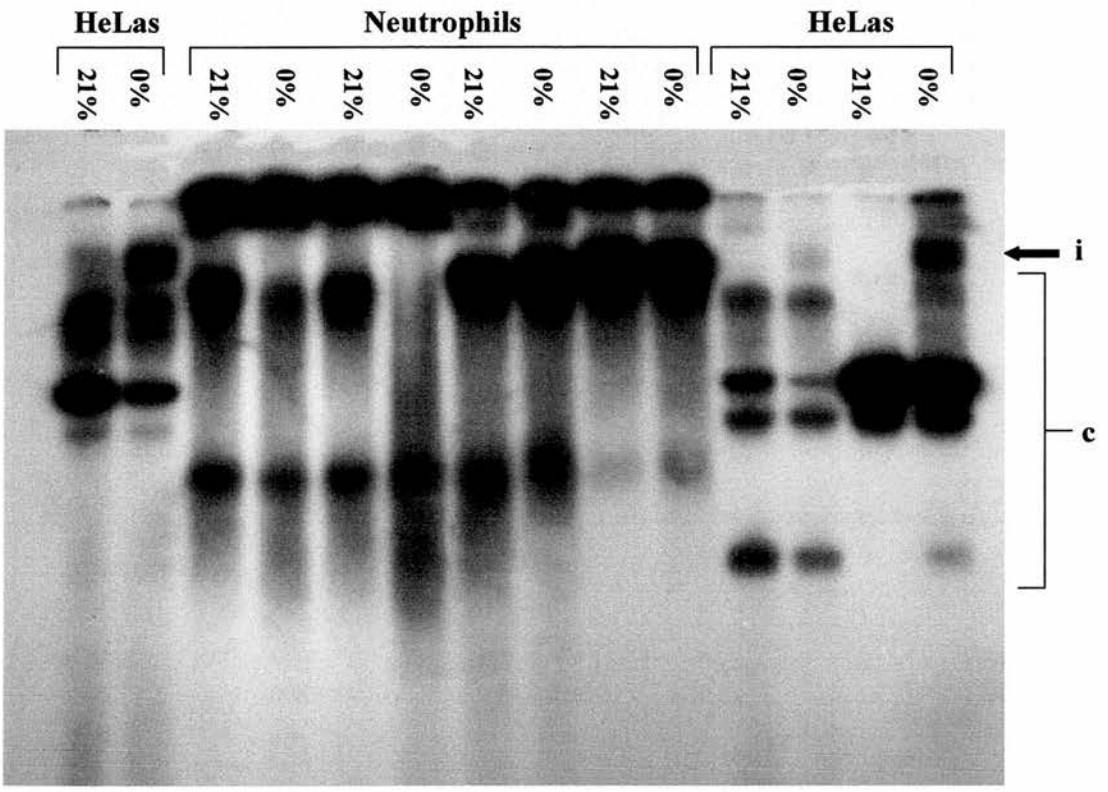


Figure 5.2.3C Electrophoretic mobility-shift assay using nuclear protein extracts from neutrophils and an oligonucleotide containing a HIF-1 binding site. Nuclear extracts were prepared from neutrophils and HeLa cells that had been exposed to either normoxia (21% oxygen) or hypoxia (0% oxygen) for 3 hours. A 5' end-labelled oligonucleotide containing a HIF-1 DNA-binding site was used as the probe. The position of the inducible complexes (**i**) and constitutive complexes (**c**) in the HeLa samples are indicated, however, these bands are not evident in the neutrophil samples. The multiple lane repeats for the neutrophils represent samples from 2 separate experiments. The data is a representative experiment of n = 3.

at present, unclear. The neutrophil bands were reduced significantly when unlabelled wildtype probe was added to the binding reaction, although there was no change when excess probe containing a mutated binding site was added (data not shown), suggesting that these bands reflect, at least partially, specific binding to the HIF-1 binding site. Thus, although we have been able to show the presence of HIF-1α mRNA in neutrophils and an increase in HIF-1α protein levels in response to hypoxia, we were unable to correlate this to an increase in HIF-1 binding activity to a recognised HIF-1 DNA binding site.

5.2.4 NF-κB binding activity in nuclear protein extracts from human neutrophils

In view of reports implicating a role for NF-κB in regulating apoptosis and data from several groups suggesting that NF-κB DNA-binding may be induced by hypoxia, we investigated the activity of this transcription factor in hypoxic neutrophils. Neutrophils (10^7/ml) were resuspended in medium that had been pre-incubated in either 0% oxygen or 21% oxygen conditions overnight, thus ensuring that cells were fully hypoxic from time 0, with parallel cells cultured in the presence of DFO (1 mM). At a range of time points up to 6 hrs, neutrophils were harvested into ice cold PBS and nuclear fractions isolated (see section 2.2.8.1 for details). The nuclear extracts were incubated with ^{32}P-labelled double stranded oligonucleotide containing the NF-κB binding site and binding activity assessed by EMSA. As shown in figure 5.2.4A, when neutrophil nuclear extracts were used, the NF-κB EMSAs indicated the presence of 3 discrete bands. The fastest (C) and slowest (A) migrating NF-κB DNA binding activities were strongly expressed as opposed to the middle complex (B), which was very weakly expressed. Other workers in the group (C.Ward, personal communications) have demonstrated, by including an excess of cold probe in the binding reaction, that only bands B and C represent specific NF-κB binding (Ward et al., 1999). In addition, known activators of NF-κB, LPS and TNFα, are able to upregulate band B while having no effect on band C, which is also expressed in freshly isolated neutrophils, leading to the proposal that band C represents a form of constitutively active NF-κB while band B is an inducible form (Ward et al., 1999). In all experiments none of the conditions detailed above caused an upregulation of

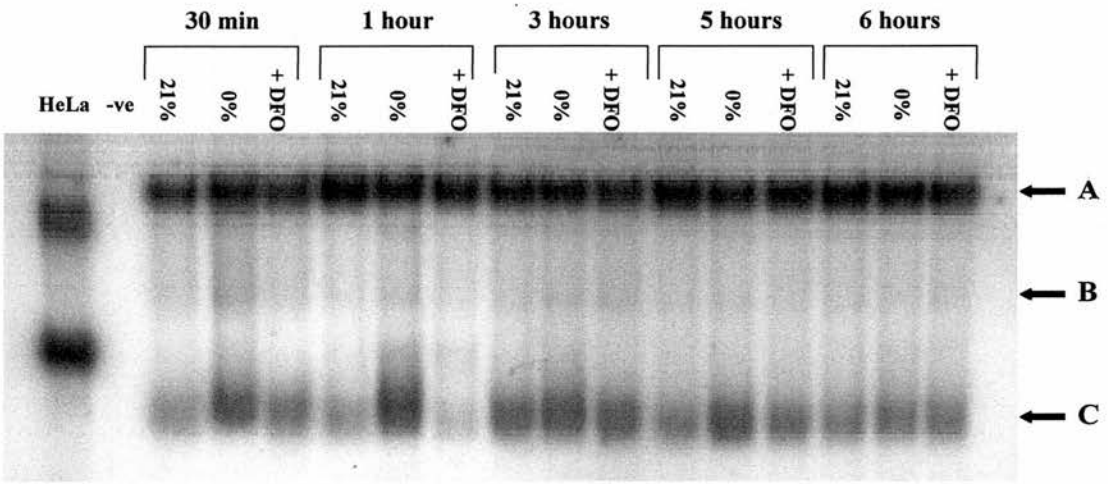


Figure 5.2.4A The effect of hypoxia and desferrioxamine on NF-κB binding in nuclear extracts from human neutrophils. Electrophoretic mobility-shift assay using nuclear protein extracts from neutrophils exposed to either, normoxia (21% oxygen), hypoxia (0% oxygen) or incubated in the presence of 1 mM desferrioxamine (DFO) for the stated times. A 5' end-labelled oligonucleotide containing an NF-κB DNA-binding site was used as the probe. The position of the three DNA-binding complexes A, B and C are indicated (see text for details). HeLa cell nuclear extract was used as a positive control; there was no shift in probe mobility in the absence of nuclear protein extract (lane 2; -ve). This gel is a representative example of 6 others.

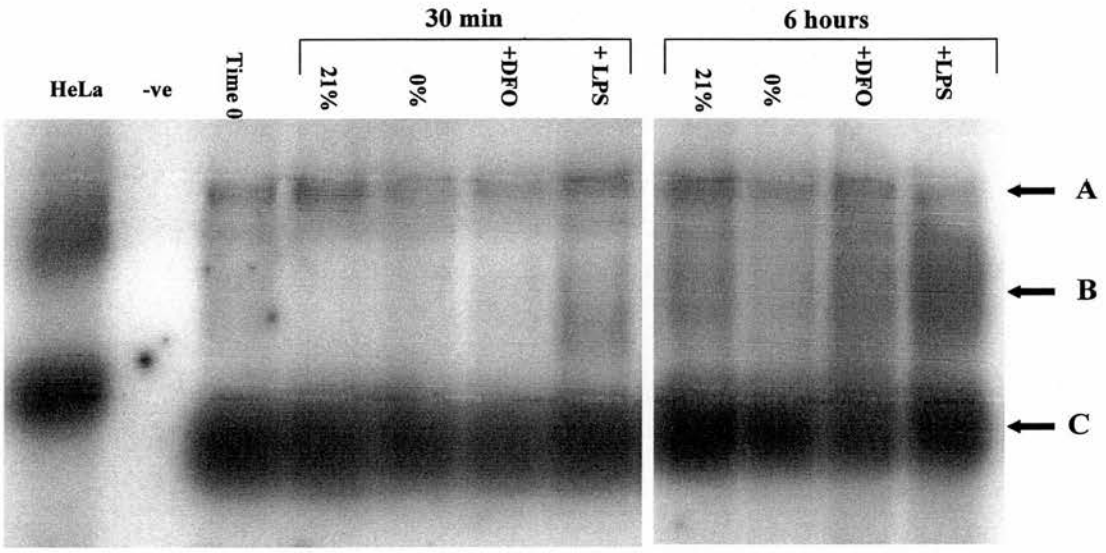


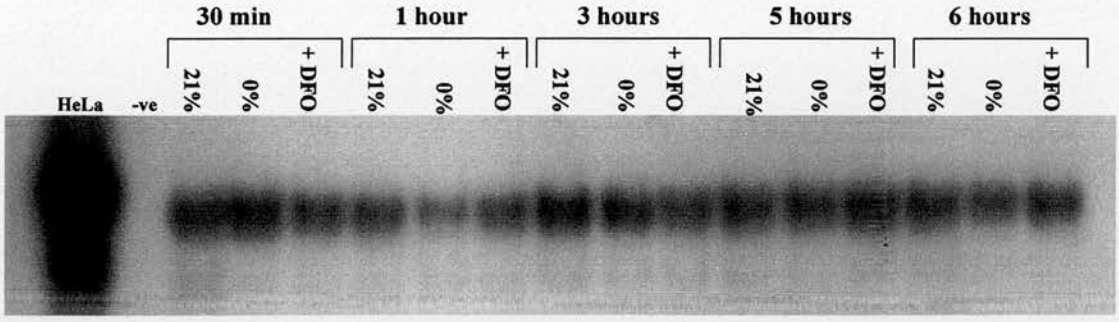
Figure 5.2.4B Upregulation of inducible NF-κB binding by LPS in human neutrophils. Electrophoretic mobility-shift assay using nuclear protein extracts from neutrophils exposed to either, normoxia (21% oxygen), hypoxia (0% oxygen) or incubated in the presence of desferrioxamine (DFO, 1 mM) or lipopolysaccharide (LPS, 1 μg/ml) for the stated times. A 5' end-labelled oligonucleotide containing an NF-κB DNA-binding site was used as the probe. The position of the three DNA-binding complexes A, B and C are indicated. HeLa cell nuclear extract was used as a positive control; there was no shift in probe mobility in the absence of nuclear protein extract (lane 2; -ve). This gel is representative of n = 2 separate experiments.

band B. However, in certain experiments hypoxia caused an increase in the density of band C, which was most evident at earlier time points. This effect appeared to be donor specific as it was not observed in all experiments. To ensure the lack of upregulation of band B was not due to experimental technique, identical experiments were performed including a fourth condition in which neutrophils were co-incubated with LPS (1 μg/ml) for 30 min or 6 hours. Figure 5.2.4B shows a representative EMSA demonstrating an obvious upregulation of band B in the LPS treated cells, an effect which was not observed with hypoxia or DFO treatment.

5.2.5 AP-1 binding activity in nuclear protein extracts from human neutrophils

Activation of the transcription factor, AP-1, has also been demonstrated in response to hypoxia. In view of this, the effect of hypoxia on AP-1 binding was investigated in human neutrophils. Neutrophil nuclear proteins from cells treated as described in section 5.2.4 were co-incubated with ^{32}P-labelled double stranded oligonucleotide containing the AP-1 binding site and binding activity assessed by EMSA. Figure 5.2.5A shows a representative EMSA from one such experiment. AP-1 binding was present in all conditions at every time point tested; there was no difference in the intensity of the bands, as assessed by densitometry, with any of the treatments or between time points (figure 5.2.5B). AP-1 binding was also found in freshly isolated cells and could not be upregulated by using LPS (1 μg/ml) (data not shown). The binding activity was shown to be specific for the AP-1 site using an excess of unlabelled probe containing the AP-1 binding site but was not affected by inclusion of unlabelled probe containing the NF-κB site (figure 5.2.5C). These data suggest that constitutive AP-1 binding is found in human neutrophils and this is not modulated by hypoxia or desferrioxamine, at least at the time points investigated in these experiments.

A



B

	30 min	1 hour	3 hours	5 hours	6 hours
Normoxia	34.3 ± 8.5	30.1 ± 9.8	31.5 ± 7.6	31.7 ± 7.6	31.4 ± 6.2
Hypoxia	36.9 ± 10.4	28.7 ± 9.5	30.3 ± 6.3	36.1 ± 5.2	27.8 ± 4.8
+ DFO	29.5 ± 9.8	28.5 ± 9.5	24.8 ± 8.3	36 ± 7.3	26.5 ± 5.0

C

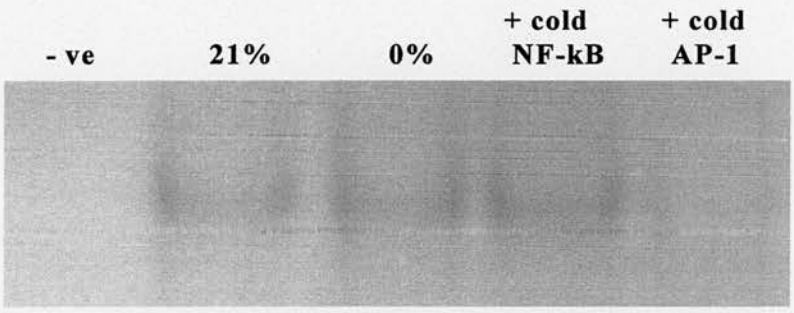


Figure 5.2.5 The effect of hypoxia and desferrioxamine on AP-1 binding in nuclear extracts from human neutrophils. A Electrophoretic mobility-shift assay using nuclear protein extracts from neutrophils exposed to either normoxia (21% oxygen), hypoxia (0% oxygen) or incubated in the presence of 1 mM desferrioxamine (DFO) for the stated times. A 5' end-labelled oligonucleotide containing an AP-1 DNA-binding site was used as the probe. HeLa cell nuclear extract was used as a positive control; there was no shift in probe mobility in the absence of nuclear protein extract (-ve). This gel is representative of n = 6 separate experiments. **B** AP-1 band density was also analysed using densitometry and mean values from 6 experiments were obtained for all conditions. No statistical difference was found between conditions ($p < 0.05$). Data are expressed as mean ± SEM. **C** Electrophoretic mobility shift assay using neutrophil nuclear extracts. AP-1 binding is evident in nuclear extracts from cells incubated in both 21% and 0% oxygen while there was no shift in probe mobility in the absence of nuclear protein extract (-ve). The addition of excess cold NF-κB probe to the binding reaction had no effect on binding to the AP-1 probe, however, addition of cold AP-1 probe displaced binding to the labelled probe suggesting that bands represent specific AP-1 binding.

