THE CENTRAL ROLE OF THE NEUTROPHIL IN ISCHAEMIC/REPERFUSION INJURY

Submitted as a Final Report for a Higher Degree by Research by Nicola Susan Buck.

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March 1998.

I would like to dedicate this thesis to John.

THANK YOU.

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Inadequate blood flow to an organ, ischaemia, may lead to both local and remote tissue injury characterized by oedema, increased microvascular permeability to protein and degradation of connective tissue components. This damage is probably caused by the accumulation and inappropriate activation of neutrophils which occurs when the tissue is reperfused.

To test this hypothesis a number of *in vitro* models of the sequential stages of ischaemia/reperfusion injury were examined. Methods were initially developed to examine the adhesion of neutrophils to monolayers of a cultured endothelial cell line (ECV304) after periods of hypoxia and reoxygenation. Neutrophil migration in response to factors secreted by the treated endothelial cells was then assessed. The genesis of an inappropriate oxidative burst by the neutrophil upon exposure to endothelial chemoattractants and adhesion molecules was also measured. Finally to appraise how tissue function might be affected by endothelial cell hypoxia the contractility of vascular smooth muscle was examined.

Neutrophil adhesion to ECV304 cells, which had been hypoxic for 4 hours and then reoxygenated for 30 minutes, was significantly increased. This response was probably initiated by reactive oxygen species (ROS) generated by the endothelial cells. Blockage of their production by allopurinol reduced the heightened adhesion. Similarly removal of ROS by superoxide dismutase or catalase also attenuated adhesion. ROS generation in turn caused the release of a soluble factor (s) which induced a conformational change on the neutrophil surface allowing it to bind to the intercellular adhesion molecule 1 (ICAM-1) on the endothelial cell.

Soluble factor (s) from hypoxia/reoxygenated endothelial cells also had a powerful neutrophil chemoattractant ability. When neutrophils were exposed to both hypoxic/reoxygenated endothelial cells and the soluble factor (s) released by them a large oxidative burst was elicited. This response was greatest immediately after reoxygenation and one hour later was diminishing suggesting at least one of the components involved was labile. Analysis of the supernatant from hypoxic/reoxygenated endothelial cell cultures and studies using inhibitors of secretion suggested platelet activating factor (PAF) may be a major component in this overall sequence of events. Lesser roles for IL-8, TNFα and LTB_4 were also suggested.

The secretory products from hypoxia/reoxygenated endothelial cells also affected smooth muscle contractility having an anti-vasoconstrictor or relaxation property, similar to that exerted by PAF.

I would like to thank Dr. Alan Perris for all his patience, encouragement and constructive criticism throughout my Ph.D. Furthermore I would like to thank Dr. John Smith and Mr Alan Richardson for all their help with the contractility work.

Table Of Contents

Table of Figures

1. INTRODUCTION

Ischaemia, inadequate blood flow to an organ, is a common clinical event which may lead to both local and remote tissue injury. Evidence indicates that this damage, manifested by oedema and increasing microvascular permeability to protein, is largely caused by activated neutrophils which accumulate when the tissue is reperfused (Mullane *et al* 1983, Romson *et al* 1986 and Linas *et al* 1988). The resulting injury is thought to be similar in most states of tissue hypoperfusion, including limb ischaemia, transplantation, strokes and hypovolaemic shock. If the mass of ischaemic tissue is large, such as the legs or gastrointestinal tract, the neutrophils activated in these localities may also migrate to and become sequestered in the lungs, inducing non-cardiogenic pulmonary oedema - the adult respiratory distress syndrome (Schmeling *et al* 1989).

Ischaemia/reperfusion injury is initiated by the production of reactive oxygen species (ROS), such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), by endothelial cells. ROS appear directly responsible for neutrophil chemotaxis (Welbourn *et al* 1991). Up-regulation of adhesion molecules and their counter ligands on the surface of the endothelial cells and neutrophils, then facilitates neutrophil adhesion. Later, once adherent to the endothelium, neutrophils mediate damage by secretion of additional reactive oxygen species as well as proteolytic enzymes, such as elastase (Ward and Varani 1990 and Kurose and Granger 1994).

1.1 The neutrophil

Neutrophils are polymorphonuclear leukocytes 10-20μm in diameter that are produced in the bone marrow before being released into the peripheral blood where they circulate for 7-10 hours before migrating into tissue. Once resident in the tissue neutrophils have a life span of 3 days (Duijvestijn and Harmann 1989). In a healthy adult neutrophils constitute an average of 70% of the total white blood cell count, $5x10^6$ cells per ml. Neutrophils are the first immune cells at sites of infection or inflammation attracted by a number of chemoattractant factors such as complement components (Junger *et al* 1993) or products derived from other leukocytes, platelets and certain bacteria. Here their predominant role is phagocytosis and their

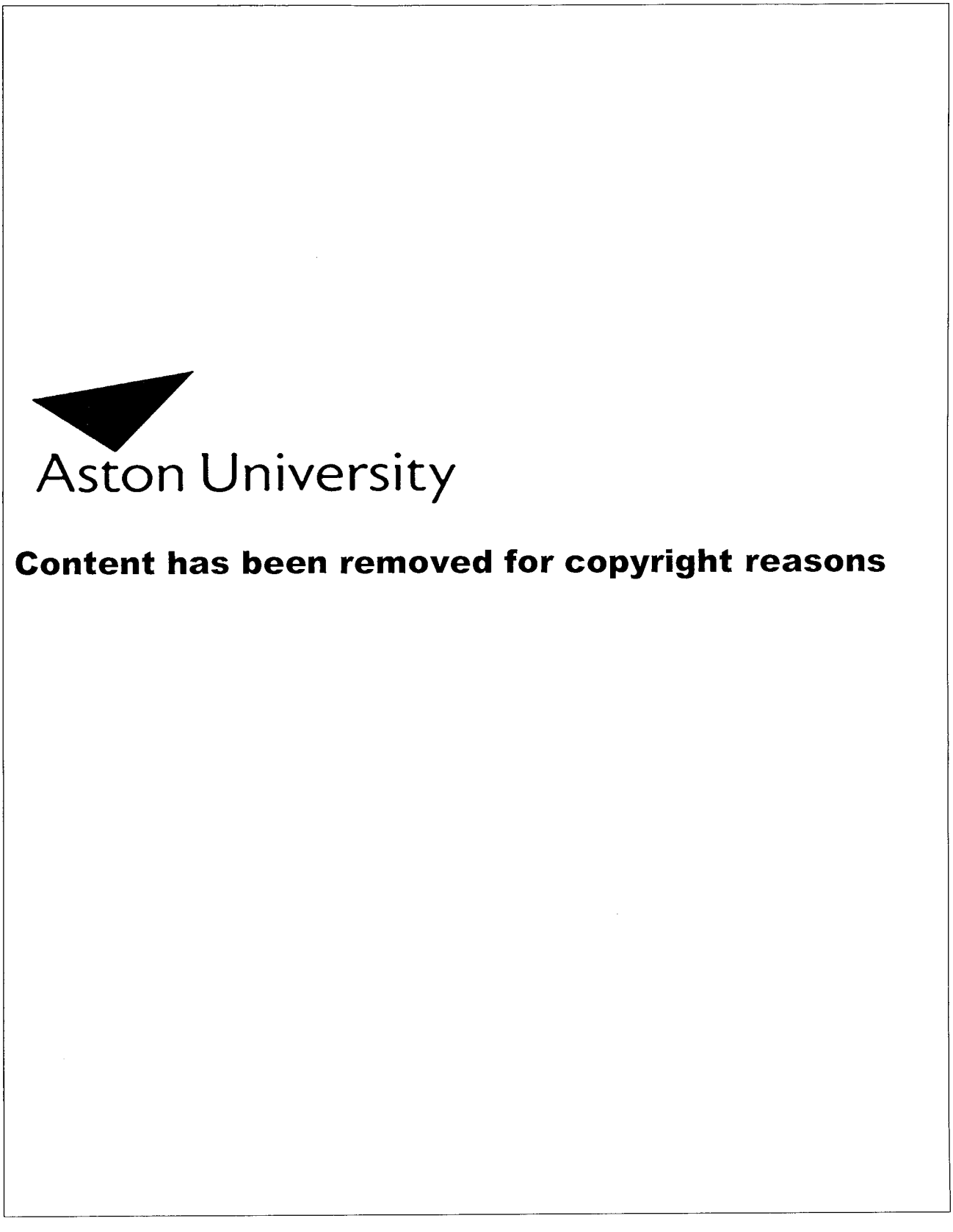
importance in protection is emphasized by the increase in susceptibility to infections found in individuals with a low circulating neutrophil numbers (Anderson 1995).

1.1.1 Neutrophil production

Production and distribution of neutrophils involves a series of dynamically interlinked pools of cells as shown in figure 1.1 (Cronkite and Fliedner 1964).