5.3 Discussion

In the above experiments we have sought to characterise the oxygen-sensing mechanism underlying the hypoxic-mediated survival of neutrophils and have examined the potential involvement of several redox-sensitive transcription factors in this phenomenon.

The molecular mechanism underlying oxygen sensing in mammalian cells has been investigated primarily in two systems: erythropoietin (EPO)-producing hepatoma-derived cell lines (Goldberg et al., 1988; Porter and Goldberg, 1993) and type 1 carotid body cells (Acker, 1994; Acker and Xue, 1995). We noted that the upregulation of EPO mRNA in response to hypoxic stimulation and the hypoxic inhibition of neutrophil apoptosis share similar characteristics, namely that both events are not mimicked by mitochondrial inhibitors, glucose deprivation or heat shock and both are sensitive to the addition of cycloheximide. In view of this, the possibility that the effect of hypoxia on these physiological processes shares a common mechanism was investigated. Early experiments with intact animals (Goldwasser et al., 1958) as well as perfused kidneys (Fisher and Langston, 1968) demonstrated that the production of EPO could also be stimulated by cobaltous chloride treatment. Moreover, when human hepatoma cells (Hep3B or HepG2) were incubated for 24 hours in the presence of $CoCl_2$, there was a concentration-dependent enhancement of EPO mRNA expression (Fandrey and Bunn, 1993). Manganese and nickel have also been shown to cause a concentration-dependent increase in EPO production, albeit at higher ion concentrations than cobalt, although other divalent cations (zinc, iron and cadium) were not able to stimulate EPO-production (Goldberg et al., 1988). In addition to these metal ions, iron chelators have been demonstrated as potent stimulators of EPO mRNA production (Wang and Semenza, 1993a). Thus, as a preliminary experiment to test the hypothesis that a similar mechanism may be involved in the inhibition of neutrophil apoptosis by hypoxia, neutrophils were incubated with $CoCl_2$ and an iron chelator, DFO. Surprisingly, $CoCl_2$ had no effect on neutrophil apoptosis, however, DFO promoted neutrophil survival in a concentration-dependent manner. The same effect was obtained with CP94, which is a member of the hydroxypyridinone group of iron chelators and, although their

primary action is also the chelation of iron, these compounds are structurally quite different from DFO (Dobbin et al., 1993). At higher concentrations these compounds inhibited apoptosis to a similar extent to hypoxia. Depending on iron availability, these chelating agents may act to chelate extracellular iron and transfer it into cells or they may enter the cell and bind iron intracellularly. However, as reported for EPO (Wang and Semenza, 1993a), the addition of a molar excess of ferrous chloride to the medium prevented the action of both iron chelators, suggesting that they are operating by the latter mechanism. Both of the iron chelators used show a very strong preference for iron over other metal ions (Porter et al., 1989; Hider et al., 1994), and the common action of the different compounds thus suggests that their ability to inhibit neutrophil apoptosis is most probably achieved by the chelation of iron.

One putative explanation for the ability of iron chelators to mimic the protective effect of hypoxia on neutrophil apoptosis is that this effect may involve a ferro-protein oxygen sensor, which is active only in its deoxy-form. In this model iron chelators would exert their effect by locking this sensor in its deoxy, active state. Haem iron is not chelatable (Rounalt et al., 1985), thus eliminating haem containing proteins as possible oxygen-sensing candidates, however, examples exist among non-haem ferroprotein sensors of labile iron atoms being susceptible to chelation (Klausner et al., 1993; Spiro et al., 1989).

A second possibility is that the effect of iron chelators on the free iron pool could effect the generation of ROS. Iron acts as the catalyst in the Haberweiss reaction:

$$O_2^{\bullet} + H_2O_2 \rightarrow O_2 + OH^{-} + HO^{\bullet}$$

Thus chelation of iron would block the catalysis of H_2O_2 resulting in reduced hydroxyl-radical concentrations. Changes in ROS levels could be involved in signalling or have a direct effect on apoptosis, however, our data (Chapter 4) showing that the antioxidants, superoxide dismutase and methionine, failed to mimic the effect of hypoxia on neutrophil apoptosis argues against this as a putative mechanism.

We also cannot discount a potential effect of iron chelators on non-haem iron containing enzymes. Two such enzymes, which have also been implicated in modulating apoptosis, are the lipoxygenases and ribonucleotide reductase (Tang et al., 1996; Anderson et al., 1994; Huang and Plunkett, 1995). Lipoxygenases catalyse the site-specific oxygenation of polyunsaturated fatty acids to produce hydroperoxides. In human neutrophils the principle lipoxygenase is 5-LPO (Ford-Hutchkinson et al., 1994), which catalyses the first two steps in the conversion of its substrate (arachidonic acid) to leukotriene A_4. Leukotriene A_4 is the precursor of the potent pro-inflammatory molecule leukotriene B_4. Indeed, hydroxypyridone iron chelators have been shown to inhibit leukotriene release from neutrophils by this mechanism (Abeysinghe et al., 1996). A second iron chelator sensitive enzyme is human ribonucleotide reductase. This enzyme catalyses the conversion of ribonucleotides to deoxyribonucleotides and, hence, iron chelators are inhibitors of DNA synthesis (Alcain et al., 1997). However, it seems unlikely that the iron chelators could be inhibiting neutrophil apoptosis via an effect on these enzymes. LTB_4 has been shown to inhibit neutrophil apoptosis in a concentration-dependent manner (Hebert et al., 1996), thus inhibiting 5-LPO might be expected to have a pro-apoptotic effect but it is difficult to explain an anti-apoptotic effect using this mechanism. In addition, DFO is too large and hydrophilic to access the active site of 5-LPO and thus does not inhibit this enzyme (Cooper and Porter, 1997). Both the hydroxypyridones and DFO have been shown to inhibit ribonucleotide reductase in neutrophils (Cooper and Porter, 1997), however, inhibitors of this enzyme have been shown to have anti-proliferation effects in dividing cells and in other cells appear to induce rather than inhibit apoptosis (Wright et al., 1996; Cory et al., 1985).

The ability of iron chelators to promote neutrophil survival to a similar extent to hypoxia is consistent with the hypothesis that a similar oxygen-sensing mechanism to that mediating the hypoxic regulation of EPO is involved. However, the lack of effect of $CoCl_2$ on the rate of neutrophil apoptosis led us to question this theory. Various models of oxygen sensing have been proposed to accommodate the effects of metal ions and chelators on EPO production. Goldberg et al. (1988) hypothesised that the oxygen sensor in this system is a haem protein and that cobalt substitutes for

the iron atom in the haem moiety. Because these metals have a low affinity for oxygen, they would lock the sensor in the deoxy conformation. They provided experimental evidence for this proposal by showing that induction of EPO by hypoxia could be blocked by carbon monoxide (CO) and by haem-synthesis inhibitors. In contrast to its effects in hypoxic cells, CO did not inhibit the induction of EPO by cobalt. This result is fully consistent with the inability of cobalt substituted haem to bind CO. If this proposal is correct, and operates in a similar way in other cell types, it is difficult to explain the lack of effect of cobalt on neutrophil apoptosis in any other way than by proposing a different oxygen sensing mechanism to the one utilised by EPO. However, to date, no such ferro-protein oxygen sensing molecule has been identified in mammalian cells. Furthermore the role of iron chelators as potent stimulators of EPO production is difficult to explain using this model. Similarly, the inhibitory effect of haem synthesis inhibitors and CO have not been found in all cells (Eckardt et al., 1993; Graven et al., 1998). The inconsistencies in this proposal has led to additional suggestions. Other models of oxygen sensing have been based on the role of oxygen as an electron acceptor in a variety of non-mitochondrial redox systems involving electron transport. For instance, the involvement of a hypoxia-sensitive, but cyanide-insensitive, cytochrome b in an electron transport chain similar to that involved in the NADPH oxidase respiratory burst has been proposed (Gorlach et al., 1993; Gorlach et al., 1994). In this system O_2 has been proposed to be reduced to ROS such as superoxide anions, H_2O_2 and hydroxyl radicals and that one or more of these molecules may be responsible for hypoxia-induced signal transduction (Ehleben et al., 1997; Fandrey et al., 1997; Ratcliffe et al., 1997). However, it is notable that the cloned neutrophil NADPH oxidase has been eliminated as an essential component of the hypoxia signal transduction pathway (Wenger et al., 1996b). Indeed, iron chelators have also been shown not to effect the respiratory burst in neutrophils (Abeysinghe et al., 1996). Involvement of an NADPH-oxidase like molecule was supported by the observation that diphenylene iodium (DPI), which inhibits NADPH oxidase by reacting with its flavin cofactor, blocked the induction of EPO expression in hypoxic cells (Gleadle et al., 1995b). A second important observation in this study was that, while DPI inhibited hypoxic induction of EPO, it was not able to block cobalt or

desferrioxamine stimulated induction, giving the first indication that these stimuli work via different mechanisms or at different points in a common pathway. In addition, whereas exposure of HEPG2 cells to either hypoxia or $CoCl_2$ induced absorption-spectrophotometric changes that suggested effects on an NADPH oxidase-like cytochrome b_{558}, DFO had no such effect, implying a different mode of action (Ehleben et al., 1997). These experimental findings suggest that the three known inducers of EPO expression each act via a different molecular pathway. Thus, it appears plausible that the inability of cobalt to inhibit neutrophil apoptosis does not rule out a role for a similar oxygen-sensing mechanism to that involved in the hypoxic regulation of EPO.

Although the precise mechanism underlying oxygen 'sensing' remains unclear, studies on the transcriptional control of EPO have been more fruitful. Cis-acting DNA sequences required for transcriptional activation in response to hypoxia were identified in the EPO 3' flanking region and a trans-acting factor that binds to the enhancer, hypoxia-inducible factor-1 (HIF-1), fulfilled criteria for a physiological regulator of EPO transcription (Semenza, 1994). EPO expression is cell type specific but induction of HIF-1 activity by hypoxia, $CoCl_2$ and DFO has been detected in many mammalian cell lines and the EPO enhancer is able to direct hypoxia-inducible transcription of reporter genes transfected into non-EPO-producing cells (Wang and Semenza, 1993b; Maxwell et al., 1993). More recently, HIF-1 has also been shown to be involved in the hypoxic regulation of several other genes, including glycolytic enzymes, certain growth factors and haem oxygenase 1 (for review see Wood and Ratcliffe, 1997). Thus, HIF-1 appears to play a very general role in mediating transcriptional responses to hypoxia. As noted, HIF-1 has been shown to be a heterodimer consisting of the HIF-1α and HIF-1β subunits (Wang and Semeza, 1995) and both subunits are basic-helix-loop-helix proteins containing a PAS domain (bHLH-PAS) (Wang et al., 1995). HIF-1β was found to be identical to ARNT, while HIF-1α represented a novel bHLH-PAS protein (Wang et al., 1995). mRNAs for HIF-1α and HIF-1β have been found in all cells and tissues examined to date (Wenger et al., 1996a; Wiener et al., 1996) and, in keeping with this, we found HIF-1α mRNA to be expressed in both human neutrophils and

monocytes. We also examined neutrophils for endothelial PAS domain protein 1 (EPAS1) mRNA expression. EPAS1 is a recently discovered member of the bHLH-PAS protein family. It shares a 48% sequence homology with HIF-1α and, like HIF-1α, can form heterodimers with ARNT and activate transcription from a HIF-1 binding site (Tian et al., 1997). EPAS1 mRNA is also widely expressed in many tissue types, although protein expression is mainly restricted to endothelial cells. However, we were unable to detect EPAS1 mRNA in neutrophils. These results are consistent with those of Tian and co-workers (1997) who found HIF-1α but not EPAS1 mRNA expressed in leukocytes.

HIF-1 activity is largely controlled by HIF-1α protein levels and we found that HIF-1α protein levels were upregulated in neutrophils upon stimulation with hypoxia and DFO suggesting (but not proving) that HIF-1 activity is also increased under these conditions. However, we were unable to show this experimentally. When a DNA-binding assay was performed using nuclear extracts from both normoxic and hypoxic neutrophils an unusual binding pattern was observed, with no visible inducible band, which bore little relation to the 'standard' HIF-1 binding pattern seen with HeLa cell nuclear extracts. The reason for this is unclear and further work is required to elucidate whether this represents an experimental artefact or a true representation of HIF-1 binding in neutrophils.

Recent experimental evidence has implicated a role for HIF-1 in hypoxia-induced apoptosis (Carmeliet et al., 1998). Hypoxia was able to induce apoptosis in wild-type (HIF-1$\alpha^{+/+}$) C4.5 cells but not in HIF-1α-deficient Ka13 Chinese hamster ovary (CHO) cells. Furthermore, a HIF-1α dependent induction of p53 and p21 and suppression of Bcl-2 was observed. It will be of considerable interest to investigate a possible association between the induction of HIF-1α by hypoxia in neutrophils and the inhibition of neutrophil apoptosis. However, at present these studies are hampered by the lack of a cell permeable and specific HIF-1 inhibitor and the inability to readily transfect neutrophils.

A second transcription factor, which, like HIF-1, has been proposed to play a role in the control of apoptosis is NF-κB. NF-κB consists of homo- or heterodimers of the Rel protein family, p50/NF-κB1, p52/NF-κB2, p65/RelA, RelB and c-Rel. Evidence suggesting that NF-κB may act to protect cells against apoptosis came from studies in which cell lines previously resistant to pro-apoptotic stimuli (TNFα, ionising radiation and chemotherapeutic drugs) became very sensitive to these agents when lacking p65 or when expressing a dominant negative form of IκBα (Beg and Baltimore, 1996; Wang et al 1996). Furthermore, experiments using gliotoxin, a potent inhibitor of NK-κB, and DNA-binding assays have implicated this transcription factor in mediating the late (>12 hrs) anti-apoptotic effect of TNFα in neutrophils (Ward et al., 1999). Unlike HIF-1, NF-κB activity is upregulated by a large range of physiological stimuli, including cytokines (TNFα and IL-1), LPS, phorbol esters, mitomycin C, calcium ionophore, lectins, ultraviolet light and ROS (Anderson et al., 1994; Schreck et al., 1991). Koong and co-workers (1994) have also proposed that hypoxia can cause NF-κB activation by a mechanism involving tyrosine phosphorylation of IκBα. We thus investigated the response of NF-κB to hypoxia in human neutrophils. As detailed above, nuclear extracts from neutrophils were found to contain 3 separate DNA-binding activities. The slowly migrating complex A is unlikely to represent specific NF-κB binding as it was not sensitive to the addition of excess unlabelled probe. However, the two other complexes (B and C) were judged to represent specific binding activities. The slower-migrating complex (B) has been designated as an inducible form of NF-κB as it is only very weakly expressed in control cells and is upregulated by known NF-κB inducing agents, while complex C appears to represent a constitutively expressed form of this transcription factor. We found no upregulation of complex B in response to either hypoxia or DFO although, as previously reported, LPS was able to induce activation (McDonald et al., 1997). The inability of DFO to effect inducible NF-κB binding is consistent with reports that iron chelators are inhibitors of NF-κB activation (Schreck et al., 1992). In contrast, we found a donor specific enhancement of the binding activity complex C by hypoxia. Due to the inability of DFO to produce this effect, and the inconsistency of these results when using neutrophils from different donors,

it appears unlikely that this NF-κB binding complex is involved in the anti-apoptotic effect of hypoxia although further studies will be required to prove this conclusively.

There is still some debate concerning the ability of hypoxia to activate NF-κB. Although degradation of IκBα and transcriptional activation of reporter constructs containing κB-binding sites has been reported in Jurkat T-cells following hypoxia (Koong et al., 1994), other studies using HeLa cells suggest that NF-κB is only upregulated following reoxygenation (Rupec and Baeuerle, 1995). The latter finding is more in keeping with the large body of evidence indicating that NF-κB is activated under pro-oxidant conditions and that antioxidants inhibit NF-κB (for review see Piette et al., 1997). Indeed, rapid activation of NF-κB during reoxygenation may explain the above neutrophil data: although we took care to harvest neutrophils into ice cold PBS immediately following removal from hypoxic conditions, activation of NF-κB by reoxygenation has been reported to be extremely rapid reaching maximal levels in less that 15 minutes.

A third transcription factor that is sensitive to intracellular redox levels is AP-1. Like the previously mentioned transcription factors, AP-1 is a dimeric protein. Its subunits consist of the products of the proto-oncogenes c-jun and c-fos and their related family members. Although AP-1 activity is largely induced at a transcriptional level by *de novo* synthesis of these subunits, c-Jun stability can also be enhanced by phosphorylation of serine and threonine residues by MAPK. In addition, phosphorylation can also increase AP-1 binding activity. Initial observations that AP-1 can be stimulated by anti-oxidants and inhibited by oxidants, suggested that AP-1 can be regulated by the redox state of the cell. Site-directed mutagenesis identified a conserved cysteine residue in the basic DNA-binding motif of both Fos and Jun; under non-reducing conditions an inter-molecular disulphide bridge is formed between the cysteine residues of each basic motif within a dimer, which prevents DNA binding (Bannister et al., 1991). It has been demonstrated that the reduction of AP-1 under physiological conditions is mediated by a ubiquitous nuclear protein called Ref-1 rather than by low-molecular weight redox compounds (Xanthoudakis and Curran, 1992). Ref-1 is itself subject to redox control. Its

activity can be augmented by thioredoxin, suggesting that AP-1 may be regulated by a redox cascade (Abate et al., 1990). In view of these findings, it is perhaps not surprising that hypoxia has also been reported to induce AP-1 binding (Bandyopadhyay et al., 1995; Rupec and Baeuerle, 1995).

Contrary to these data, we were unable to detect an enhancement of AP-1 binding in response to hypoxia in human neutrophils. In HeLa cells, Rupec and co-workers (1995) found that hypoxia induced a biphasic activation of AP-1 with upregulation first noticeable at 15 minutes and peaking at 40 minutes and a second increase in activity seen at 5 hours. Although we investigated similar time points and used a nearly identical level of hypoxia, there was no evidence that hypoxia influenced AP-1 binding in neutrophils. The ability of DFO to lower intracellular ROS levels is well documented and, thus, this agent may be expected to reduce the redox potential of cells, however, as with hypoxia, DFO did not affect AP-1 binding in neutrophils.

Hypoxic induction of AP-1 appears to be cell type-dependent to the extent that, while AP-1 binding was increased 4-6 fold over basal levels in HeLa cells, it was elevated much more modestly in endothelial and Hep-3B cells (Bandyopadhyay et al., 1995). While activation of AP-1 binding in response to LPS has been reported in neutrophils (Sugita et al., 1998), we were unable to mimic this effect in our system. However, the AP-1 response to LPS appears to be very concentration-sensitive and it is possible that the single concentration of LPS (1 μg/ml) used was too high. An additional difference is that Sugita and co-workers incubated cells in the absence of serum, while our system includes 10% autologous serum. Hence, we cannot exclude the possibility that serum is responsible for the high basal levels of AP-1 binding observed in our experiments and that this precluded us observing any further stimulation with LPS or, indeed, hypoxia. We also found this constitutive AP-1 binding in freshly isolated (t_0) cells but, as neutrophils come into contact with platelet poor plasma during several steps in the isolation process, it is again feasible that AP-1 binding activity is induced during cell preparation. While it would be of interest to repeat these experiments in the absence of serum, the inability of both hypoxia and desferrioxamine to modulate AP-1 binding under culture conditions in

which these agents are able to profoundly inhibit neutrophil apoptosis suggests that AP-1 binding is not a key step in this process.
It is also notable that, although AP-1 binding has been reported following hypoxia, this effect has not always been sufficient to activate AP-1 inducible genes, suggesting that some other factor is also required (Bandyopadhyay et al., 1995). Indeed transcriptional activation of vascular-endothelial growth factor (VEGF) by hypoxia in C6 glioma cells requires binding of both AP-1 and HIF-1 (Damert et al., 1997). Hence, AP-1 activation may play an important 'permissive' role in allowing HIF-1 mediated gene regulation.

Several reports have implicated a role for certain AP-1 subunits in apoptosis. For example, an increase in c-jun or c-fos expression was noted in cells that were induced to undergo apoptosis following exposure to various stresses (Colotta et al., 1992b) and it has been proposed that c-Jun is necessary for both growth factor withdrawal induced apoptosis in cultured neurons (Ham et al., 1995) and ceramide-induced apoptosis in human tumour cell lines (Verheij et al., 1996). However, again these observations implicate a pro-apoptotic role for AP-1. Furthermore, although AP-1 may be involved in apoptosis induced by specific stimuli in certain cell types, there is now substantial evidence against a universal role for AP-1 in apoptosis. c-fos $^{-/-}$ primary cells isolated from c-fos null mice are able to undergo normal apoptosis (Gajate et al., 1996) and neither c-Fos or c-Jun are essential for normal *in vivo* development (Roffler-Tarlov et al., 1996).

To conclude, these data suggest that the oxygen-sensor involved in the inhibition of neutrophil apoptosis by hypoxia may be a ferro-protein and that the oxygen-sensing mechanism is similar to that involved in the hypoxic-induction of EPO mRNA in other cell types. Consistent with this proposal, we have demonstrated that neutrophils are capable of upregulating HIF-1α protein in response to the anti-apoptotic stimuli, hypoxia and DFO. Thus, it is possible that HIF-1 may be involved in the upregulation of gene(s) which serve an anti-apoptotic function in neutrophils. However, further work will be required to substantiate this hypothesis. Our data also

suggest that the transcription factors AP-1 and NF-κB do not play an important role in the inhibition of neutrophil apoptosis by hypoxia.

CHAPTER 6

THE ROLE OF OXIDATIVE PATHWAYS IN TUMOUR NECROSIS FACTOR-α-STIMULATED APOPTOSIS IN HUMAN NEUTROPHILS

6.1 INTRODUCTION

Tumour necrosis factor-α (TNFα) is a cytokine that was originally described as a peptide capable of inducing necrosis of tumours in experimental animal models (Carswell et al., 1975). Consistent with this finding, TNFα has also been shown to be cytotoxic to certain cell types *in vitro* (Niitsu et al., 1988; Watanabe et al., 1988; Helson et al., 1975) and, depending on the cell type, can induce either necrosis or apoptosis. The role of TNFα as a powerful priming agent in neutrophils is also well documented (Klebanoff et al., 1986; Shalaby et al., 1985; Atkinson et al., 1988), and like other pro-inflammatory agents, TNFα was initially reported to have the capacity to delay neutrophil apoptosis (Colotta et al., 1992a). However, recent reports have demonstrated that TNFα induces apoptosis in neutrophils at early time points (2-12 hours) in culture (Takeda et al., 1993; Murray et al., 1996), a finding which sets TNFα apart from other neutrophil priming agents, such as LPS, G-CSF and GM-CSF.

Two distinct receptor subtypes for TNFα, with molecular masses of 55 kD (TNFRI/TNFR55) and 75 kD (TNFRII/TNFR75), have now been identified (Hohmann et al., 1990; Brockhaus et al., 1990) and human and mouse cDNAs corresponding to both receptor subtypes have been isolated and characterised (Loetscher et al., 1990; Schall et al., 1990; Smith et al., 1990; Lewis et al., 1991; Goodwin et al., 1991). These receptors are members of a superfamily of receptors referred to as the NGF/TNF receptor family. This family is characterised by functional trimerization and the presence of one to six cysteine-rich repeats of approximately 40 amino acids in the extracellular domain, which provide the ligand binding motif. The extracellular domains of the two human TNFRs are 28% identical but, of note, have no more homology to each other than to other members of the NGF/TNF receptor superfamily. Likewise, there is no significant homology

between the intracellular domains of the two TNFRs, indicating that these receptors almost certainly serve discrete functions and employ different mechanisms of action (Lewis et al., 1991).

TNFR55 contains a classical intracellular death-domain sequence (Tartaglia et al., 1993), and has been reported to be the sole mediator of the TNFα-triggered death signal in most non-haematopoietic cell types. Despite this, the TNFR75, which lacks such a death domain sequence, has been proposed to play a key role in mediating TNFα-induced cytotoxicity in murine $CD8^+$ peripheral T-cells (Zheng et al., 1995), and recently a requirement for both TNFRs in TNFα-mediated induction of neutrophil apoptosis has been reported (Murray et al., 1997).

Despite the characterisation of the TNFRs, the intracellular mechanisms regulating TNFα cytotoxicity are not fully understood. Studies demonstrating that TNFα results in a rapid rise in the levels of intracellular reactive oxygen species (ROS) (Larrick et al., 1990; Matthews et al., 1987) led to proposals that TNFα may mediate its cytotoxic effects in certain cell types through a free-radical based mechanism. Consistent with this, in some model systems TNFα-mediated apoptosis can be inhibited by the anti-oxidants, thioredoxin (Matsuda et al., 1991) and N-acetylcysteine (Chang et al., 1992). The mitochondria, a prime source of ROS, have also been implicated in playing a role in TNFα induced cell killing. Alterations in mitochondrial ultrastructure have been observed following treatment with TNFα and TNFα cytotoxicity can be affected by treatment of cells with mitochondrial inhibitors (Schulze-Osthoff et al., 1992). Furthermore, it has been reported that cellular sensitivity or resistance to TNFα correlates with decreased or increased levels of mitochondrial superoxide dismutase respectively (Hirose et al., 1993).

The aim of the work presented in this chapter was to investigate the potential involvement, and role, of ROS in TNFα-induced apoptosis in human neutrophils. If ROS are involved in the pro-apoptotic process then TNFα-stimulated apoptosis ought to be reduced by (a) anaerobic conditions (if oxygen-based radicals are

involved) (b) free-radical scavengers and (c) inhibitors of pathways which generate free radicals. Thus, experiments were designed to investigate the effect of these conditions/agents on TNFα-induced neutrophil apoptosis.

6.2 Results

6.2.1 Characterisation of TNFα-stimulated apoptosis in neutrophils

Studies performed by Dr. J. Murray in the lab demonstrated that co-incubation of human neutrophils with TNFα caused a significant inhibition of neutrophil apoptosis when assessed after 20 hours in culture (figure 6.2.1A), an effect common to many other neutrophil priming agents. However, consistent with the observation that TNFα is cytotoxic to many other cell types, this cytokine was shown to cause a significant increase in morphological apoptosis at earlier time points (up to 12 hours), when the constitutive rate of apoptosis was still low (figure 6.2.1A). Figure 6.2.1B shows a more detailed time course of TNFα-induced apoptosis. The early pro-apoptotic effect of TNFα was rapid (observed in all experiments by 2 hr), time-dependent, and maximal in terms of the ratio of apoptotic to non-apoptotic cells at 6 hours. To explore the concentration-dependence of TNFα-stimulated apoptosis at 6 hours, neutrophils were incubated in the presence of increasing concentrations of TNFα (0-100 ng/ml). Figure 6.2.1C shows that the TNFα effect is nearly maximal by 10 ng/ml with an EC_{50} value of 2.8 ng/ml. In view of these data, in all subsequent experiments the effect of TNFα was examined after a 6 hour incubation period using a, just maximal, TNFα concentration of 25 ng/ml.

6.2.2 Effect of hypoxia on TNFα-stimulated apoptosis

In certain cell lines the cytotoxic effect of TNFα has been abrogated using antioxidants and mitochondrial inhibitors and this has led to the hypothesis that TNFα mediates its cytotoxic effect via an increase in intracellular ROS levels. To investigate whether this could be the case in neutrophils, we examined the effect of anoxia on the pro-apoptotic effect of TNFα in this cell type. Neutrophils were cultured in the presence or absence of TNFα under normoxic (21% oxygen) or 'anoxic' conditions using medium that had been fully deoxygenated before use. The pro-apoptotic effect of TNFα was greatly reduced under anoxic conditions (figure 6.2.2A), implicating an essential role for molecular oxygen in the cytocidal effect of TNFα. In a second set of experiments, neutrophils were cultured under anoxic conditions but using medium thar had not been deoxygenated prior to TNFα

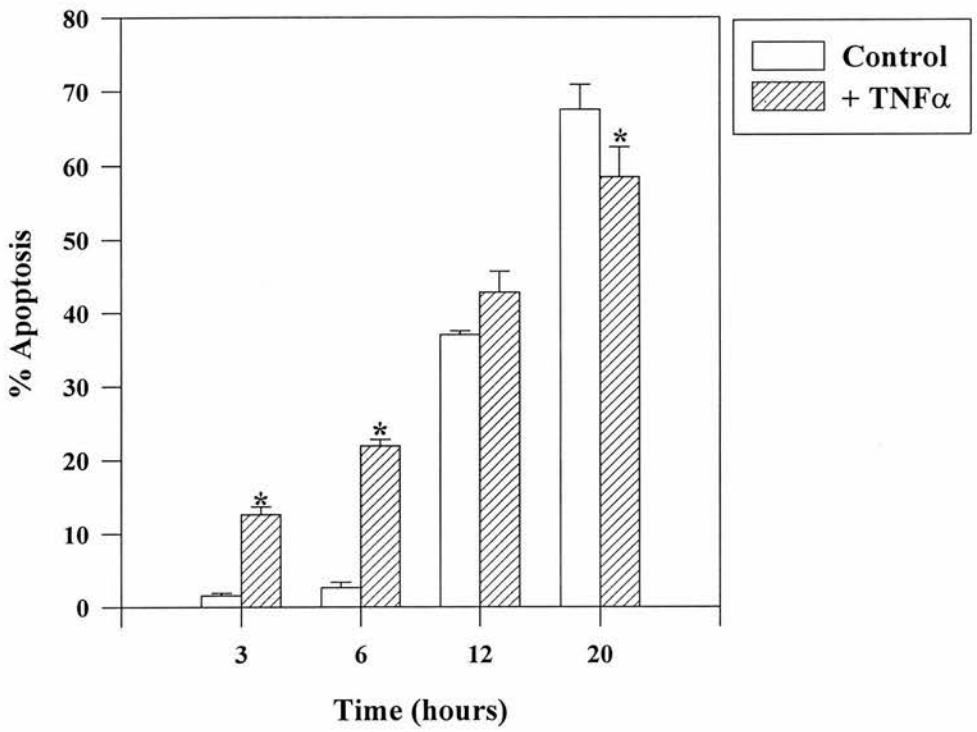


Figure 6.2.1A Time course for the effect of TNFα on apoptosis in neutrophils. Human neutrophils (5 x 10^6/ml) were incubated in Iscove's MDM supplemented with 10% autologous serum in the presence or absence of TNFα (25 ng/ml). At the time points indicated, the cells were harvested and assessed for the morphological features of apoptosis. Data represent mean ± SEM of 6 determinations from 2 independent experiments (*$p<0.05$ compared with control values) (experimental data provided by Dr. J Murray).