The progenitor is the most primitive pool and contains stem cells and other progenitors not totally committed to neutrophil production. These start with stem cells and develop into progressively more committed cells such as CFU-GEMM (colony forming unit granulocyte, erythrocyte, monocyte, megakaryocyte), CFU-GM (colony forming unit-granulocyte macrophage), through to CFU-G (colony forming unit-granulocyte). Haematopoietic stem cells are pluripotential cells with the capacity for self replication as well as differentiation. They are thought to originate in the embryonic yolk sac subsequently migrating to the liver and spleen and eventually resting in the bone marrow (Christesein 1989). The neutrophil proliferative pool (NPP) is more mature and contains committed neutrophil precursors capable of cell division, i.e. myeloblasts, promyelocytes and myelocytes. These cells together with the progenitor pool, constitute the neutrophil proliferative pool. The non-dividing stages of myelopoiesis constitute the neutrophil storage pool (NSP). This pool consists of metamyelocytes and neutrophils. As a neutrophil matures it alters its surface charge and acquires new cell membrane receptors and demonstrates motility (Wallace *et al* 1987). It has been proposed that the acquisition of these properties helps the mature neutrophil actively migrate or 'egress' through *trans*-endothelial pores in the marrow sinusoids to reach the bloodstream. Interleukin-1 (IL-1) and complement factors appear to act as positive modulators for this process (Shigeoka *et al* 1988). Athens *et al* (1981) demonstrated that neutrophils in the peripheral blood consist of a freely circulating pool (CNP) and a marginated pool (MNP). Both pools are of approximately the same size. Neutrophils from the MNP can be released into the general circulation by a suitable stimulus such as IL-6 though the exact mechanism by which neutrophils leave the MNP to enter the CNP remains unclear.

Figure 1.1 The maturational process of myeloid progenitor and mature effector neutrophil pools. Taken from Cronkite and Fliedner 1964.



1.1.2 Neutrophil function

As soon as microbes invade the tissues, circulating neutrophils leave the blood stream, adhere to activated endothelial cells, and move through the endothelial barrier to the site of the infection.

1.2 Neutrophil adhesion

The mature neutrophil has receptors for complement: CR_1 and CR_3, as well as Fc receptors for IgG namely, Fc gamma RI, II and III. CR_3 receptors are a family of at least three different glycoproteins each consisting of an identical beta subunit (CD18) non-covalently linked to different alpha subunits (CD11a, CD11b, CD11c, corresponding to LFA-1, MAC-1 and p150,95). Glasser and Fiederleir (1987) have studied the appearance of receptors during myelopoiesis. CR_1 is widely represented in mature neutrophils. CR_3, however, is first detected at the promyelocyte stage and is present in over half of metamyelocytes, and almost all band and mature neutrophils. Brainton *et al* (1987) have suggested that CD11a/CD18 may be the earliest receptor to appear. Intracellular pools containing MAC-1 and p150,95 are first detected at the myelocyte stage. FcR gamma III is present on one-third of all promyelocytes, one-half of all metamyelocytes and four-fifths of band and mature neutrophils.

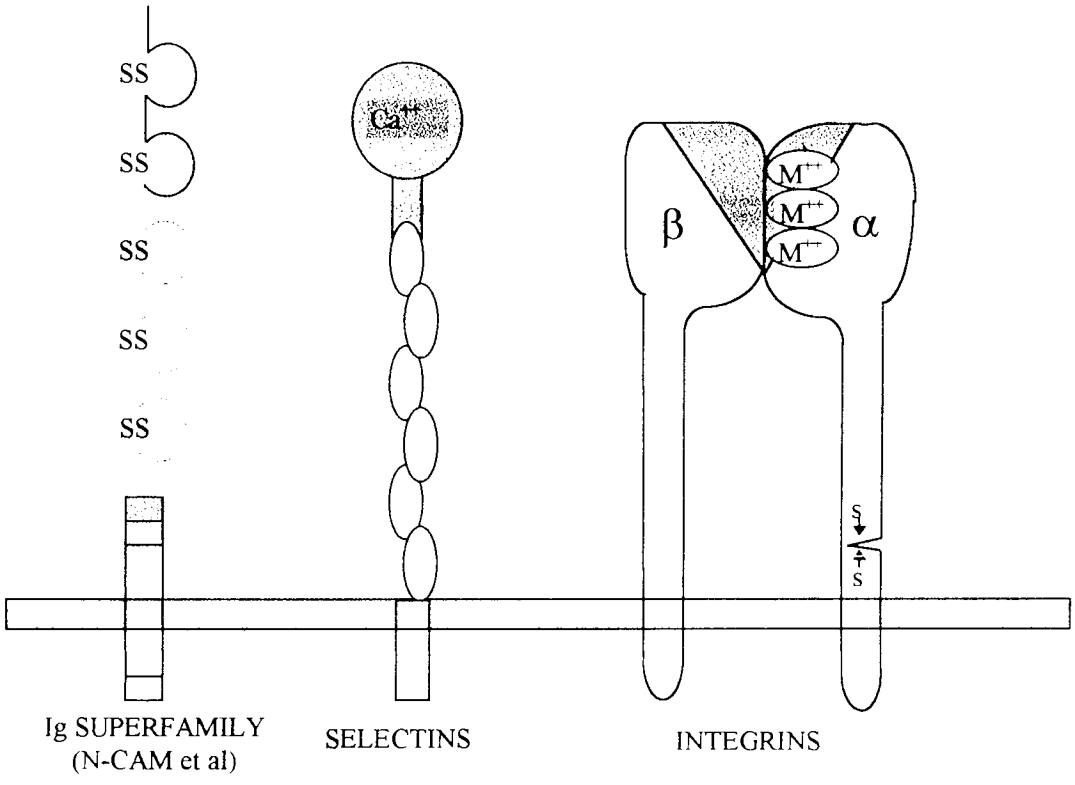
1.2.1 Adhesion molecules

Neutrophil adhesion to the endothelium, extravasation from the blood vessel and migration into the underlying tissue is an active, dynamic process involving a variety of cell-cell and cell-endothelium adhesion molecules (Figure 1.2). These events must be accurately regulated so that adhesion occurs only at the site of infection or inflammation (Hynes and Lander 1992). Extravasation is mediated by at least three sequential steps (as summarized in Figure 1.3);

- Initial rolling of neutrophils along the endothelium.

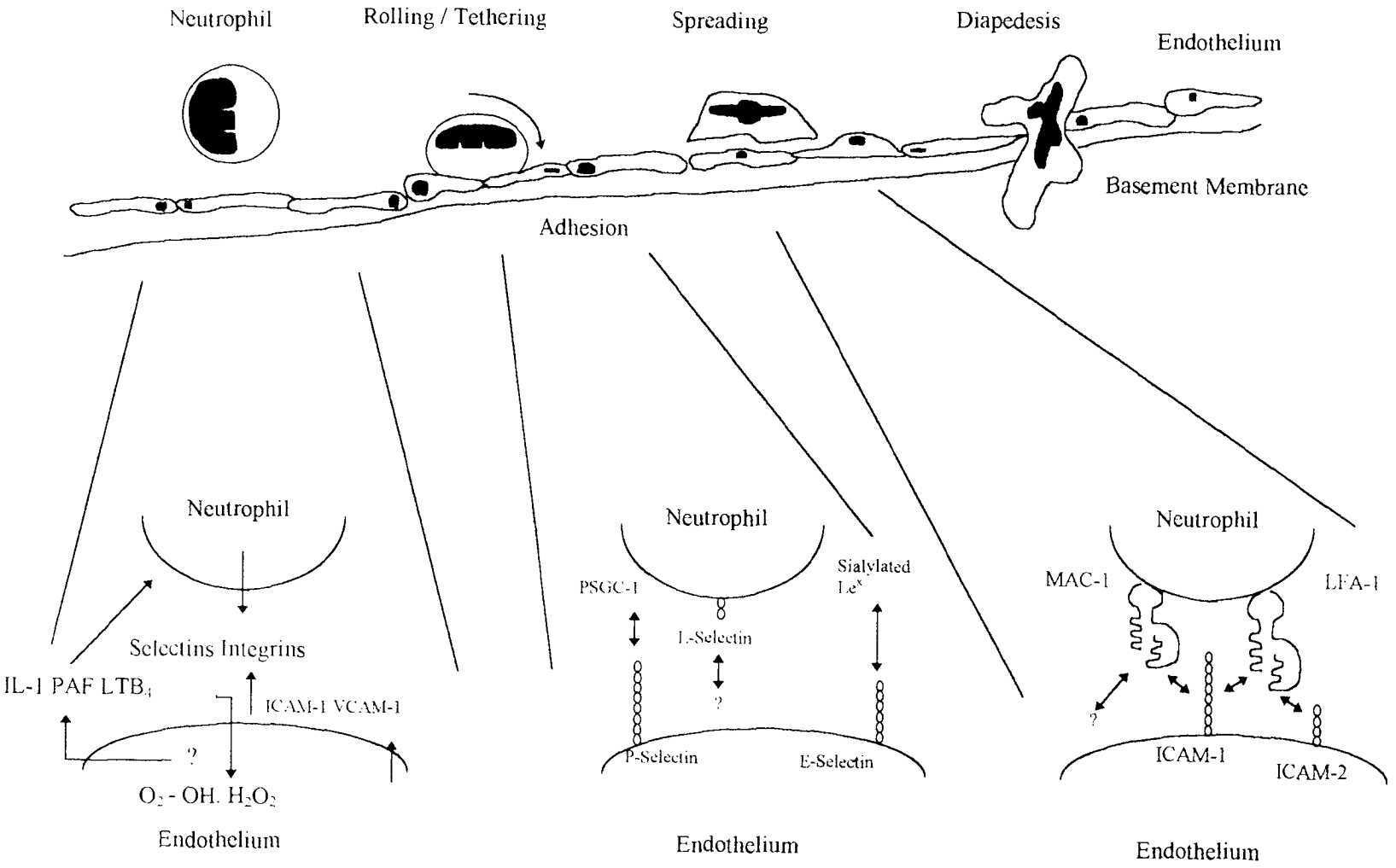
- Neutrophil activation, strengthening of adhesion, and cessation of rolling.