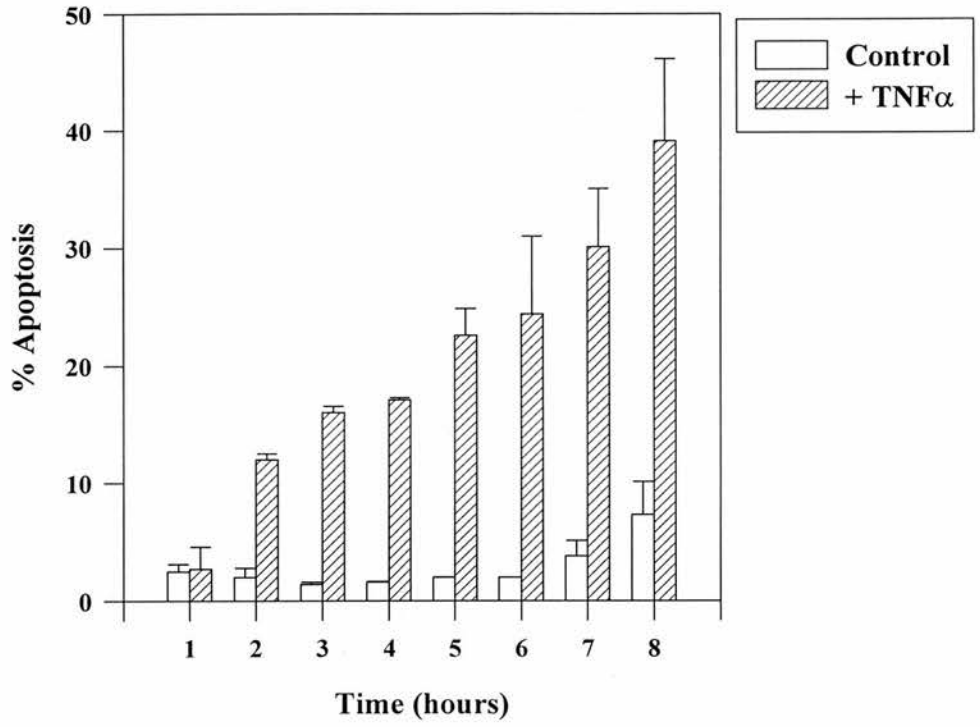


Figure 6.2.1B TNFα induces neutrophil apoptosis at early time points.
Human neutrophils (5 x 10^6/ml) were cultured in serum-supplemented Iscove's MDM in the absence or presence of 25 ng/ml TNFα. Neutrophils were harvested hourly for 8 hours and apoptosis assessed morphologically. Data represent mean ± SEM of 3 separate experiments, each performed in triplicate (experimental data provided by Dr. J. Murray).

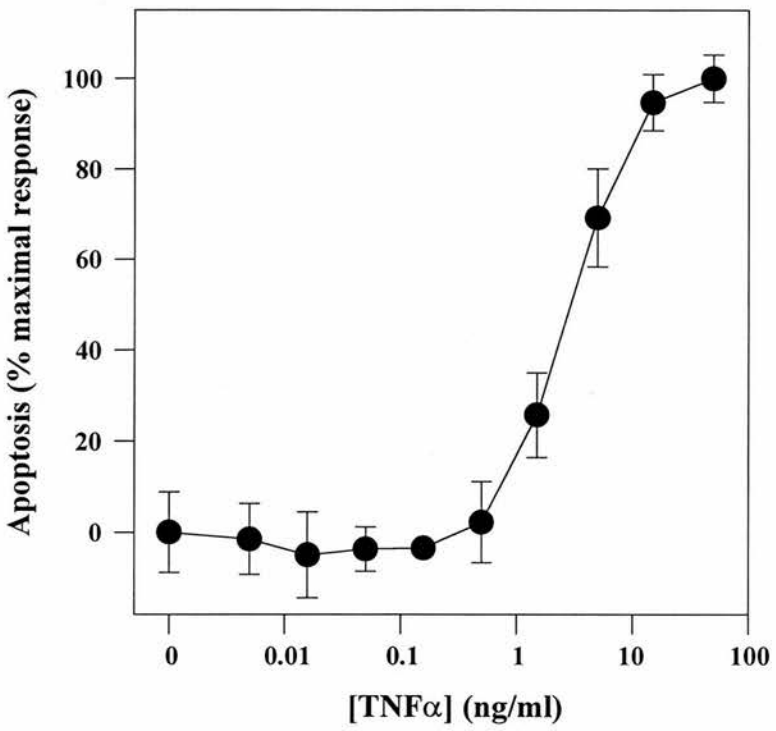


Figure 6.2.1C Concentration-response curve for TNFα-induced apoptosis.
Human neutrophils were incubated at 5 x 10^6/ml with increasing concentrations of TNFα (0-100 ng/ml). After 6 hours, neutrophils were harvested and apoptosis assessed morphologically. Data represent mean ± SEM from 3 separate experiments, each performed in triplicate (experimental data provided by Dr. J. Murray).

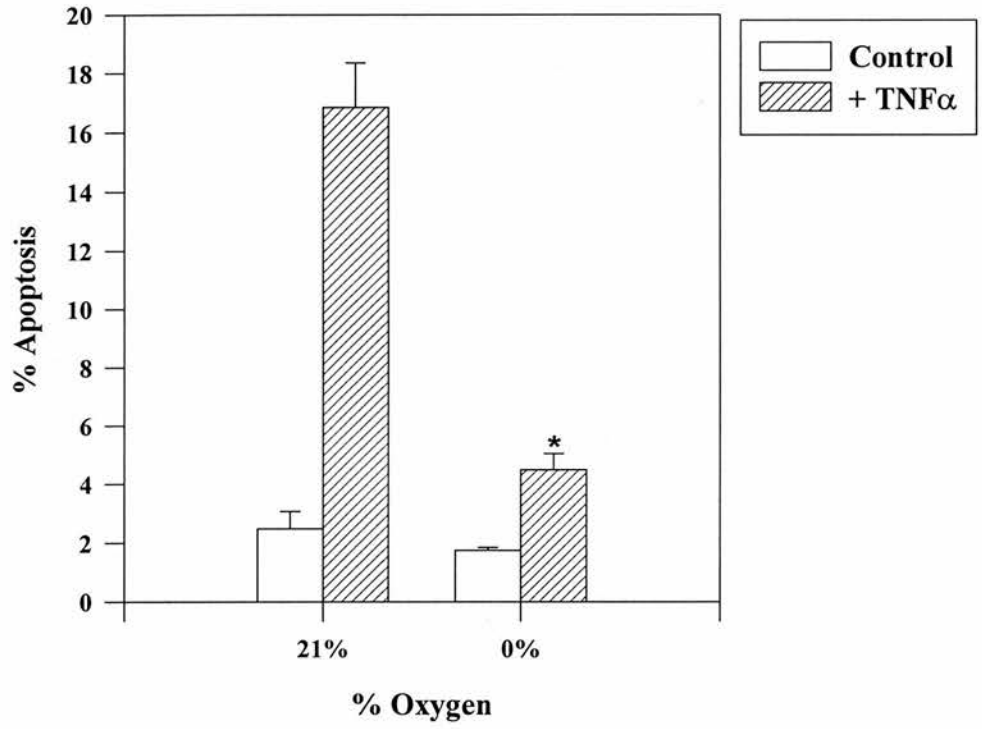


Figure 6.2.2A Hypoxia inhibits TNFα-stimulated apoptosis in neutrophils.
Neutrophils (5 x 10^6/ml) were resuspended in medium that had been incubated overnight in either 0% or 21% oxygen. Cells were then cultured in the presence or absence of TNFα (25 ng/ml). After 6 hours, neutrophils were harvested and apoptosis assessed morphologically. Data represent mean ± SEM from 5 separate experiments, each performed in triplicate (*$p<0.05$ compared with 21% oxygen + TNFα values).

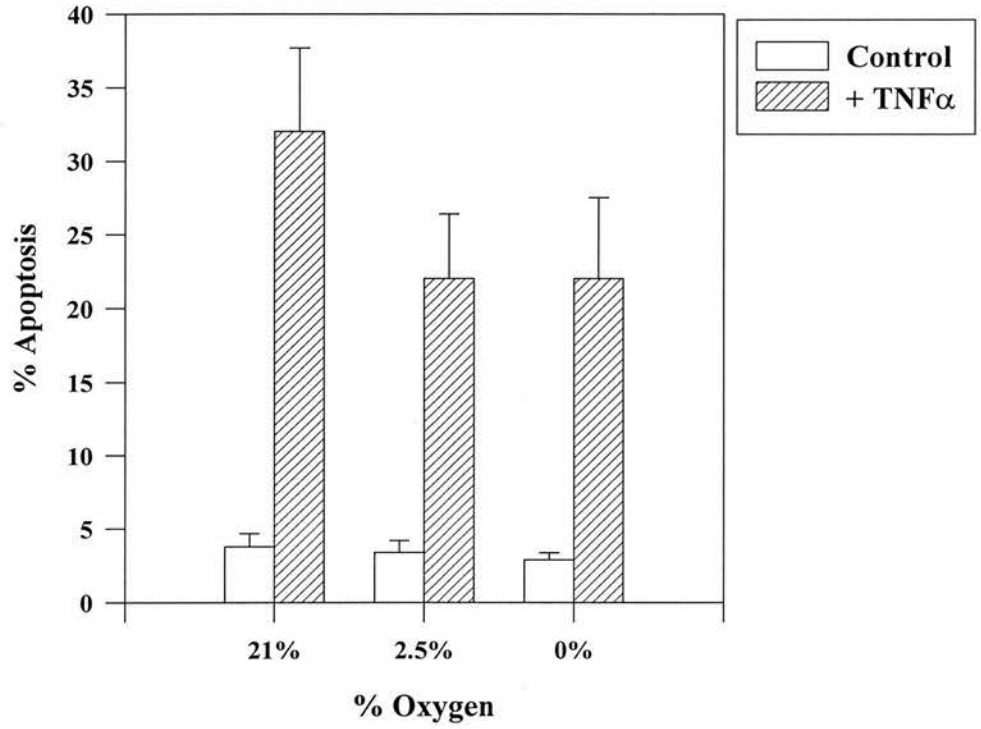


Figure 6.2.2B Requirement for culture medium to be fully deoxygenated in order to inhibit TNFα-stimulated apoptosis. Neutrophils (5 x 10^6/ml) were cultured in the presence or absence of TNFα in medium that had not been initially deoxygenated. Cells were then incubated in atmospheres containing 21, 2.5 or 0% oxygen for 6 hours. At this time, neutrophils were harvested and apoptosis assessed morphologically. Data represent mean ± SEM from 5 separate experiments, each performed in triplicate.

stimulation. By serial monitoring of the medium pO_2 we were able to demonstrate that, under these conditions, the medium did not fully equilibrate with the anoxic atmosphere until at least 30 minutes into the incubation period (data not shown). In these experiments TNFα-induced neutrophil apoptosis occurred at a rate not significantly different ($p < 0.05$) from that seen under normoxic conditions (figure 6.2.2B), indicating that the (oxygen-dependent) commitment of cells to undergo apoptosis in response to TNFα is likely to be extremely rapid, occurring within 30 minutes of exposure to this cytokine.

6.2.3 Effect of hypoxia on TNFα receptor expression

The pro-apoptotic effect of TNFα in neutrophils requires the presence of both the p50 and p75 TNFα receptor subtypes (Murray et al., 1997). To ensure that hypoxia was not inhibiting TNFα-induced apoptosis by influencing TNFR expression, we quantified, using flow cytometric analysis, TNFR55 and TNFR75 expression in neutrophils incubated in the presence and absence of TNRα under normoxic and 'anoxic' conditions (using medium which had been fully deoxygenated before addition of TNFα or normoxic, control, medium). These experiments (figure 6.2.3), although preliminary, demonstrated no major difference in the extent of TNFR55 and TNFR75 loss observed during a 30 min incubation in flexiwell plates between normoxic or anoxic medium. The addition of TNFα did not increase the extent of either TNFR55 or TNFR75 loss under these conditions, but again no differences were observed in TNFR expression between hypoxic and normoxic conditions.

6.2.4 Effect of antioxidants on TNFα-stimulated apoptosis

To further investigate the role of oxygen in the pro-apoptotic effect of TNFα, the effect of antioxidants on this phenomenon was examined. In view of the previous experiments showing that the first half hour of TNFα exposure is critical in determining the fate of the cells, neutrophils were pre-incubated with antioxidants for 30 minutes before being exposed to TNFα. The effects of a wide range of antioxidants were examined, namely: N-acetyl-L-cysteine (NAC, 5 and 10 mM); dimethylsulfoxide (DMSO, 400 mM); reduced glutathione (5mM); taurine (100

A

	Time 0	21% control	21% + TNF	0% control	0% + TNF
TNFR55	3.27 ± 0.01	1.69 ± 0.06	1.58 ± 0.13	2.04 ± 0.12	1.62 ± 0.15
TNFR75	3.42 ± 0.42	1.66 ± 0.02	1.67 ± 0.18	1.81 ± 0.09	1.72 ± 0.23

B

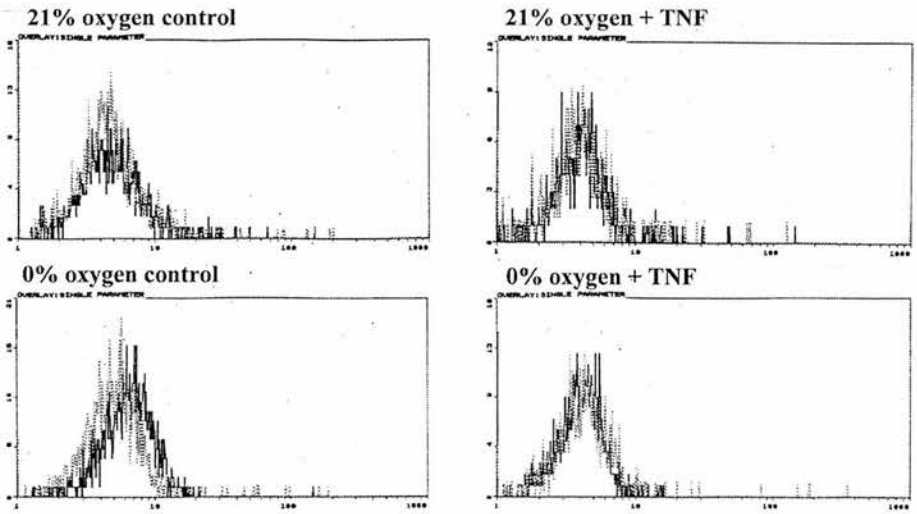


Figure 6.2.3 Effect of hypoxia on TNFR55 and TNFR75 expression in neutrophils. Human neutrophils (5 x 10^6/ml) were resuspended in either anoxic (0% oxygen) or normoxic (21% oxygen) medium prior to incubation in the presence or absence of TNFα for 30 minutes. At this time point neutrophils were harvested and incubated at 4°C for 30 min with a saturating concentration of mouse anti-human TNFR55, TNFR75 or CD2 (negative control) mAbs and subsequently with FITC-conjugated goat anti-mouse immunoglobin (30 min). TNFR expression was then quantified using flow cytometry. TNFR expression was also assessed on freshly isolated cells. **(A)** Data are expressed as mean fluorescence relative to the negative control and is the mean ± SEM of 3 separate experiments. **(B)** Representative flow-cytometry (EPICS Profile II) histograms (cell count vs log fluorescence) showing the profile of treated neutrophils after labelling with TNFR55 antibody (black outline) and TNFR75 antibody (dashed outline).

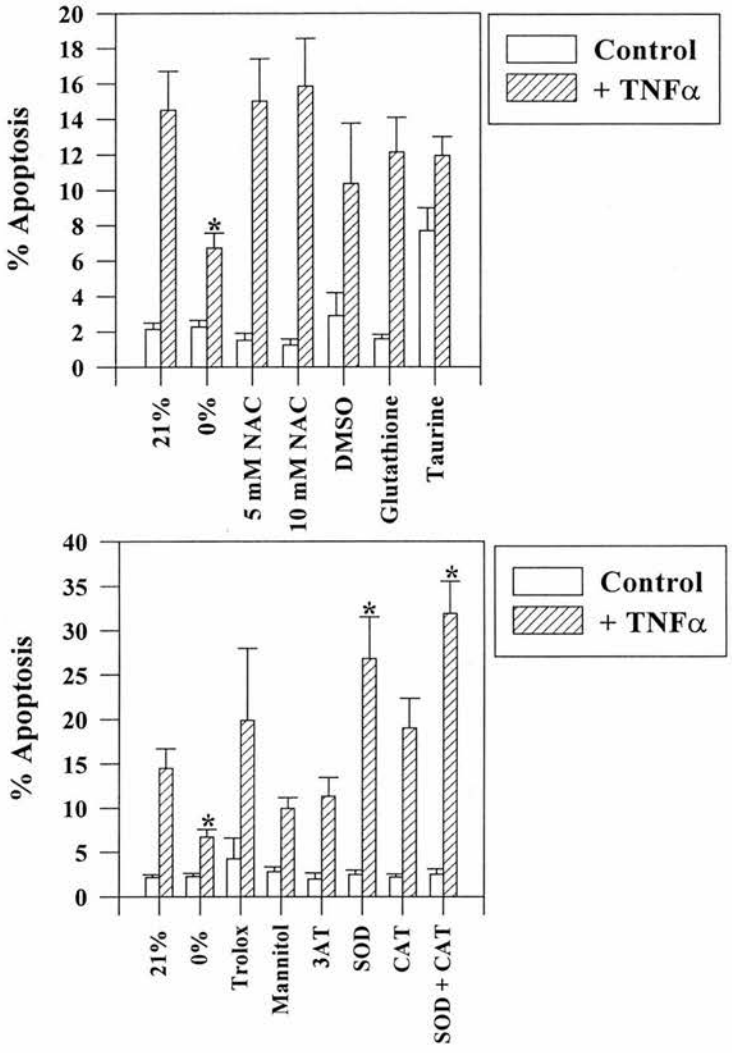


Figure 6.2.4 Antioxidants do not inhibit TNFα-stimulated apoptosis. Neutrophils (5 x 10^6/ml) were pre-incubated for 30 min in the presence of the antioxidants: N-acetyl-L-cysteine (NAC, 5 and 10 mM); dimethylsulfoxide (DMSO, 400 mM); reduced glutatione (5mM); taurine (100 mM); trolox (10 mM); mannitol (200 mM); 3-amino-1,2,4,-triazole (3AT, 12.5 mM); superoxide dismutase (SOD, 200 μg/ml); catalase (CAT, 250 μg/ml); and both superoxide dismutase (200 μg/ml) and catalase (250 μg/ml). While control cells were pre-incubated in culture medium alone. Treated cells were then cultured in the presence or absence of 25 ng/ml TNFα for 6 hours in 21% oxygen. Control cells were incubated in the presence or absence of TNFα under both normoxic (21%) and hypoxic (0%) conditions. Data represents mean ± SEM of 4 separate experiments each performed in triplicate (*$p<0.05$ compared with 21% + TNFα values).

mM); trolox (10 mM); mannitol (200 mM); superoxide dismutase (SOD, 200 μg/ml); catalase (CAT, 250 μg/ml); and both superoxide dismutase (200 μg/ml) and catalase (250 μg/ml). In addition, the effect of the catalase inhibitor, 3-amino-1,2,4,-triazole (3AT, 12.5 mM), was investigated.

No significant inhibitory effect on the induction of apoptosis by TNFα was observed with any of the agents tested (Figure 6.2.4). However, SOD both alone and in conjunction with catalase, significantly enhanced TNFα-induced apoptosis without affecting the constitutive rate of apoptosis.

6.2.5 Effect of hydrogen peroxide on TNFα-stimulated apoptosis

SOD catalyses the reaction: $2H^+ + 2\ O_2^- \rightarrow H_2O_2 + 2O_2$. Thus, to investigate the possibility that SOD was enhancing the pro-apoptotic effect of TNFα by increasing intracellular hydrogen peroxide levels, we examined the direct effect of exogenously applied H_2O_2 on neutrophil apoptosis. Neutrophils were co-incubated with TNFα and increasing concentrations of H_2O_2. An initial set of experiments using a wide range of H_2O_2 concentrations (0.05 – 10 mM) suggested that a concentration of H_2O_2 that might enhance the levels of TNFα-stimulated apoptosis, without affecting basal apoptosis lay between 1 – 5 mM (figure 6.2.5A). Thus, a second set of experiments examined the effect of 1 – 5 mM H_2O_2 on TNFα stimulated apoptosis (Figure 6.2.5B). Only 5 mM H_2O_2 significantly increased basal apoptosis, while concentrations of 3 mM H_2O_2 and above significantly enhanced TNFα-stimulated apoptosis. These data suggest that TNFα-induced apoptosis can be augmented by, and is more sensitive to, H_2O_2 than basal apoptosis.

6.2.6 Effect of mitochondrial inhibitors and iron chelators on TNFα-stimulated apoptosis

An important cellular source of ROS are the mitochondria and, in order to investigate the putative involvement of these organelles in TNFα-stimulated apoptosis, a range of mitochondrial inhibitors were utilised. Neutrophils were pre-incubated with the mitochondrial inhibitors: rotenone (0.1 μg/ml), a complex I inhibitor;

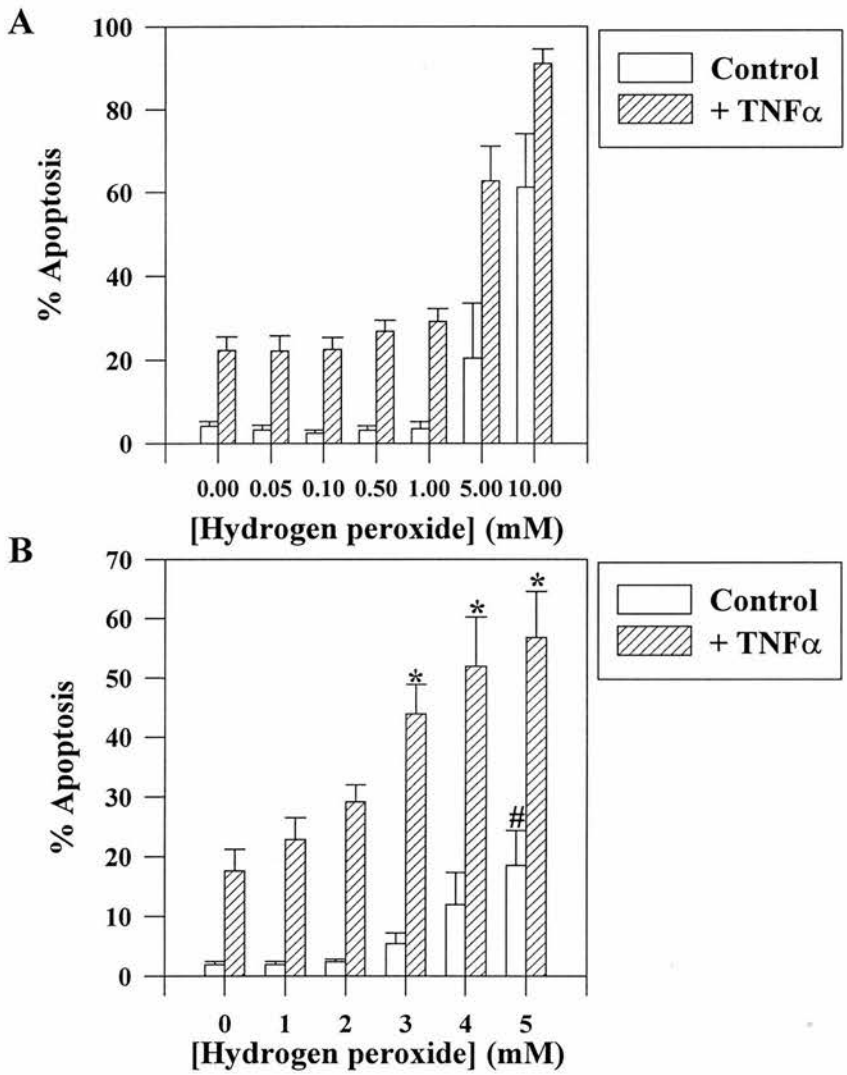


Figure 6.2.5 The effect of hydrogen peroxide on TNFα stimulated apoptosis. Neutrophils (5 x 10^6/ml) were incubated with a range of concentrations of hydrogen peroxide (0.05 – 10 mM) **(A)** or a narrow range between 1-5 mM **(B)** in the presence or absence of TNFα for 6 hours. At this time, neutrophils were harvested and apoptosis assessed morphologically. Data represents mean ± SEM from n = 4 separate experiments, each performed in triplicate (*$p<0.05$ compared with + TNFα values measured in the absence in the absence of H_2O_2 and # $p<0.05$ compared with control, - TNFα, values).

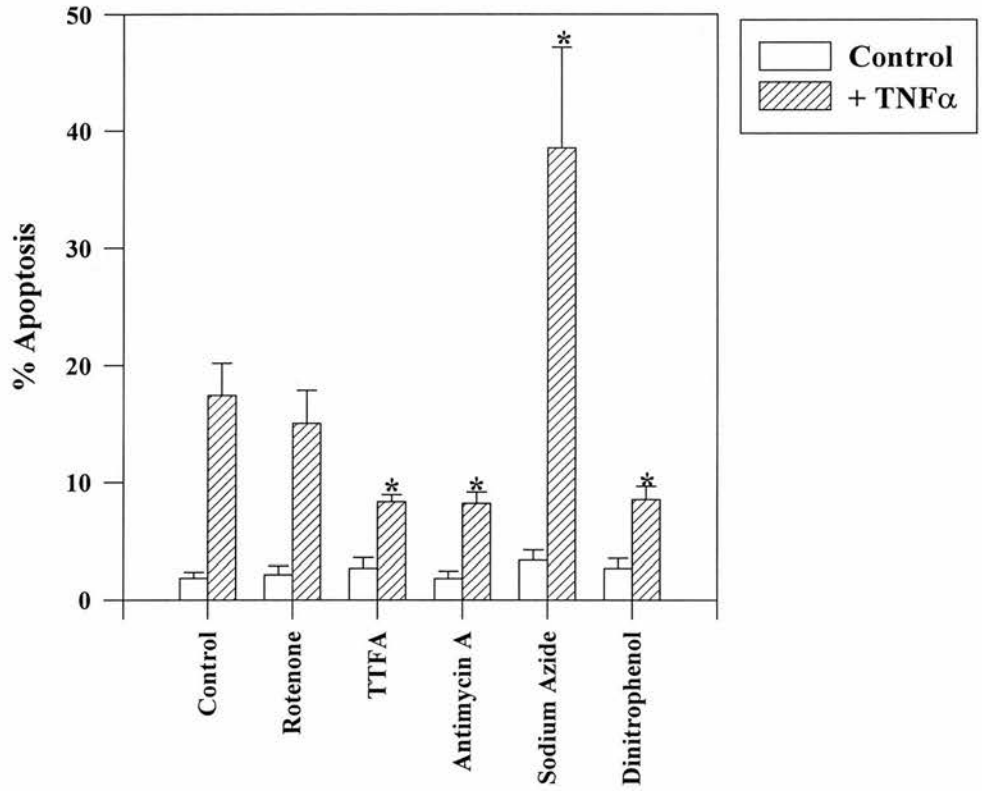


Figure 6.2.6A The effect of mitochondrial inhibitors on TNFα-stimulated apoptosis. Neutrophils (5 x 10^6/ml) were pre-incubated with the mitochondrial inhibitors: rotenone (0.1 μg/ml); thenoyltrifluoroacetone (TTFA, 250 μM); antimycin A (50 μM); sodium azide (1 mM) and the uncoupler dinitrophenol (1 mM) for 30 minutes before being cultured in the presence or absence of 25 ng/ml TNFα for 6 hours. Parallel control cells were also prepared. At this time point, neutrophils were harvested and apoptosis assessed morphologically. Data represent mean ± SEM from n = 4 separate experiments each performed in triplicate (*$p<0.05$ compared with values obtained with cells incubated with TNFα alone).

thenoyltrifluoroacetone (TTFA, 250 μM), a complex II inhibitor; antimycin A (50 μM), a complex III inhibitor; sodium azide (1 mM), a complex IV inhibitor; and the uncoupler dinitrophenol (1 mM) for 30 minutes before being incubated in the presence or absence of TNFα. While none of these agents effected basal apoptosis, both TTFA and antimycin A significantly inhibited the induction of apoptosis by TNFα. A similar effect was also seen with dinitrophenol. In contrast, sodium azide significantly enhanced TNFα-induced apoptosis compared with control values, while no affect was seen with rotenone (figure 6.2.6A).