Figure 1.2. Summary of the receptors involved in neutrophil adhesion to the endothelium .



Group	Structure	Name	Cell Distribution
Immunoglobulin Gene Superfamily	Contain at least on large domain 70-110 animo acids long. Disulphide bridges spanning between 50-70 give a tertiary structure like an antibody.	Intercellular Adhesion Molecules (ICAM-1 and ICAM-2).	Endothelium.
		Vascular Cell Adhesion Molecule (VCAM-1)	Endothelium.
Integrins	Composed of two gene products (α and β) linked non-covalently. Very stable. 10α and 8β chains. Any β chain can combine with more than one α.	Very Late Activation Antigen (VLA) (β_1).	?Presence on neutrophils.
		Leukocyte Cell Adhesion Molecules (Leu CAMs) (β_2)	All white blood cells.
		1. Lymphocyte Function-Associated Antigen (LFA-1)	
		2. Macrophage Function-Associated Antigen-1 (MAC-1)	
		3. Glycoprotein (gp) 150,95	Low levels on white blood cells
Selectins	Terminally contain a lectin-like domain followed by a domain with homology to EGF and repeats of a cysteine rich domain homologous to complement regulatory protein.	L-Selectin	Lymphocytes/ Neutrophils.
		P-Selectin	Endothelium/ Platelets.
		E-Selectin	Endothelium

Figure 1.3 Schematic diagram summarizing neutrophil extravasation to sites of infection or inflammation, as described in the text.



- Transendothelial migration (McEver 1992, Adams and Shaw 1994 and Springer 1994).

Neutrophil rolling is mediated by the selectin family of molecules: E-selectin (endothelium), L-selectin (lymphocyte) and P-selectin (platelet) (Bevilacaqua 1993). The selectin family of adhesion molecules all contain a C-type lectin domain, an EGF-like segment and a series of complement repeats. Selectins bind to sialylated carbohydrates related to sialyl Lewis^X and sialyl Lewis^a, although the exact identities of specific ligands of individual selectins have not been completely defined (Lasky 1992 and Bevilacaqua 1993). L-selectin is constitively expressed on the surface of neutrophils but is rapidly shed after neutrophil activation (Kishimoto *et al* 1989). E-selectin is expressed on endothelial cells after stimulation by inflammatory cytokines such as TNFα (Bevilacqua *et al* 1989). P-selectin is stored in platelets and Weibel-Palade bodies of endothelial cells and is rapidly mobilized to the endothelial cell surface in response to various inflammatory stimuli such as thrombin, histamine and oxygen-derived free radicals (Lorant *et al* 1991 and Patel *et al* 1991). The bonds between selectins and their counterligands are weak and at physiological flow rates shear forces initiate selectin-mediated rolling and tethering of circulating neutrophils to the endothelial surface. This facilitates exposure to various neutrophil activators such as PAF (Prescott *et al* 1984, Lewis *et al* 1988 and Zimmerman *et al* 1994) and IL-8 (Huber *et al* 1991, Rot 1992 and Raigner *et al* 1997). At physiological flow rates these events promote neutrophil recruitment by the local microenvironment and provide the basis of activation-induced adhesion through B_2 integrins (Von Adrian 1991 and Springer 1994).

Firm attachment of neutrophils to the endothelial cell and direction of transendothelial migration are mediated by neutrophil B_2 integrins (LFA-1 and MAC-1). These adhesion molecules are surface-associated heterodimeric glycoproteins possessing a common B_2 chain (CD18) and one of three separate α chains (CD11a, CD11b and CD11c) (Arnould 1993). Neutrophils constitutively express B_2 integrins, and chemotactic stimulation results in a rapid but transient conformational changes of CD11b/CD18, which is a prerequisite for firm neutrophil attachment to the endothelium and subsequent migration (McEver 1992, Adams and Shaw 1994, Springer 1994 and Raigner *et al* 1997). Integrins bind to endothelial cell immunoglobulin-like counterligands, namely intercellular adhesion molecule 1

(ICAM-1), which constitutes the principal ligand for neutrophil CD11b/CD18 (Mac-1) and vascular cell adhesion molecule (VCAM-1). CD11b/CD18 is also the receptor for CR3, one of the breakdown products of the third component of complement C3. ICAM-1 is upregulated by cytokine stimulation (Bevilacaqua *et al* 1985).

1.3 Neutrophil chemotaxis

The process of neutrophil migration under guidance is called chemotaxis and is defined as directed cell movement in one direction in response to an agent which signals and induces the cell to move. Generally while neutrophil migration is occurring the microbes are opsonized, that is their surface is coated with antibody and complement factors for recognition by neutrophils (Brown 1991). Neutrophils have receptors specifically designed to bind to the Fc fragment of the IgG molecule present on the surface of the opsonized bacteria and the other receptors designed to bind to the activated complement factors (Anderson *et al* 1990). The complement factors and the antibody molecules are ligands that promote attachment of the microbe to the cell enhancing otherwise inefficient microbe-neutrophil interactions like phagocytosis.

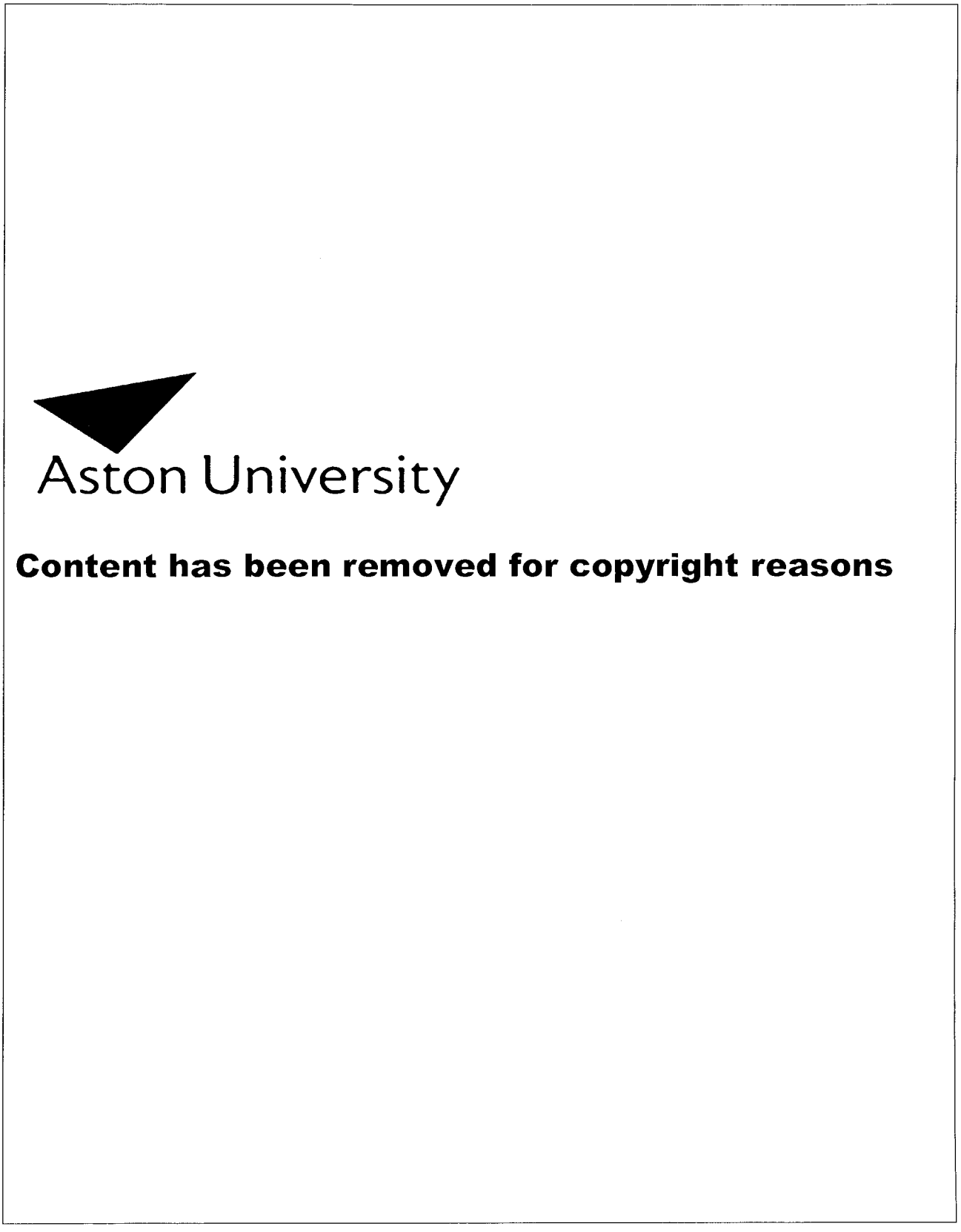
The best known of the numerous neutrophil chemoattractants is C5a, one of the cleaved protein fragments produced by activation of both the alternative and classical pathways of the complement cascade. Others include formylmethionyl peptides (fmlp) of bacterial origin, platelet-activating factor (PAF) and leukotriene B4 (LTB_4) (Harvath 1991). Interleukin 8 (IL-8), a recently characterized proinflammatory cytokine, is the most specific and potent for neutrophils (Kunkel *et al* 1991 and Rainger *et al* 1995). It is a 6.5kDa protein produced by tissue macrophages in response to endotoxin. IL-8 is also produced by fibroblasts and endothelial cells in response to other cytokines, such as tumor necrosis factor (TNFα) and interleukin-1 (IL-1). This networking permits the establishment of a chemoattractant gradient across the endothelium along which neutrophils will migrate (Baggiolini *et al* 1989).