The ROS produced by the mitochondria are changed into the more reactive $OH^{\bullet}$ molecule via the iron dependent Fenton reaction. Thus, to investigate the hypothesis that TNFα induces apoptosis by enhancing ROS release from the mitochondria, the effect of iron chelators on TNFα-stimulated apoptosis was examined. As in previous experiments, neutrophils were pre-incubated with the iron chelators desferrioxamine (DFO) and CP94 for 30 minutes before being exposed to TNFα. No effect was observed with either 1 or 10 mM DFO or 1 mM CP94 and, although 10 mM CP94 did partially inhibit TNFα-induced apoptosis, this concentration also increased basal apoptosis, suggesting a toxic effect (figure 6.2.6B).

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6.2.7 Role of CPP32 in TNFα-stimulated apoptosis

Release of cytochrome c from the mitochondria has also been implicated as an important step in precipitating apoptosis in other cell types. There is strong evidence to indicate that cytochrome c is an important co-factor in the auto-proteolysis required for the cleavage of ICE protease, caspase 9. Caspase 9 is then able to process, thus activating, CPP32 (caspase 3). Activation of this enzyme has been shown to be alone sufficient to induce apoptosis. In view of this, we investigated the effect of the specific CPP32 inhibitor, Z-DEVD-FMK, on TNFα-stimulated apoptosis in neutrophils. Z-DEVD-FMK (100 μM) significantly reduced the cytocidal effect of TNFα, although not to the same extent as anoxia (figure 6.2.7). A higher concentration of this inhibitor (200 μM) was unable to further abrogate the effect of TNFα (data not shown). However, the more general, or broad spectrum,

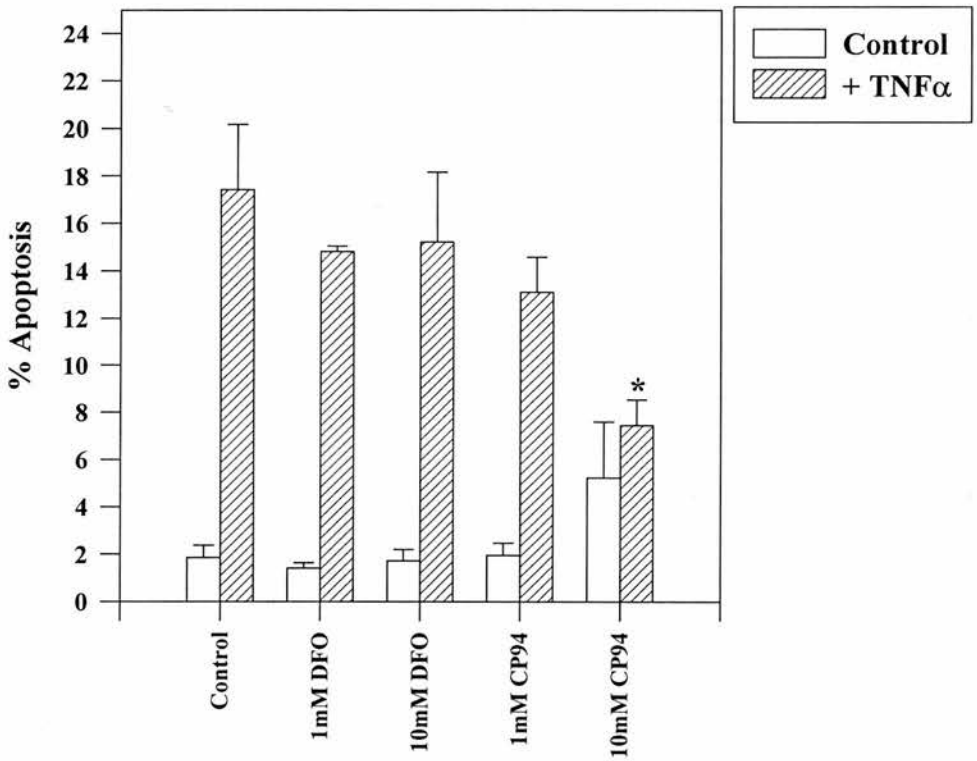

Figure 6.2.6B The effect of iron chelators on TNFα stimulated-apoptosis.
Neutrophils (5 x 10^6/ml) were pre-incubated with the iron chelators desferrioxamine (DFO, 1 and 10 mM) and CP94 (1 and 10 mM) for 30 minutes before being cultured in the presence or absence of TNFα for 6 hours. Parallel control cells were also prepared. At this time point, neutrophils were harvested and apoptosis assessed morphologically. Data represent mean ± SEM of n = 4 separate experiments, each performed in triplicate (*$p<0.05$ compared with 21% apoptotic values in control TNFα treated cells).

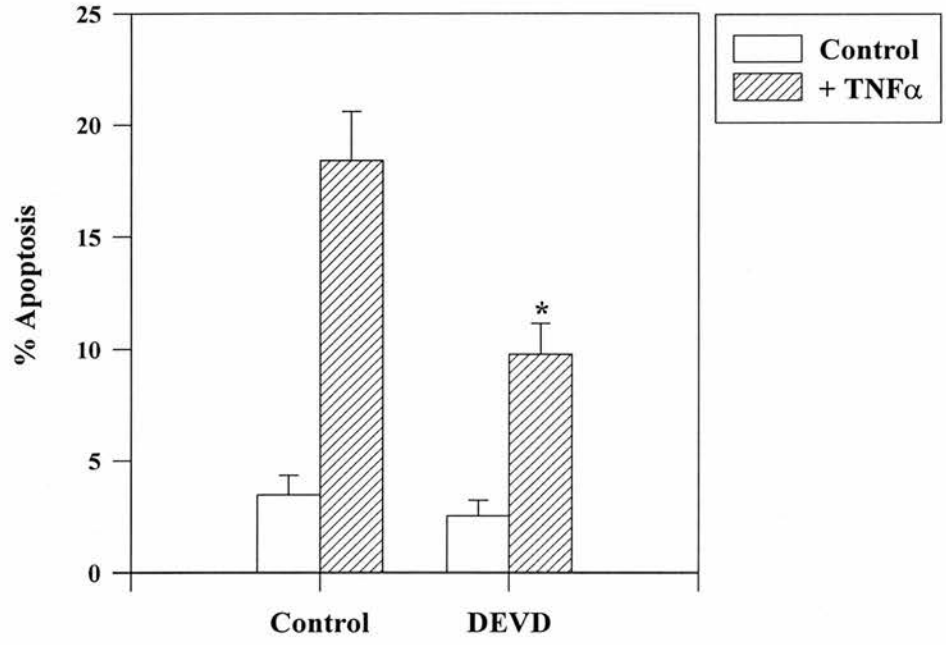

Figure 6.2.7 The effect of a CPP32 inhibitor on TNFα-stimulated apoptosis. Neutrophils (5 x 10^6/ml) were incubated with the CPP32 inhibitor, Z-DEVD-FMK, (DEVD, 100 μM) in the presence and absence of TNFα (25 ng/ml). Parallel control cells were also prepared. After 6 hours, neutrophils were harvested and apoptosis assessed morphologically. Data represent mean ± SEM from n = 8 separate experiments each performed in triplicate (*$p<0.05$ compared with cells incubated with TNFα alone).

caspase inhibitor, Z-VAD-FMK, had a more potent effect, reducing TNFα-induced apoptosis to control levels (C. Ward, personal communications).

6.3 Discussion

In our initial experiments we confirmed the previous observations of our group (Murray et al., 1997), which found that TNFα has an apparently unique bimodal effect on the rate of neutrophil apoptosis *in vitro*, with the capacity to induce early apoptosis (< 12 hours) in addition to decreasing the extent of apoptosis at a much later time point (20 hours). We also demonstrated that the pro-apoptotic effect of TNFα is concentration-dependent with an EC_{50} value of 2.8 ng/ml.

Although many mechanisms have been proposed to explain how TNFα may mediate its cytotoxic effect, there is no one theory which appears to hold true for all cell types. In addition, different pathways may be employed to induce apoptosis and necrosis. One hypothesis suggests that an increase in intracellular ROS levels may play a critical role in TNFα-induced cytotoxicity and the experiments described in this chapter were designed to investigate whether this could be the case in neutrophils. Preliminary work found that if neutrophils were made anoxic prior to TNFα exposure, the pro-apoptotic effect of TNF-α was greatly reduced. These data suggest that the pro-apoptotic effect of TNFα in neutrophils may involve oxidative pathways and are consistent with the observations of Matthews and colleagues (1987), who found that TNFα-mediated cytolysis of L929 cells was inhibited by anaerobic conditions. However, when neutrophils were exposed to TNFα in medium that was not fully anoxic until 30 minutes into the incubation period, TNFα was still able to induce apoptosis. These data suggest that oxygen is required at a very early stage and that the commitment of these cells to undergo apoptosis is extremely rapid, occurring within 30 minutes of TNFα exposure.

Reports showing an adverse effect of hypoxia on the expression of certain receptor types in neutrophils (Simms and D'Amico, 1993) prompted us to investigate TNFR55 and TNFR75 expression in neutrophils incubated under anoxic conditions. The time-dependent decrease in both TNFR55 and TNFR75 observed during neutrophil culture is consistent with the demonstration of adherence-induced TNFR shedding by Lantz et al (1994); however, we found no difference in the rate of receptor shedding observed between neutrophils incubated under normoxic and

anoxic conditions, either in the presence or absence of TNFα. These findings are in agreement with those of Scannell et al. (1995), who, despite observing an upregulation of TNFR expression in response to chemical hypoxia (induced with sodium cyanide), found no difference between TNFR expression in control cells and neutrophils treated with physical hypoxia. Thus, the inhibition of TNFα-stimulated apoptosis in neutrophils cannot be explained by a direct effect of hypoxia on TNFR expression.

To investigate further the role of ROS in TNFα-induced apoptosis, neutrophils were pre-treated with a wide range of compounds possessing antioxidant properties before being exposed to TNFα. Both mannitol, which quenches hydroxyl radicals, and DMSO, a free radical scavenger, exhibited some inhibitory effects, although when grouped, these effects were not significant. NAC is a well established thiol antioxidant, which after cellular uptake, deacylation and conversion to glutathione, functions both as an intracellular redox buffer and, in conjunction with glutathione peroxidase, as a ROS scavenger (Droge et al., 1992; Roederer et al., 1992). NAC has been shown, using a similar experimental protocol, to block TNFα-induced cytotoxicity in several cell types, including L929 fibroblasts and oligodendrocytes (Mayer and Noble, 1994). 10 mM NAC has previously been shown to completely inhibit PMA-stimulated superoxide anion and hydrogen peroxide production in neutrophils, as well as protecting these cells from PMA-induced cytotoxicity (Takei et al., 1996). However, in our system NAC, at both 5 and 10 mM, failed to protect against TNFα induced apoptosis and, consistent with this result, no effect was observed with reduced glutathione. The vitamin E analogue, trolox, and anti-oxidant, taurine, also failed to abrogate TNFα-induced killing. It remains possible, however, that the lack of effect of antioxidants may be due to an inability of these compounds to penetrate to the site of ROS generation. We have already demonstrated the commitment to undergo TNFα-induced apoptosis is extremely rapid, and, although neutrophils were pre-incubated with the antioxidants for 30 minutes before exposure to TNFα, this pre-incubation time may not have been adequate. However, due to the extent of spontaneous TNFR shedding that occurs in

neutrophils cultured under such conditions (Murray et al., 1997), it was not possible to extend this incubation time.

The antioxidant enzymes, superoxide dismutase (SOD) and catalase, catalyse the reactions $2H^+ + 2O_2^- \rightarrow H_2O_2 + O_2$ and $2H_2O_2 \rightarrow H_2O + O_2$ respectively. A role for endogenous mitochondrial (Mn) SOD in TNFα-mediated cytotoxicity has been demonstrated by Wong et al (1989) studying a human embryonic kidney cell line. In this system, overexpression of Mn SOD resulted in improved survival after TNFα treatment for 24 hours. TNFα can itself upregulate Mn SOD mRNA, as shown in a murine adipogenic cell line and in a pulmonary adenocarcinoma cell line (Warner et al., 1991). Previous data suggest that neutrophils do not upregulate Mn-SOD in response to TNFα stimulation and, in addition, that exogenous superoxide dismutase does not affect apoptosis induced by TNFα (Kettritz et al., 1997). Contrary to these findings, we found that exogenous SOD markedly enhanced TNFα-stimulated apoptosis. The discrepancy between our findings and those of Kettritz and co-workers (1997) may be because of differences in methodology; we used a higher concentration of SOD and included a pre-incubation step in our experiments.

As discussed above, SOD promotes the formation of H_2O_2 from superoxide anions. Thus, although the synergistic killing effect of SOD and TNFα was unexpected, we postulated that it might be explained if H_2O_2 was the critical ROS involved in the TNFα effect. In order to investigate this possibility, the effect of H_2O_2 on basal and TNFα induced neutrophil apoptosis was examined. The ability of H_2O_2 to induce apoptosis in certain cell types is well documented (Lennon et al., 1991) and concentrations as low as 0.1 mM have previously been shown to enhance basal apoptosis in neutrophils (Hannah et al., 1995). As an initial experiment, we investigated the effect of a broad range of H_2O_2 concentrations (0.05 – 10 mM) on both TNFα-induced and basal apoptosis. At concentrations of 1 mM and below, no effect on either TNFα-induced or constitutive apoptosis was observed, while apoptosis under both conditions was induced by 5 and 10 mM H_2O_2. The greater resistance of neutrophils to H_2O_2 treatment observed in our experiments compared

with those of Hannah and co-workers (1995) can probably be explained by the increased amount of serum present in our cell culture medium (10% as opposed to 2%). However, when the effect of 1 – 5 mM H_2O_2 was examined, we found that although H_2O_2 increased both basal and TNFα-induced apoptosis in a concentration-dependent manner, TNFα-stimulated cell killing was more sensitive to H_2O_2 than basal apoptosis. Indeed, 3 and 4 mM H_2O_2 were able to enhance TNFα-induced apoptosis without significantly affecting control levels. These data may suggest a role for H_2O_2 in TNFα-stimulated apoptosis in neutrophils. However, the concentrations required to enhance TNFα-induced killing are undoubtedly supra-physiological, although the 10% serum present in the culture medium may have had a neutralising effect on the H_2O_2. Furthermore, the inability of catalase (an H_2O_2 scavenger) to protect against TNFα killing and the lack of effect of the catalase inhibitor (3-AT), which would be expected to enhance H_2O_2 levels, again argues against a role for this ROS in TNFα-stimulated neutrophil apoptosis.