1.3.1 The classical view of chemoattractant signalling

Neutrophil chemoattractant receptors have multiple functions. They not only direct migration, but also activate integrin adhesiveness and stimulate degranulation, shape change, actin polymerization and the respiratory burst. The now "classical" view of chemoattractant receptor signalling stemmed from the recognition of the importance of protein kinase C activity in neutrophil activation as well as the finding that the ability of chemoattractants to stimulate neutrophil function was blocked by pertussis toxin and that chemoattractant receptors were coupled to heterotrimeric GTP-binding proteins (G proteins) (Omann *et al* 1987, Sha'afi 1988 and Cockroft 1992).

A variety of chemoattractant receptors have now been cloned, and these exhibit the seven trans-membrane-spanning structure typical of G-protein-coupled receptors (Thelen *et al* 1993, Gerard and Gerard 1994 and Murphy 1994). The binding of a chemoattractant to its receptor results in the activation of the associated G protein, as shown in Figure 1.4. The majority of neutrophil responses induced by chemoattractants can be inhibited by pertussis toxin, and, consistent with this Gi_2 and Gi_3 are the primary transduction partners associated with these receptors. Upon activation by the ligand-bound receptor Gi dissociates into GTP-bound Gα subunit and the ßγ subunit complex (Gilman 1987). Although the Giα subunit was originally thought to interact with the effector enzyme phospholipase C it is actually the Gβγ complex that regulates phospholipase Cβ isoforms in neutrophils (Katz *et al* 1992). Phospholipase Cβ activation results in the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) to form inositol trisphosphate (IP_3) and diacylglycerol (DAG). Both have important roles as intracellular secondary messengers, with IP_3 acting to mobilize Ca^{2+} from intracellular stores and DAG acting in conjunction with Ca^{2+} to activate various isoforms of protein kinase C (PKC). Activation of PKC, as well as various Ca^{2+}-sensitive protein kinases, catalyzes protein phosphorylation, and this is believed to account for activation of various neutrophil functions, such as chemotaxis and activation (Rossi 1986). The ability of chemoattractants to stimulate phospholipase A_2 and D is also known, but whether these are downstream events resulting from PKC activation and Ca^{2+} mobilization or events regulated by distinct pathways remains unclear (Lew 1990 and Billah 1993).

Figure 1.4 Classical view of neutrophil chemoattractant signalling. Taken from Bokoch 1995.



Although the classical view of neutrophil chemoattractant signalling provides a substantial insight into neutrophil responses to chemoattractants it does not adequately explain all aspects of the activation process. A number of studies (see Section 1.3.2) provide evidence for PKC and Ca^{2+}-independent mechanisms of neutrophil activation, making use of alternative signalling pathways involving kinases and phosphatases, adapter molecules, and small GTP-binding proteins (summarized in Figure 1.5).

1.3.2 Alternative views of chemoattractant signalling

1.3.2.1 Tyrosine phosphorylation

Many studies have shown that neutrophil responses to chemoattractants can be blocked with inhibitors of tyrosine phosphorylation, indicating that tyrosine phosphorylation may also play an important role in chemoattractant signalling (Naccahe *et al* 1990). Fmlp and other chemotactic agents increase the tyrosine phosphorylation of a number of proteins in human neutrophils. This appears to be caused by both the activation of tyrosine kinases as well as by inhibition of tyrosine phosphatases though the phosphatases and kinases involved have not been well characterized (Bokoch 1995). A number of nonreceptor tyrosine kinases of the Src family have recently been shown to participate in neutrophil cell signalling responses to growth factors and to IgG binding to Fc receptors. These include Lyn, Yes, Hck, Fgr, c-Src as well as the non-Src kinase, Syk (Gee *et al* 1986, Willman *et al* 1991, Agarwal *et al* 1993, Corey *et al* 1993, Hamada *et al* 1993, Berton *et al* 1994, Durden *et al* 1994, Ravetch *et al* 1994 and Zhou *et al* 1995,). In light of the importance of these kinases, it is perhaps not surprising that chemoattractant receptors also appear able to use these mechanisms for regulating cell function. The fmlp receptor has recently been shown to activate the Lyn tyrosine kinase, stimulating autophosphorylation and activity towards exogenous targets (Stephens *et al* 1994). One of these phosphorylation targets is the Shc adapter protein. Shc is a member of a growing family of adapter molecules made up largely of SH2 and SH3 protein-binding motifs that serve to link together other proteins involved in cellular signalling

Figure 1.5. Updated view of chemoattractant signalling. Taken from Bokoch 1995.

An updated view of chemoattractant receptor signalling: Multiple pathways. Schematic diagram summarizing the known signalling pathways and effectors used by leukocyte chemoattractant receptors, as described in the text.

(Pawson and Schlessinger 1994). Associated with the Lyn-Shc kinase, formed during chemoattractant stimulation is PI 3-kinase, an important signalling enzyme whose activity may be regulated by this interaction (Pawson and Schlessinger 1994 and Pleinman *et al* 1994). Shc is also known to link receptors and Src-related kinases to a second adapter molecule, Grb2. This is of interest because a fraction of Grb2 in cells is normally constitutively bound to the mammalian Ras guanine nucleotide exchange regulator termed mSOS (the mammalian homologue of the yeast Son of Sevenless gene) (Rozakis-Adcock *et al* 1992 and Egan *et al* (1993)). Thus, the interaction of chemoattractant receptors with Shc can potentially lead to activation of the small GTPase, Ras.

1.3.2.2 The Ras/MAPK Cascade

Ras is indeed activated acutely in chemoattractant-stimulated human neutrophils, with the level of GTP-bound active Ras rapidly increased in stimulated cells (Worthen *et al* 1994). Activation of Ras is a component of normal cell growth in response to growth factors, as well as of malignant growth induced by oncogenes. Ras activation in these instances leads to stimulation of a cascade of protein kinases that ultimately phosphorylate and activate mitogen-activated protein kinases (MAPK) (Davis 1993).

Ras that has been activated either by tyrosine kinase receptors and/or G protein-coupled receptors can initiate the MAPK cascade by binding to the serine/threonine kinase Raf. Recent work has determined that Ras serves to translocate Raf from the cytoplasm to the plasma membrane where it becomes activated in a Ras-independent manner (Freed *et al* 1994 and Stokoe *et al* 1994). A second family of kinases, termed MEKKs (MAPK/ERK kinase) can also be regulated through a process involving Ras and heterotrimeric G proteins (Winitz *et al* 1993 and Johnson *et al* 1994). Both Raf and MEKK phosphorylate and activate MAPK/ERK, which is a dual function kinase that in turn catalyzes both the threonine and tyrosine phosphorylation of MAPK/ERK itself, activating the enzyme and leading to the regulation of a variety of downstream targets. The basic components of this kinase cascade have been shown to be present and active in neutrophils during chemoattractant stimulation. Raf-1 and B-Raf are both activated by the C5a receptor (Buhl *et al* 1994). Activation of both enzymes leads to the phosphorylation of MEK-1. Neutrophils express both MEK-1 and MEK-2, but only MEK-1 has been shown to undergo activation by chemoattractants

(Worthen *et al* 1994 and Grinstein *et al* 1994). Activation of all these responses is blocked by pertussis toxin, indicating that they are mediated through Gi. Both PKC-dependent and -independent activation mechanisms appear to be operative (Buhl *et al* 1994, Grinstein *et al* 1994 and Worthen *et al* 1994)although the exact mechanism through which Ras becomes activated remains to be fully elucidated. MEKK has been shown to be stimulated by TNFα in mouse macrophages (Winston *et al* 1995) though its activation in response to chemoattractants has not yet been reported However, the indications are that chemoattractant receptors can acutely regulate activity of the Ras/MAPK pathway, and that this regulation is likely to play an important role in the early signalling events leading to cell activation.

1.3.2.3 Other Serine/Threonine Kinases

Neutrophil stimulation by N-formyl peptides induces the rapid and transient activation of a group of ser/thr kinases of approximately 40, 49, 63 and 69KD (Grinstein *et al* 1993). These kinases exhibit the ability to be renatured after gel electrophoresis, and retain their activation state under these circumstances. Activation is inhibited by pertussis toxin but is not induced by phorbol myristate acetate (PMA) or blocked by staurosporine. Interestingly, activation of these kinases is also blocked by wortmannin (Ding and Badivey 1994) and Ly294002, inhibitors of PI-3 kinase, suggesting that the activities of the renaturable kinases may be dependent on the lipid messengers generated by PI 3-kinase (Ding and Badivey 1994). The renaturable kinases remain incompletely characterized, with their structure and regulatory properties still unknown. The identification of neutrophil p21-activated kinases (Paks) as members of this group suggests that low-molecular-weight GTP-binding proteins (LMWG) (Knaus *et al* 1995) are involved in the regulation of these signalling enzymes. The close correlation between activation of the renaturable kinases and acute neutrophil stimulation by chemoattractants makes it likely that they are participants in regulating early events in pathways leading to activation of the respiratory burst, cytoskeletal assembly and motility, and possible vesicle secretion.