Investigations on TNFα cytolysis of L929 tumour cells demonstrated that cells treated with TNFα showed gross abnormalities in their mitochondrial structure. It was reported that these organelles looked swollen and had fewer cristae compared to mitochondria from control cells (Schulze-Osthoff et al., 1992). In addition, mitochondrial inhibitors have been able to effect TNFα-induced cytotoxicity in several cell types, thus, implicating a role for the mitochondria in TNFα-mediated cell killing. Coupled with this, it has long been recognised that the mitochondria are an important source of ROS under certain conditions. According to Boveris and Chance (1973) about 1-2% of the oxygen consumed in state 4 (resting respiration) is utilised by the formation of ROS. In the presence of various drugs (i.e. mitochondrial inhibitors and quinoid compounds) mitochondrial generation of ROS can increase several fold.

Two sites in the mitochondrial respiratory chain have been identified as sources responsible for the formation of ROS. One is dependent on the auto-oxidation of the flavin mononucleotide from the NADH-dehydrogenase (complex I), whereas the other, probably more relevant site, depends on auto-oxidation of the unstable

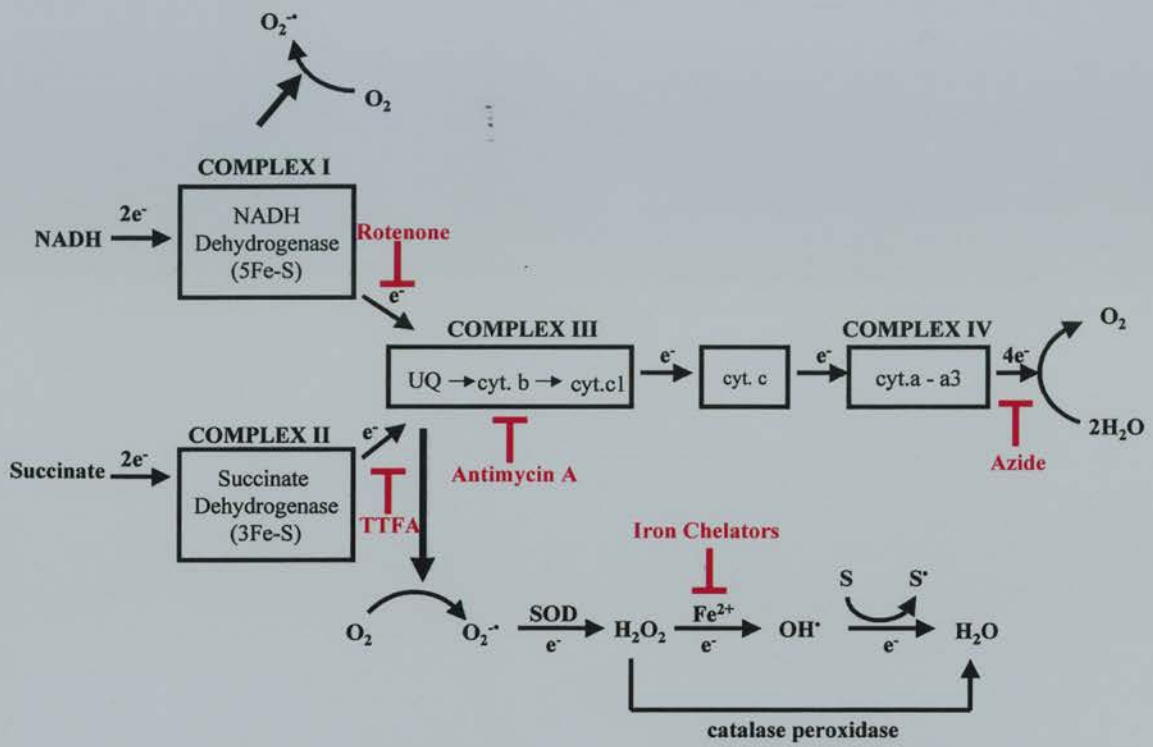

Figure 6.3.1 Production of superoxide radicals by the mitochondrial respiratory chain. Succinate and NADH are oxidized by succinate dhydrogenase and NADH dehydrogenase respectively. The two electrons (e^-) are then passes on to ubiquinone (UQ). From UQ, e^- pass through different cytochromes (cyt). Finally, reduced cytochrome c is re-oxidised by the multi-enzyme complex cytochrome oxidase. For every four e^- taken in by this complex, one oxygen molecule becomes fully reduced to two molecules of water. 1 to 5% of oxygen leaks from this pathway, mainly at the UQ site, and undergoes stepwise univalent reduction. The one e^- reduction of molecular oxygen generates the superoxide radical ($O_2^{-\bullet}$). This can subsequently be reduced to hydrogen peroxide (H_2O_2), most commonly in a dismutation reaction catalysed by superoxide dismutase (SOD). H_2O_2 can be directly detoxified to water by a catalase or peroxidase-catalysed reduction. Alternatively H_2O_2 can be reduced, usually in an iron-catalyzed reaction, to the hydroxyl radical ($OH^\bullet$), which can react with almost every type of organic substrate (S) in living cells. The site of action of the mitochondrial inhibitors and iron chelators used in the experiments described in the text are also indicated.

ubisemiquinone (complex III), which is an intermediate of the Q-cycle reaction (Turrens and Boveris, 1980; Turrens et al., 1985). Mitochondrial substrates and inhibitors have been described as effective modulators of ROS and the production of oxygen radicals at the ubiquinone site is diminished by complex I and II inhibitors (Cino and Del Maestro, 1989; Konstantinov et al., 1987; Cadenas and Boveris, 1980). On the other hand, formation of ROS increases several fold in the presence of the complex III inhibitor, antimycin A, proposing ubisemiquinone as the main reductant site of oxygen (Boveris et al., 1976). Thus, if TNFα activates ROS production at the ubisemiquinone site in mitochondria to induce apoptosis, we would expect the complex I and II inhibitors rotenone and TTFA, to inhibit TNFα-induced apoptosis and the complex III inhibitor, antimycin A, to cause enhancement. Such an effect has indeed been reported to be the case in both L929 and WEHI mouse fibrosarcoma cells (Schulze-Osthoff et al., 1992). However, we found the complex I inhibitor, rotenone, had no effect on TNFα-induced apoptosis in neutrophils (a finding in agreement with that of Matthews et al (1987) who found this inhibitor did not effect TNFα cytolysis of L929 cells). Both TTFA (complex II) and antimycin A (complex III) significantly reduced the TNFα pro-apoptotic effect, however, the respiratory chain uncoupler, dinitrophenol, displayed a similar anti-apoptotic effect, implying that the TNFα effect may simply be ATP-dependent. In contrast, the complex IV inhibitor, sodium azide, markedly enhanced the pro-apoptotic effect of TNFα.

The first oxygen reduction product generated in the mitochondria, under both physiological and pathological conditions, appears to be the superoxide radical, which is converted to H_2O_2 (Loschen et al., 1974). Dismutation of O_2^- and, subsequently, H_2O_2 results in the production of more toxic ROS, namely the hydroxyl radical and singlet oxygen. Conversion of superoxide and hydrogen peroxide to these harmful oxygen intermediates is catalysed by transition metals such as iron ions in the Fenton reaction and the (metal catalysed) Haber-Weiss reaction (Freeman and Crapo, 1982; Halliwell and Gutteridge, 1990). Thus again, if TNFα is causing upregulation of ROS generation from the mitochondria, and this process was coupled to the induction of apoptosis, then iron chelators could be expected to

abrogate TNFα killing. We therefore investigated the effect of two cell permeable iron chelators, DFO and CP94, on the TNFα pro-apoptotic effect. Neither concentration of DFO tested affected TNFα-induced apoptosis and, although the higher concentration of CP94 (10 mM) did partially inhibit, it also induced basal apoptosis suggesting that this concentration was toxic in neutrophils.

Together, these data do not support the hypothesis that the early induction of apoptosis by TNFα reflects an effect of this cytokine, either at the ubiquinone site or through enhanced free radical production, but does implicate the mitochondria in having some mediatory role in the induction of apoptosis by TNFα. Our data, showing that complex II and III inhibitors abrogate the TNFα effect, while a complex IV inhibitor enhances TNFα-induced apoptosis, suggest that such a regulatory step may lie between complexes III and IV. This suggestion is supported by the knowledge that cytochrome c functions as the mediator between these two complexes and, notably, that cytosolic cytochrome c has been shown in many circumstances to be necessary for the initiation of the apoptotic program in other cell types (Yang et al., 1997).

Cytochrome c is encoded by a nuclear gene and translated by cytosolic ribosomes as apopcytochrome c (Gonzales and Neupert, 1990). Apopcytochrome c is subsequently translocated to the mitochondria where a haem group is attached covalently to form holocytochrome c (Gonzales and Neupert, 1990). Holocytochrome c can be released from the mitochondria into the cytoplasm and this event has been shown to be required for full activation of CPP32 (caspase 3), a member of the family of interleukin-1β-converting enzyme (ICE)/ced-3 cysteine proteases (Liu et al., 1996; Yang et al., 1997). ICE is a cysteine protease, identified as a mammalian homologue of the *Caenorhabditis elegans* cell death protein Ced-3 (Yuan et al., 1993). All known caspases occur as zymogens and become activated after proteolytical processing (Henkart, 1996). The ICE family of proteases (or caspases) are thought to be potential positive regulators of apoptosis since apoptosis is induced by overexpression of these protease genes and is inhibited by ICE/ced-3 protease-specific inhibitors (for review see Zhivotovsky et al., 1996).

Recent reports have implicated a potential role for CPP32 and the mitochondria in TNFα cytotoxicity. Activation of CPP32 like proteases has been implicated in TNFα-induced hepatic parenchymal cell apoptosis (Jaeschke et al., 1998). Dong and co-workers (1998) demonstrated that, in a human monocytic leukaemia U937 subclone resistant to TNFα-induced apoptosis, CPP32 activation in response to this cytokine was not observed, unlike the TNFα-sensitive parental cell line. In addition, the parental cell line also demonstrated a change in mitochondrial $\Delta\Psi_m$ after TNFα treatment, an effect not observed in the TNFα-resistant cells, suggesting that the mutant cell line may have a functional defect in apoptosis signalling between the TNFR55 and the mitochondria. Similarly, experiments using mitochondrial respiratory chain-deficient clones of a leukaemic cell line demonstrated resistance to TNFα-induced apoptosis and CPP32 activation. However, these cells were sensitive to staurosporine-induced apoptosis, an effect which could be inhibited by a CPP32 inhibitor, suggesting that, while certain agents can activate CPP32 directly, TNFα requires the mitochondria to mediate this activation (Higuchi et al., 1997). Caspase inhibitors were also able to completely abrogate TNFα-induced apoptosis in TNFα-sensitive HeLa cells and KYM cells (Vercammen et al., 1998). However, it is important to note that the same inhibitors also sensitise L929 cells to TNFα-induced necrosis, an effect that is more clearly dependent on enhanced ROS production. Such data provide additional evidence to support the view that TNFα-induced necrosis and apoptosis may be mediated via independent mechanisms. Finally, expression of CPP32 was found to be reduced in TNFα-resistant inflammatory lung neutrophils when compared to TNFα-sensitive peripheral blood neutrophils, however, it should be noted that TNFR expression was also significantly less in the inflammatory lung neutrophils (Watson et al, 1997).

We thus investigated a potential role for CPP32 in TNFα-induced apoptosis in neutrophils by utilising the specific inhibitor of this enzyme, Z-DEVD-FMK. We found this inhibitor was able to significantly attenuate TNFα-stimulated killing, although this effect was not as pronounced as that of hypoxia. However, a more

general caspase inhibitor (Z-VAD-FMK) was able to completely inhibit TNFα-induced apoptosis (C. Ward, personal communications). These results imply a role for CPP32, amongst other caspases, in TNFα-stimulated apoptosis in neutrophils. Clearly, the events occurring upstream of CPP32 activation, including a potential role of the mitochondria, and the down-stream substrates of this enzyme still require further investigation.

Recently the role that cytochrome c plays in CPP32 activation has been clarified. The cytoplasmic partner of cytochrome c in its pro-apoptotic capacity has been identified as Apaf-1 (apoptosis activating factor-1) (Zou et al., 1997; Li et al., 1997). This protein was found to be homologous to the nematode protein Ced-4, a factor that is also capable of activating pro-caspase 3. The above authors have now demonstrated that the addition of Apaf-1, cytosolic cytochrome c (previously Apaf-2) and dATP to pro-caspase 9 (previously Apaf-3) results in the activation of caspase 9 which can then cause cleavage of pro-caspase 3 (CPP32) to its active form.

A second pathway that has been reported to cause CPP32 activation involves stimulation of receptors from the TNF/NGF receptor superfamily. Recent reports demonstrate that, following TNFR55 activation, two proteins, TNF-receptor associated death-domain-containing protein (TRADD) and Fas-associated death-domain-containing protein (FADD) are complexed to the intracellular domain of the TNFR55 at the region involved in receptor-induced cell death (death domain) (Hsu et al., 1995; Chinnaiyan et al., 1996). The TRADD-FADD complex recruits another protein called FLICE/MACH (caspase 8) by means of the interaction of their respective death-effector domains. This protein complex is referred to as the death binding signal complex (DISC). Upon binding to the receptor cluster, caspase 8 becomes activated by auto-proteolysis and thereby is able to cleave and activate downstream effector caspases (Muzio et al., 1996).

Despite *in vitro* evidence that caspase 8 can process and thus activate other caspases including CPP32 (Fernandes-Alnemri et al., 1996; Muzio et al., 1997), more recent evidence suggests that caspase 8 may not always act through a direct caspase-caspase

cascade. Using a Xenopus cell-free system, Kuwana and co-workers (1998) observed that, in the absence of mitochondria, caspase 8 was only able to produce a partial apoptotic phenotype in nuclei added to the extracts. However, when mitochondria were present full nuclear apoptosis occurred, the critical step being the release of cytochrome c from mitochondria into the cytosol. Further evidence supporting the existence of a direct and an indirect pathway involving caspase 8 comes from a study by Scaffidi et al. (1998) who investigated the role of caspase 8 in Fas-mediated apoptosis. In a similar fashion to TNFR55, cross-linking of the Fas receptor by engagement of the Fas-ligand results in the formation of a DISC that, in the case of Fas, consists of the adapter proteins FADD/MORT-1 and caspase 8. Scaffidi and colleagues identified two cell types, which each activate apoptosis through one of two different Fas signalling pathways. In the so-called Fas type 1 cells, receptor activation was followed by the recruitment of a relatively large amount of caspase 8 to the DISC. The activated caspase 8 initiated the apoptotic pathway by processing downstream effector caspases into their active counterparts as well as causing mitochondrial damage that also initiated a proteolytic cascade. In the so-called Fas type II cells, less caspase 8 was recruited to the DISC in response to receptor cross-linking and the activated caspase 8 induced apoptosis predominantly by causing mitochondrial damage. Recently, two groups have reported that a critical step lying between caspase 8 activation and mitochondrial damage is the cleavage of BID, a BH3 domain containing pro-apoptotic Bcl-2 family member (Li et al., 1998; Luo et al., 1998). Full length BID is located in the cytosol, however, following cleavage by caspase 8 into its truncated (and active) form (tBID), it translocates to the mitochondria. Once located on these organelles, tBID induces cytochrome c release and thus can initiate a pro-apoptotic cascade.

Although signal pathways resulting in CPP32 activation are rapidly emerging, the physiological substrate(s) and/or apoptotic 'effector' mechanism of this enzyme are, as yet, unknown. One well characterised potential substrate of CPP32 is the DNA repair enzyme poly(ADP-ribose) polymerase (PARP). However, the observation that mice lacking the PARP gene are still capable of efficient DNA repair (Wang et al., 1995) makes it difficult to consider cleavage of PARP as an important early event in

the apoptotic process. Furthermore, this is supported by a report suggesting that PARP is not found in neutrophils (Sanghavi et al., 1998).

Putative recognition sequences for caspase 3-related proteins are also present in the amino-acid sequences of NF-κB p65 (RelA) and NF-κB p50 (Nicholson et al., 1995; Nolan et al., 1991; Ghosh et al., 1990). Indeed, repression of NF-κB activity by proteolytic cleavage of these subunits in Jurkat T cells could be prevented by inhibitors of caspase 3 like proteases (Ravi et al., 1998). Gelsolin, an actin filament-capping protein, has also been identified as a substrate for CPP32 and caspase cleaved gelsolin has been shown to sever actin filaments (Kothakota et al., 1997). Interestingly, neutrophils contain high levels of gelsolin and treatment of these cells with TNFα and cycloheximide has been shown to result in an augmented rate of gelsolin cleavage. Furthermore neutrophils isolated from gelsolin$^{-/-}$ mice demonstrate a delay in morphological aspects of apoptosis in response to various apoptotic stimuli including co-incubation with TNFα and cycloheximide (Kothakota et al., 1997).

To conclude, we have presented data that implicate a role for both the mitochondria and the caspase family of proteases, in particular CPP32, in TNFα-stimulated apoptosis in neutrophils, findings that are supported by other reports in the literature. However, further investigations are required to link these two steps. Taking into account recent findings in other systems, we propose the following hypothesis: TNFα acts via its cell surface receptors to activate the caspase protease cascade both directly, via proteolysis of FLICE, and indirectly by exerting a detrimental effect on the mitochondrial respiratory chain (MRC) (perhaps through BID). Our data would predict that this latter event causes release of cytochrome c into the cytoplasm, which would then act as a necessary co-factor for the cleavage of pro-caspase 9, thus leading to an augmented activation of the effector caspases, including CPP32. In this model hypoxia could exert a protective effect on the MRC either by reducing damage caused by ROS or by preventing loss of the mitochondrial $\Delta\Psi_m$, thus preventing the release of cytochrome c.

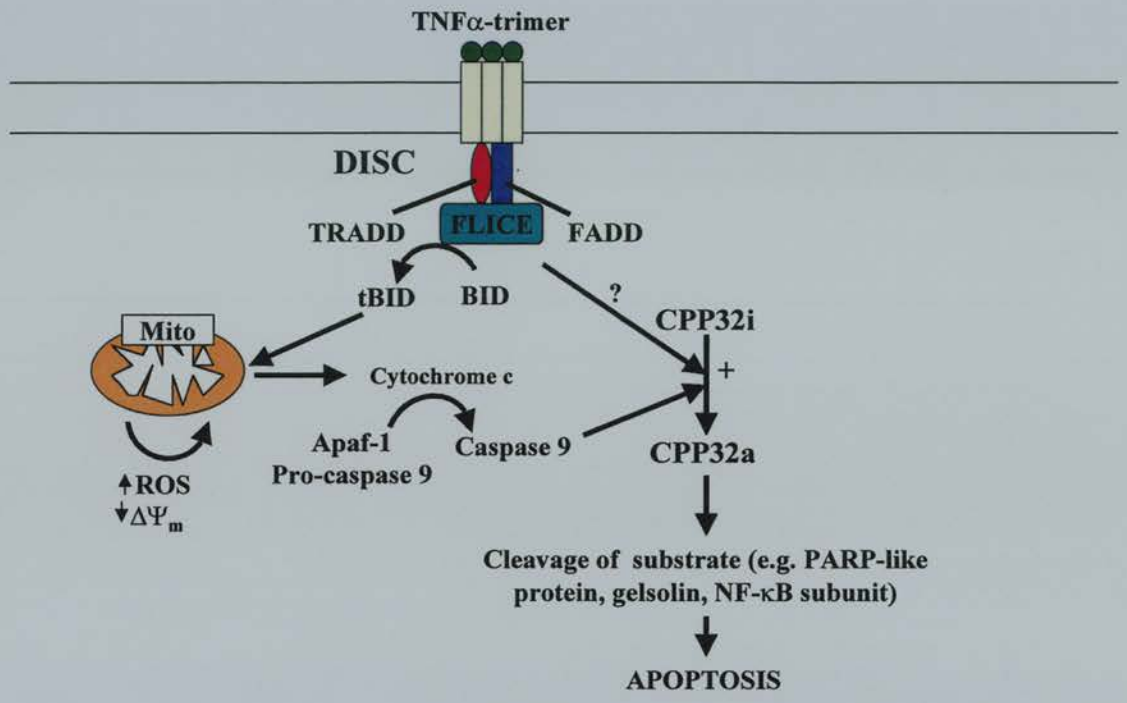

Figure 6.3.2 Proposed mechanism underlying TNFα-induced apoptosis in human neutrophils. Simplified diagram showing one putative mode of action of TNFα. In this model TNFα binds to the TNFRs. Following TNFR activation TNF-receptor associated death-domain-containing protein (TRADD) and Fas-associated death-domain-containing protein (FADD) are complexed to the TNFR55. This receptor complex then recruits FLICE (MACH/caspase8), a protein complex termed the death binding signal complex (DISC). Subsequently, FLICE is activated by autoproteolysis and in turn cleaves and activates both effector caspases, including CPP32 (caspase 3), and BID. The truncated (active form) of BID (tBID) then translocates to the mitochondria where it causes cytochrome c release into the cytosol. Cytosolic cytochrome c would act as a necessary co-factor in caspase 9 activation thus augmenting activation of CPP32. The mechanism by which CPP32 induces apoptosis is, as yet, unclear. However putative substrates include the actin filament cleaving protein, gelsolin, poly (ADP-ribose) polymerase (PARP) and the NF-κB subunits p50 and p65. See text for more detail.

CHAPTER 7

SUMMARY

Neutrophil apoptosis has been proposed as a major mechanism underlying the removal of redundant neutrophils from an inflammatory focus. Although the intracellular processes underlying neutrophil apoptosis are not yet fully defined, the ability of many pro-inflammatory agents to modulate this event *in vitro* is well documented. For example, LPS, GM-CSF, G-CSF and the interleukins 1, 6 and 8 all delay neutrophil apoptosis, while TNFα is able to induce apoptosis in these cells. Several of these agents also act to prime the functional responsiveness of neutrophils. Thus, it appears that the balance of inflammatory mediators present at an inflamed site may hold the potential to either up or downregulate neutrophil function and longevity. We were interested to examine the possibility that other changes in the neutrophil's environment could also affect the functional life span of these cells.

Clinically, the effect of hypoxia on neutrophil apoptosis is particularly relevant. Neutrophils are implicated in the pathogenesis of many inflammatory diseases, such as, emphysema, acute respiratory distress syndrome, idiopathic pulmonary fibrosis, glomerulonephritis and reperfusion injury following myocardial infarction. In these conditions, the accumulation of neutrophils is associated with destruction of tissue or deposition of scar tissue, which can lead to catastrophic loss of organ function. In the majority of these diseases, the sites of inflammation, and hence neutrophil accumulation, will be relatively hypoxic. This is because of reduced blood flow to the site of inflammation or, in the case of pulmonary disease, reduced gas exchange due to lung damage. Furthermore, tissue hypoxia following haemorrhage and trauma is thought to be a possible initiating factor of the generalised inflammatory response seen after shock. Thus, the aim of the work presented in this thesis was to examine the effect of hypoxia on neutrophil apoptosis.

We have shown that, while hypoxia had no effect at early time points (< 6 hr) when the constitutive rate of apoptosis is still low, at later times (≥ 10 hrs) hypoxia was

able to markedly inhibit neutrophil apoptosis. The anti-apoptotic effect of hypoxia in neutrophils was confirmed by annexin V binding and propidium iodide staining and was shown to be concentration-dependent. Furthermore, while neutrophils had to be hypoxic from time 0 in order for hypoxia to achieve its full protective action, a delayed hypoxic challenge was still able to provide some inhibitory effect, even at a late stage in the apoptotic 'program'. The effect of re-oxygenation on neutrophil apoptosis was also examined. When hypoxic neutrophils (3 – 18 hr) were returned to a normoxic environment they underwent apoptosis at the same rate as control cells. However, if the hypoxic incubation time was more prolonged (20 hr), the re-oxygenated neutrophils became resistant to spontaneous apoptosis for a considerable time period after they were returned to a normoxic environment.

We were unable to mimic the inhibitory effect of hypoxia on neutrophil apoptosis using anti-oxidants, suggesting that the effect of hypoxia was not mediated solely by the inhibition of ROS generation. The rate of neutrophil apoptosis was also not sensitive to changes in glucose concentration or heat shock treatment, indicating that the enhanced survival of neutrophils under reduced oxygen conditions is specific for hypoxia, and is not sensitive to other cellular stresses such as cellular ATP depletion. These results also argue against a role for the GRP and HSP families of stress proteins in the inhibition of neutrophil apoptosis by hypoxia. Experiments indicating that neutrophils consitutively expressed Hsp 70 under the culture conditions studied and, moreover, that neither heat shock or hypoxic treatments caused further upregulation of this protein in neutrophils, also indicate that HSP expression is not an important factor in the inhibition of neutrophil apoptosis by hypoxia. The inhibitory effect of hypoxia on neutrophil apoptosis was additive to the anti-apoptotic action of the cytokine, GM-CSF, implying these effects are discrete and act via independent mechanisms. Furthermore, neither of these protective effects was associated with expression of the proto-oncogene Bcl-2. p38 MAP kinase has been reported to be activated upon hypoxic stimulation and has been implicated in some forms of apoptosis. However, the anti-apoptotic effect of hypoxia was not significantly affected by a p38 MAP kinase inhibitor, suggesting that this kinase does not play a major role in the signalling pathway underlying hypoxic inhibition of neutrophil

apoptosis. Treatment of neutrophils with mitochondrial inhibitors also failed to mimic the anti-apoptotic effect of hypoxia. Thus, it would seem that the hypoxic inhibition of neutrophil apoptosis is specific for hypoxia, and cannot be induced by other cellular stresses associated with making the cells hypoxic, namely the compromise of oxidative metabolism. However, the hypoxic-mediated inhibition of neutrophil apoptosis was found to be sensitive to the protein synthesis inhibitor, cycloheximide, even at very low concentrations that did not influence the rate of constitutive apoptosis. These observations strongly implicate *de novo* protein synthesis as a requirement for the inhibition of neutrophil apoptosis by hypoxia and imply that hypoxia may be inducing upregulation of a survival protein(s).

In order to investigate the oxygen-sensing mechanism involved in the anti-apoptotic effect of hypoxia, neutrophils were incubated in the presence of the iron chelators, DFO and CP94. Both iron chelators mimicked the effect of hypoxia in a concentration-dependent manner. Moreover, the inhibitory effects of CP94 and DFO could be overcome by the addition of a molar excess of $FeCl_2$, indicating these compounds were acting via the chelation of intracellular iron. Thus, chelatable iron may be closely involved in the oxygen-sensing mechanism underlying the hypoxic inhibition of neutrophil apoptosis and leads to the proposal that the control mechanism may involve the interaction of oxygen with a ferro-protein sensor. These data also suggested that the inhibition of neutrophil apoptosis by hypoxia can be induced by stimuli similar to those that are known to induce activation of the transcription factor HIF-1. For instance, other groups have shown that HIF-1 cannot be induced by mitochondrial inhibitors, heat shock or glucose deprivation but is activated when cells are made hypoxic or treated with iron chelators. HIF-1 activity is also induced in many cell types by cobalt ions, however, incubation of neutrophils with cobaltous chloride had no effect on the rate of apoptosis, indicating that the oxygen-sensing mechanisms underlying these effects may not be identical.

HIF-1 is a heterodimer consisting of the HIF-1α and HIF-1β subunits, with HIF-1 activity being largely regulated by hypoxic-modulation of HIF-1α protein levels. In order to investigate a potential role for HIF-1 in the hypoxic inhibition of neutrophil

apoptosis, HIF-1α mRNA and protein expression was examined. HIF-1α mRNA was found to be present in untreated neutrophils, while HIF-1α protein was expressed in hypoxic or DFO stimulated cells but was not present in control neutrophils, suggesting that HIF-1 activity is also increased under these conditions. However, in preliminary studies we were unable to demonstrate this experimentally using a DNA-binding assay for HIF-1.

We also examined the putative activation of two other redox-sensitive transcription factors in hypoxic and DFO-stimulated neutrophils. A constitutive form of NF-κB binding was found to be upregulated in response to hypoxia; however, this effect was extremely donor specific and the iron chelator, DFO, did not cause a similar enhancement. AP-1 DNA binding activity was found to be present constitutively in neutrophils, and treatment of cells with hypoxia or DFO failed to further modulate binding of this transcription factor. These data indicate that neither NF-κB nor AP-1 play an important role in the inhibition of neutrophil apoptosis by hypoxia.

Finally, the role of oxidant pathways in TNFα-stimulated apoptosis in neutrophils was investigated. Incubation of neutrophils under anoxic conditions resulted in a major abrogation of the pro-apoptotic effect of TNFα, implicating an oxygen-dependent step in TNFα-mediated apoptosis. TNFR55 and TNFR75 expression in neutrophils was not affected by incubation under anoxic conditions compared to control cells, suggesting that hypoxia was not inhibiting TNFα-induced apoptosis by influencing TNFR expression. Co-incubation of neutrophils with mitochondrial inhibitors and TNFα demonstrated that pharmacological inhibition of the respiratory chain at complexes II and III attenuated the pro-apoptotic effect of TNFα, while inhibition of complex IV enhanced the efficacy of TNFα. In contrast, antioxidants and iron chelators did not affect the ability of TNFα to induce neutrophil apoptosis, implying that the mitochondria, but not ROS, may play a role in the enhancement of neutrophil apoptosis by TNFα. Inhibition of the pro-apoptotic enzyme CPP32 (caspase 3) also protected neutrophils from TNFα-induced apoptosis suggesting, that activation of this enzyme may be an important effector step in this process.

The discovery that neutrophil apoptosis can be markedly inhibited by hypoxia indicates that neutrophils may remain alive and viable for considerably longer periods when recruited to an inflammatory focus (e.g. an empyema cavity), which often exists in a state of low oxygen tension. The ability of extreme tissue hypoxia to prolong neutrophil longevity and, thus delay neutrophil clearance, could potentially exacerbate the disease state and result in additional neutrophil-mediated tissue damage. Since the neutrophil is becoming increasingly associated with a number of pathological states, such as rheumatoid arthritis and glomerulonephritis, the recognition that the oxygen tension of the inflammatory site may potentiate the destructive capacity of the neutrophil (by increasing neutrophil longevity) suggests that increasing tissue oxygenation may aid in the treatment of such conditions.

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