1.3.2.4 Phosphatidylinositol 3-Kinase (PI3K)

The enzyme PI3K catalyses the addition of a phosphate group to the D3-position of phosphatidylinositol lipids, i.e., phosphorylation of phosphatidylinositol bisphosphate (PIP_2) generates phosphatidylinositol trisphosphate (PIP_3) (Cockroft 1992). The lipid products of PI3K have been implicated in signalling pathways leading to cell growth and cytoskeletal assembly (Cantley *et al* 1991). In the human neutrophil PI3K activity is rapidly stimulated by chemoattractants (Stephens *et al* 1991). Formation of PIP_3 correlates with actin assembly in fmlp-stimulated neutrophils and it has been suggested to be involved in this process (Eberle *et al* 1990). More recently the availability of inhibitors of PI3K (wortmannin and Ly294002) have enabled its role in neutrophil activation to be better defined. The major effect of PI3K inhibition appears to be blockade of chemoattractant stimulation of NADPH oxidase (Okada *et al* 1994). Overall actin polymerization is not blocked but wortmannin can induce oscillations in F-actin content (Wymann *et al* 1989). In many other cells PI3K appears to be involved with the cytoskeletal changes accompanying membrane ruffling, a process involving the localized polymerization of actin filaments to produce short, highly cross-linked membrane-associated fibers (Ridley *et al* 1992). Wortmannin blocked fmlp-stimulated granule secretion in neutrophils (Dewald *et al* 1988).

PI3K is a heterodimeric protein made up of an 85-kD regulatory subunit and a 110-KD catalytic subunit. The mechanisms of regulation are not well defined. PI3K is recruited to phosphotyrosine residues on activated tyrosine kinase receptors and other tyrosine-phosphorylated proteins via the SH2 motifs present on the p85 subunit. Activation appears to result from these binding interactions (Pawson and Schlessinger 1993). The mechanism by which PI3K is controlled by G-protein-coupled chemoattractant receptors does not appear to be the result of a direct recruitment of PI3K or tyrosine phosphorylation (Vlahos and Matter 1992). Regulation may involve the Src-related tyrosine kinase Lyn, which is activated by the fmlp receptor and which binds PI3K. It has also been recently demonstrated that PI3K activity can be regulated by Ras, with GTP-bound Ras binding directly to the 110-kD catalytic subunit (*Kodaki et al* 1993 and Gout *et al* 1994). Interactions of PI3K with other small GTP-binding proteins have also been described though the relative

contributions of the multiple forms of PI3K to neutrophil activation remain to be defined (Zhang *et al* 1993 and Zhang *et al* 1994).

The signalling cascades thus far identified are extremely complex and must interact with the mechanisms responsible for neutrophil chemotaxis, adhesion, phagocytosis, respiratory burst and granule discharge.

1.4 Neutrophil activation

After attachment to a microbe or particle neutrophils engulf the microbe/particle and ingestion takes place. Once a microbe is ingested by a neutrophil it is usually rapidly killed and digested (Thomas and Lehrer 1988). Most micro-organisms are digested after being killed and the bacterial components are rapidly degraded by the neutrophil's numerous granule-associated enzymes. The degree of digestion depends on the structure of the bacterial cell envelope and on the presence of digestive enzymes in the phagocyte.

Phagocytosis starts with receptor-ligand binding between the neutrophil and microbe. Neutrophils may attach to microorganisms via their non-specific cell surface receptors or, if the organism is opsonized with a fragment of the third complement component (C3b), through their cell surface receptors for C3b. The receptor-ligand interaction activates the ingestion phase involving actin, myosin and actin-binding proteins. Actin microfilaments underlying the site of attachment undergo polymerisation. This polymerization leads to puckering of the plasma membrane at the site of contact, because the microfilaments attach to the membrane. The membrane envelops the particle and new particle-membrane contacts result. Pseudopodia, finger like projections of the plasma membrane, are produced, the particle becomes surrounded by these pseudopodia and a phagocytic vacuole , the phagosome, occurs (Sawyer *et al* 1989). While this is happening, enzyme-containing granules in the cytoplasm fuse with the phagosome membrane, and a new phagolysosome is produced. Neutrophils possess two main types of granules (Boxer and Smolen 1988). The primary (azurophilic) granules (lysosomes) contain acid hydrolases, myeloperoxidase and muramidase (lysozyme) whilst the secondary or specific granules contain lactoferrin in addition to lysozyme. Primary granules are first seen in the promyelocyte while

secondary granules appear in both myelocytes and metamyelocytes (Bainton *et al* 1971).

Neutrophils are able to kill micro-organisms within the phagosome by two distinct mechanisms. One system is oxygen dependent, while the other can kill bacteria in the absence of oxygen. Extracellular release of granules and cytotoxic substances may also occur on stimulation, via complement or Fc receptors, causing damage. Such release may be important in immune complex diseases such as type III hypersensitivity (Elsbach and Weiss 1985).

The oxygen-dependent mechanism of killing is set in motion when neutrophils undergo an oxidative burst consuming molecular oxygen to generate reactive oxygen species (ROS) such as O_2^-, $OH^·$ and H_2O_2. The enzyme responsible for this is NADPH oxidase which remains inactive until the neutrophil is stimulated by engagement of receptors for chemoattractants, cytokines or receptors mediating phagocytosis (Baker *et al* 1986). Active NADPH oxidase appears to be a multi-component enzyme system, consisting of four proteins: the heterodimeric cytochrome b_{558} and the two cytosolic proteins, p47-*phox* and p67-*phox* (Clark 1990) . The active enzyme complex is membrane-associated and appears oriented to interact with NADPH on one side of the membrane and O_2 on the other (McPhail and Snyderman 1983). Cytochrome b_{558} is thought to be the terminal component in the presumed electron transport chain and transfers electrons directly to O_2 (Segal 1989). Although genes for the two subunits of cytochrome b558 have been cloned the structural properties of the heterodimer responsible for its functional activity are not yet understood (Segal 1989 and Clark 1990). Genes for the two cytosolic components have also been cloned and neither predicted sequence reveals a possible electron transfer function. Therefore, the contribution of these proteins to the structure and function of the assembled oxidase is not clear.

The signalling processes induced result in the rapid appearance of NADPH oxidase activity. The active enzyme catalyses the following reaction:

$$NADPH + 2O_2 \longrightarrow NADP^· + 2O_2^- + H^·$$

The activation of NADPH oxidase thus accounts for the burst of O_2 consumption during cell stimulation (Curnutte 1985). Increased levels of $NADP^{\cdot}$ activate the pentose phosphate pathway (hexose monophosphate shunt) and NADPH is regenerated. The $O_2^{\cdot}$ formed by NADPH oxidase activity can be rapidly converted to H_2O_2 and other toxic species and these are responsible for injury to micro-organisms and the surrounding tissue (Weiss 1989). H_2O_2 is a powerful oxidant but it reacts sluggishly with biological materials and many micro-organisms contain enzymes that detoxify it, such as catalase (Thomas and Lehrer 1988). However H_2O_2 and $O_2^{\cdot}$ can give rise to more potent agents in the presence of ferric ions or Fe^{3+} chelates such as hydroxyl radicals $OH^{\cdot}$ and HOCl (Klebanoff and Walterdorph 1990). Because $OH^{\cdot}$ reacts rapidly with chemical bonds of all kinds, it causes damage near the site where it is produced. HOCl is also a potent microbicidal agent (Cohen *et al* 1988). It reacts rapidly with ammonia to yield monochloramine and with amines to yield mono and dichloroamines (Britigan and Edeker 1991). These oxidants not only cause direct vascular injury but they also up regulate P and E selectins on the endothelial cells (Anderson 1995).

In addition to these factors neutrophil granules contain additional antimicrobial agents that are released into phagolysosomes and do not require the production of oxidants for activity. These agents include proteases, other hydrolytic enzymes, such as phospholipases, glycosidases and lysozymes, and other proteins and peptides that disrupt microbial functions or structural components. It is likely that all of these agents must bind to the microbial cell surface to have antimicrobial activity. The activity of each agent is limited to certain micro-organisms. Three well-characterized granule components known as B/PI (bactericidal/permeability-increasing protein), CLCP (chymotrypsin-like cationic protein; cathepsin G) and defensins have microbial activity in *vitro* (Selsted *et al* 1985, Thomas and Lehrer 1988, Sawyer *et al* 1989, and Wasiluk *et al* 1991). B/PI is a 58 kDa protein found in the primary granules. It is responsible for the neutrophils ability to kill gram-negative bacteria by increasing their outer membrane permeability to hydrophobic molecules and by activation of enzymes that are able to degrade peptidoglycan and outer membrane phospholipids. Other granular agents that contribute to the bactericidal activity of neutrophils are elastase and lactoferrin. Lactoferrin is bacteriostatic as it binds iron and bacteria do not multiply in the absence of iron. Lactoferrin also alters the cell wall of the bacteria

so that it becomes more accessible to lysozyme. Thus, lactoferrin and lysozyme act synergistically (Ellison and Giehl 1991).

1.4.1 Neutrophil priming

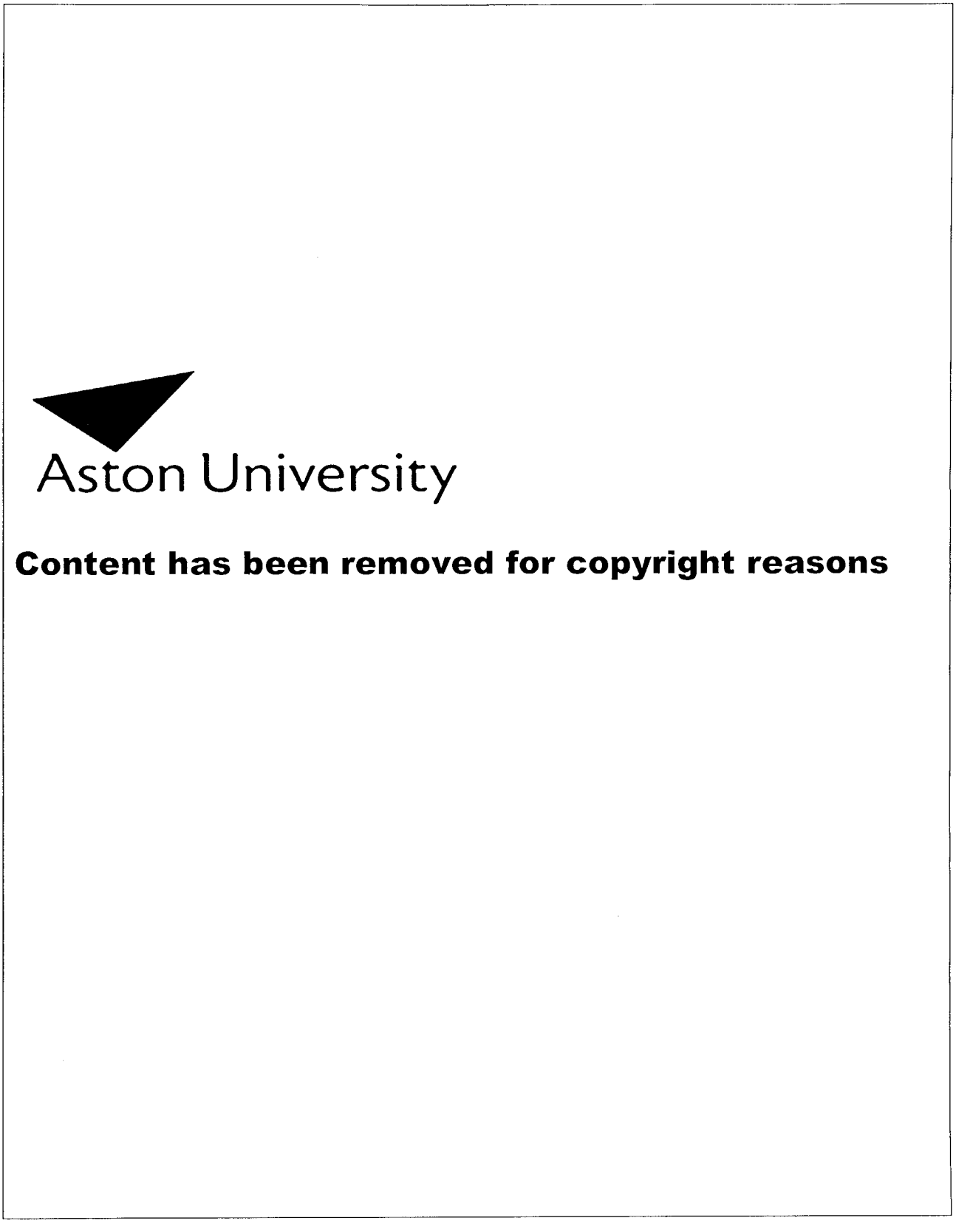
When neutrophils are exposed to 1μM of PAF there is only a small release of superoxide anion. However, when these PAF-exposed cells are then stimulated by fmlp they produce 3-4 times the amount of O_2^- when compared to naive cells stimulated with fmlp (Walker *et al* 1991). This enhanced response is referred to as 'priming'.

Priming exerts a dramatic control over neutrophils. Circulating unprimed neutrophils have a limited capacity to damage tissues or kill microorganisms that they may encounter by chance. During inflammation endothelial cells are modified following contact with mediators such as cytokines (TNFα and IL-1) which upregulate adhesion molecules that promote neutrophil adherence. In tandem mediators are released that diffuse from the site of production into the surrounding tissues producing a concentration gradient. Neutrophils as already discussed have the capacity to 'sense' the chemotactic factors within these gradients and move towards the inflammation site moving through increasingly higher concentrations of the mediators (Colditz 1985). At low concentrations some of these would prime the cells. Closer to the centre of the inflammation focus, the mediator concentration might be sufficient to initiate the release of both cytotoxic proteins and oxygen-free radicals. Priming of neutrophils may also occur within the intravascular space if priming agents are released into the blood in high enough concentrations (Tennenbeg and Solomkin 1988). The most notable circulating priming agent is bacterial endotoxin (Guthrie *et al* 1984 and Forehand *et al* 1989).

1.4.2.1 Priming agents

A variety of agents have the capacity to prime neutrophils (See Figure 1.6). Although several signal transduction pathways have been described for agents that have a priming activity, the biochemical mechanisms responsible for priming are not known. In an

Figure 1.6 Neutrophil priming agents. Taken from Walker and Ward 1992.



attempt to understand the priming response one can group these agents into two main categories: receptor-dependent and receptor-independent. Receptor-dependent priming agents are probably physiologically relevant immunomodulators. The priming of neutrophils by receptor-ligand interactions can be further subdivided into two groups based upon the pattern of their activity. In the first group are the agents that produce a rapid onset of the primed state, with a near maximal effect achieved with 1-2 minutes of cell exposure. These agonists all produce transient increases in intracellular calcium, with responses being mediated by a pertussis toxin-sensitive signal transduction pathway. Included with this group are PAF, LTB_4, fmlp, IL-8, ATP and UTP (Ward *et al* 1988). The second group of priming agents is slower in onset, requiring 15-30 minutes for a maximal primed response. These agents producing priming without increases in calcium include endotoxin and the cytokines TNFα, IL-1, GM-CSF and IL-6 (Guthrie *et al* 1984, Gay *et al* 1986 and Ward *et al* 1988). For both groups the primed state persists for greater than 15 minutes even after the removal of the priming agent.

1.4.2.2 Physiological features of priming

The term priming can be applied to the synergistic increase in any functional or biochemical event. With reference to neutrophils, priming most commonly refers to the enhanced production of O_2^- generated from a membrane associated NADPH oxidase. However priming has also been associated with release of granule-associated enzymes (Dularay *et al* 1990), phagocytosis (Shalaby *et al* 1985 and Detmers *et al* 1991), chemotaxis (Gallin *et al* 1978), microbicidal activity (Djeu *et al* 1990), depolarization (Zimmerli *et al* 1990) and PAF production (De Nichilo *et al* 1991).

It has been suggested that exposure to priming doses of A23187 or PMA causes fusion of specific granules with the plasma membrane. These granules have 'cryptic' fmlp receptors on the inner surface of their membranes and these receptors can be adducted to the external cell membrane of the primed neutrophil (Fletcher *et al* 1983). Granule fusion brings the inner surface of the granule into continuity with the plasma membrane's outer surface. In this way the inner cryptic receptors are recruited to the plasma membrane, resulting in an increase in the total fmlp receptor number. The increase in fmlp receptor number is proposed to result in the primed

response to fmlp and has been suggested for many other agonists (Finkel *et al* 1987). If neutrophils are depleted of their granules by ultracentrifugation in the presence of cytochalasin B, the cells are resistant to the priming effect. Receptor upregulation is also variably reported for priming by endotoxin though receptor regulation has not been associated with priming by TNFα (Guthrie *et al* 1984 and O'Flaherty *et al* 1991)

While there is evidence that receptor upregulation is a key component in the priming response, there are still some unanswered questions. In some studies the increase in receptor number associated with priming is only seen over a narrow concentration range of agonist (Gallin *et al* 1978). At higher doses both PMA and A23187 cause receptor downregulation. Also priming is seen between PMA and A23187, both of which are receptor-independent agonists, suggesting that these agents have effects distal to the plasma membrane events (Dahlgren 1989). PAF also primes the neutrophil to respond to PMA and generate ROS (Tennenberg and Solomkin 1990). Finally receptor upregulation has not been shown for the priming of neutrophils by ATP, endotoxin and priming by TNFα of the LTB_4 induced enzyme release occurs in spite of receptor downregulation (Berkow *et al* 1987). Thus changes in fmlp receptor number does not predictably correlate with the primed response.

Clearly chemotaxis and the respiratory burst share portions of the signal transduction machinery. Initiation of both responses proceeds via chemoattractant binding to its receptor and subsequent participation of one or more pertussis toxin-sensitive G proteins (section 1.3.1). However, a variety of studies indicate that differences exist in the signalling mechanisms that trigger chemotaxis and the respiratory burst. Certain pharmacological agents differentially affect the two responses. General inhibitors of protein kinases, such as the isoquinolinesulphonamides, reversibly inhibit neutrophil chemotaxis to fmlp and C5a, but do not inhibit the respiratory burst under similar conditions (Harvath *et al* 1987). These results suggest that the kinases controlling migration and the respiratory burst are different.

In conclusion neutrophils are the cornerstone of host defense. Without adequate numbers of neutrophils or properly functioning neutrophils, patients suffer from infections. The basic processes that are involved in the elimination of invading micro-organisms are chemotaxis, opsonization, digestion and killing. However, there is now strong evidence that neutrophils are also an important source of injury following ischaemia/reperfusion.

1.5 Biochemistry of ischaemia/reperfusion

Reperfusion injury is initiated by biochemical events during ischaemia which result in the generation of ROS such as $OH^{\cdot}$, HOCl and H_2O_2. The initial step in production of reactive oxygen species is a build up of xanthine oxidase (XO) in endothelial cells and subendothelial tissue. XO is the rate limiting enzyme in nucleic acid degradation through which all purines are channeled for terminal oxidation. Normally XO exists in non-ischaemic cells as an NAD+-dependent dehydrogenase (XDH) which, using NAD as an electron acceptor, oxidizes hypoxanthine to xanthine (Budd *et al* 1990).

$$\text{Hypoxanthine} + H_2O_2 + NAD^{+} \xrightarrow{\text{XDH}} \text{Xanthine} + NADH + H^{+}$$

During ischaemia XDH is converted to XO (Kurose and Granger 1994). Parkes *et al* (1988) were able to demonstrate that the amount of XDH converted to XO is directly proportional to the length of hypoxia.

In ischaemic tissue, therefore, there is a build up of XO and hypoxanthine as XDH is not present to convert it to xanthine and though XO can oxidize hypoxanthine it uses oxygen (O_2) not NAD as its substrate. On reintroduction of oxygen XO converts hypoxanthine to xanthine generating superoxide anion and hydrogen peroxide. XO is also able to oxidize xanthine producing further superoxide anion and hydrogen peroxide.

$$\text{Hypoxanthine} + 2O_2 + H_2O \xrightarrow{\text{XO}} \text{Xanthine} + O_2^{\cdot-} + H_2O_2$$

$$\text{Xanthine} + 2O_2 + H_2O \xrightarrow{\text{XO}} \text{Uric Acid} + O_2^{\cdot-} + H_2O_2$$

In the presence of iron (Fe^{2+}) or copper (Cu^{2+}) ions hydroxyl radicals ($OH^{\cdot}$) can also be produced by the reaction of superoxide anions and hydrogen peroxide (Harber-Weiss Reaction) (Welbourn *et al* 1991). This large burst of superoxide ROS by the endothelium can be detected 2-4 minutes after reperfusion (Lefer and Lefer 1993).

The postischaemic neutrophil itself is also another potential source of reactive oxygen species. Neutrophils contain an NADPH oxidase that reduces molecular oxygen to

superoxide anion. Neutrophils, activated to adhere by soluble factors produced by postischaemic endothelium, also secrete the enzyme myeloperoxidase (MPO) which catalyzes the production of hypochlorous acid (HOCl) from hydrogen peroxide and chloride ions (Cl) (Anderson 1995).

$$H_2O_2 + Cl^- + H^+ \xrightarrow{MPO} HOCl + H_2O$$

The hydrogen peroxide needed to form HOCl is derived from the spontaneous dismutation of NADH-derived superoxide. HOCl is a potent oxidizing and chlorinating agent that is approximately x100 more potent than hydrogen peroxide. It reacts rapidly with primary amines (RNH2) to produce N-chloro derivatives (RNHCl), which have oxidizing equivalence of hydrogen peroxide and hypochlorous acid (Zimmerman and Granger 1994).

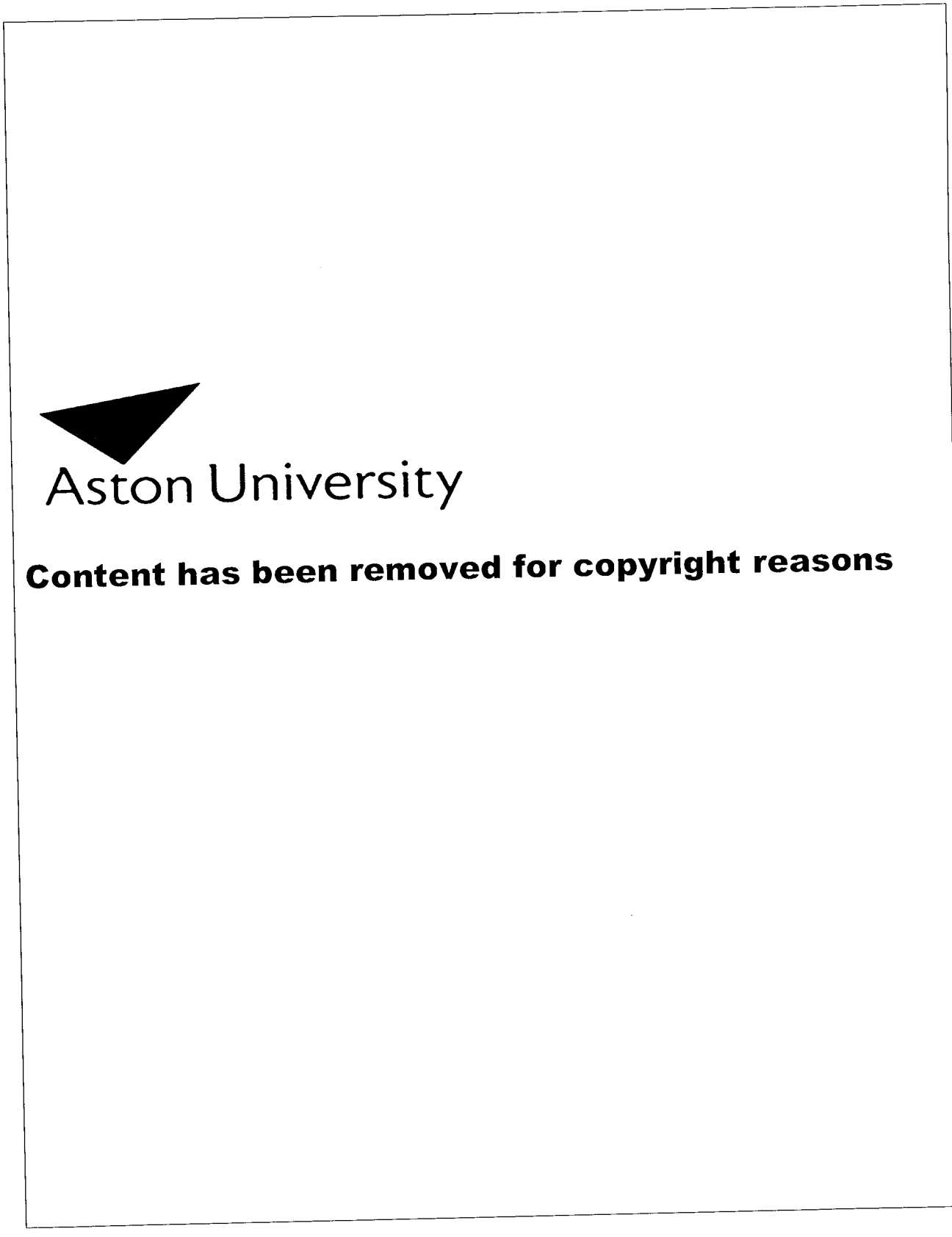
$$HOCl + RNH_2 \longrightarrow H_2O + RNHCl$$

Figure 1.7 taken from Kurose and Granger (1994) summarizes the mechanisms of neutrophil and endothelial cell mediated oxygen radical formation following ischaemia/reperfusion injury. Production of neutrophil-generated oxygen-derived free radicals can increase the endothelial damage caused by endothelial oxygen radical formation. However, this initial dysfunction may be amplified further by neutrophil/endothelial derived factors such as cytokines, proteases and lipid mediators (Lefer and Lefer 1993).

1.5.1 Reactive oxygen species and neutrophil accumulation

Estimates of neutrophil infiltration into the postischaemic intestinal mucosal have been derived from biochemical determinations of tissue MPO activity. Zimmerman *et al* (1990) demonstrated that a five to seven fold increase in MPO activity was observed during the ischaemic period, whereas reperfusion produced an 18-fold enhancement of activity. Treatment with either superoxide dismutase (SOD) (superoxide scavenger) or allopurinol (a xanthine oxidase inhibitor) significantly attenuated this

Figure 1.7 A summary of neutrophil and endothelial cell mediated oxygen radical formation following Ischaemia/reperfusion injury. Taken from Kurose and Granger 1994.



mucosal MPO activity observed after reperfusion. The neutrophil accumulation initiated by reperfusion was also significantly attenuated by treatment with either catalase (reduces H_2O_2 to H_2O and O_2), and dimethylthiourea ($OH^{\cdot}$ scavenger). The ability of XO inhibitors, oxygen radical scavengers, and or iron chelator to interfere with reperfusion-induced neutrophil infiltration suggests that XO-derived oxidants play a role in the recruitment of neutrophils in postischaemic intestine. It is postulated that XO-derived oxidants, produced in the epithelial and endothelial cells, initiate the production and release of proinflammatory agents, which subsequently attract and activate neutrophils. This hypothesis would explain why agents such as allopurinol, superoxide dismutase, catalase, dimethylthiourea, and deferoxamine attenuate both the neutrophil infiltration and the microvascular injury induced by reperfusion of ischaemic tissue (Kurose and Granger 1994).

The precise steps linking xanthine oxidase and reactive oxygen metabolites to chemotactic activity are not well understood. Moreover, the exact nature of the chemotactic agents operative in different settings of ischaemia are unknown, although arachidonic acid products and complement fragments appear to be of key importance (Pickard *et al* 1980). Breakdown products of arachidonic acid are found in high concentrations in plasma soon after reperfusion of ischaemic tissue. One consequence of oxygen radical release is that intracellular free calcium rises dramatically, and this is thought to be a crucial step in the activation of plasma membrane phospholipase A_2 and subsequent generation of products of arachidonic acid from the endothelium (Ernster 1988). Certainly inhibitors of oxygen radicals with scavenging enzymes prevents the release of arachidonic acid metabolites after reperfusion (Klausner *et al* 1989), and the superoxide ion seems to be a prerequisite for the generation of neutrophil chemotactic activity (Petione *et al* 1980). The main pathways of arachidonic acid breakdown are shown in figure 1.8. Evidence is lacking that arachidonic acid metabolites induce endothelial injury in ischaemia and reperfusion independently of neutrophils. There are, however, three mechanisms by which arachidonic acid products might influence neutrophils in reperfusion injury. Firstly, they could act as chemoattractants and induce neutrophil adhesion to endothelium. The lipoxygenase product LTB_4 and the cyclooxygenase product thromboxane A_2 are potent chemo-attractants known to do this (Gimbrome *et al* 1984 and Spangnuolo *et al* 1980). Evidence suggests that LTB_4 is generated in

Figure 1.8 The arachidonic acid cascade showing essential products and intermediates. LT, leukotriene; Tx, thromboxane; PG, prostaglandin. Taken from Welbourn *et al* (1991).

sufficient quantity in ischaemia reperfusion to induce neutrophil diapedesis (Welbourn *et al* 1990). Other observations indicate that lipoxygenase products are of central importance in determining reperfusion injury. Their inhibition reduces neutrophil accumulation and injury in the myocardium after infarction (Mullane *et al* 1984) and following hind limb tourniquet ischaemia (Klausner *et al* 1988). Secondly, arachidonic acid products may activate neutrophils to produce more oxygen radicals and proteolytic enzymes. LTB_4 is a potent stimulus for neutrophil generation of hydrogen peroxide and elastase (Smedly *et al 1986)*, which has been shown to induce endothelial permeability *in vitro* and *in vivo* (Arfors *et al* 1987). Thromboxane A_2 also activates neutrophils and mediates their H_2O_2 production following ischaemia (Paterson *et al* 1989).

Thirdly, leukotrienes and thromboxane affect blood flow and therefore tissue perfusion by direct action on the microvasculature. For example, thromboxane-mediated vasoconstriction exacerbates poor capillary flow after reperfusion (Ogletree 1987). A demonstration of this phenomenon occurs in ischaemia and reperfusion of the kidney where there is debate as to whether reperfusion injury is in fact neutrophil-dependent (Klausner 1989 and Thornton 1989). It is thought that the major mechanism of this injury is the development of an imbalance between vasodilating and vasoconstricting agents. The importance of thromboxane is indicated by the ability of vasodilating prostaglandins to attenuate damage, but only if thromboxane is inhibited at the same time (Tobimatsiu 1987 and Klausner 1989). The kidney's need for vasodilating prostaglandins is shown clinically by the potential for renal damage following administration of cyclooxygenase inhibitors such as ibuprofen or aspirin, even though these drugs inhibit thromboxane synthesis (Clive and Stoff 1984). The renal injury is due to inhibition of prostaglandin synthesis which limits the ability of the renal vasculature to vasodilate. It is not known whether leukotrienes C_4 and D_4 are important in ischaemia, although they are potent vasoconstrictors (Samuelsson 1983).

1.6 Neutrophil adhesion to ischaemic/reperfused endothelium

Monoclonal antibodies (Mabs) directed against various neutrophil and endothelial cell adhesion molecules have been used to define the molecular determinants of the

neutrophil adhesion induced by ischaemia/reperfusion. Pre-treatment with CD18 specific MAbs completely prevents the neutrophil adhesion and emigration normally observed in feline mesentery exposed to ischaemia and reperfusion (Oliver *et al* 1991). The effectiveness of CD18 specific MAbs in reducing leukocyte adhesion stem in part from the fact that these antibodies immunoneutralize the common B-subunit of all three heterodimers of CD11/CD18. Studies employing Mabs directed against α-subunits of CD11/CD18 reveal that anti-LFA-1 (CD11a) and anti-Mac-1 (CD11b) antibodies reduce reperfusion injury in feline mesentery by 60-65% and emigration by 45-50%. An ICAM-1 specific antibody reduced reperfusion-induced adherence by 40% and emigration by 50%. These results indicate that the neutrophil adherence and emigration observed in postcapillary venules exposed to ischaemia and subsequently reperfused are mediated by both LFA-1 and Mac-1 on neutrophils and that at least in part ICAM-1 on endothelial cells acts as the ligand for both heterodimers of CD11/CD18 (Oliver *et al* 1991).

An *in vitro* model of hypoxia-reoxygenation-induced neutrophil adherence to endothelial monolayers has also been used to assess the contribution of different adhesion molecules (Yoshida *et al* 1992). The adherence of human neutrophils to monolayers of human umbilical vein endothelial cells under normoxic conditions was inhibited by MAbs against CD11a and CD18. Inhibition was also observed with MAbs directed against ICAM-1, but not against E-selectin. Thirty minutes of hypoxia followed by 30 mins of reoxygenation resulted in a 2.7 fold increase in neutrophil adherence to the cultured endothelial cells. The enhanced adhesion was also inhibitable by MAbs against CD11a, CD11b or CD18. The inhibitory effects of anti-CD11a and CD11b specific antibodies were additive and equivalent, in combination, to that observed with CD18 specific MAb, which completely prevented the hyperadhesivity. Furthermore Yoshida *et al* (1992) illustrated that the supernatants recovered from endothelial cells exposed to hypoxia/reoxygenation increased the expression of CD11b and CD18, but not CD11a, on the surface of neutrophils. This suggests that an inflammatory mediator was released from endothelial cells exposed to hypoxia/reoxygenation.

The results obtained from this *in vitro* model are both qualitatively and quantitatively consistent with the data obtained from *in vivo* models. Furthermore, the *in vitro* data indicates that endothelial cells exposed to hypoxia/reoxygenation release a soluble

factor that results in the expression and/or activation of CD11b/CD18 on neutrophils. The counterligand on the endothelial cell surface is likely to be ICAM-1 as enzyme immunoassay studies indicate that ICAM-1, but not E-selectin, is constitutively expressed on endothelial cells and that hypoxia/reoxygenation does not increase the expression of either adhesion glycoproteins (Yoshida *et al* 1992).

1.7 Complement, chemokines and other chemotatic factors possibly involved in ischaemic/reperfusion injury

During myocardial ischaemia and reperfusion, the complement cascade may be activated after complement proteolysis by myocardial proteases or by interaction between complement component C1 and heart mitochondrial membranes released from disrupted myocytes (Rossen *et al* 1988). In addition neutrophils can directly activate complement by actions of proteases or reactive oxygen species (Perzel *et al* 1983 and Shigu and Nobunaga 1984). Complement fixation has been demonstrated in ischaemic myocardium and appears to correlate with the localization of neutrophil accumulation. Evidence indicated that experimental myocardial ischaemia rapidly induces complement activation and the ability of postischaemic cardiac lymph to stimulate isolated neutrophils is neutralized by anti-C5a antiserum (Dreyer *et al* 1989 and Dreyer *et al* 1992).

The role of the complement system in myocardial ischaemia has been recently reviewed (Kilgore *et al* 1994). C5a is a strong neutrophil chemoattractant, and generation of C3bi on the endothelial surface *in vitro* elicits rapid CD11b/CD18-dependent neutrophil adhesion (Marks *et al* 1989). In pigs, intracoronary administration of C5a reduces coronary blood flow and myocardial contractile function by mechanisms dependent on neutrophil accumulation and production of thromboxane A2 and leukotrienes (Ho *et al* 1990). The canine coronary vasculature may be less responsive to thromboxanes, and C5a appears to dilate canine coronary arteries *in vivo* and *in vitro* (Schumacher *et al* 1991). In addition to recruitment and activation of neutrophils within the ischaemic-reperfused myocardium, complement-derived factors can directly contribute to myocardial injury by neutrophil independent mechanisms. C3a can decrease left ventricular contraction and coronary flow in isolated guinea pig hearts (Del Balzo *et al* 1992), and similar alterations, myocardial oedema, and release of creatine kinase, have been observed in isolated rabbit hearts

perfused with human plasma, a situation eliciting complement activation (Homeister *et al* 1992). In a recent study perfusion with neutrophils and plasma or neutrophils and C5a reduced ventricular function and coronary flow after global ischaemia in an isolated rat heart model, whereas reperfusion with only plasma, neutrophils, complement-activated plasma, or C5a failed to induce significant alterations. In addition, electron paramagnetic resonance spectroscopy measurements indicated that reperfusion with neutrophils and plasma resulted in marked prolongation in the duration of oxygen free radical generation (Shandelyo *et al* 1993).

Although the complement system is believed to be one of the most important sources of inflammatory mediators after myocardial ischaemia and reperfusion, a novel superfamily of low-molecular-weight chemotactic cytokines known as chemokines have recently been defined; chemokines are secreted by several types of cells in response to inflammatory stimuli *in vitro* (Baggiolini 1993). Chemokines are subdivided into a and b subfamilies on the basis of the presence or absence of an intervening amino acid between the first two of four conserved cysteines, and the two subfamilies differ in their target cell selectivity i.e., a or C-X-C chemokines primarily stimulate neutrophils, whereas b or C-C chemokines predominantly act on monocytes, basophils, eosinophils, and T cells. Specifically, IL-8 synthesized by endothelial cells after stimulation with TNFα or IL-1 is a strong neutrophil chemoattractant (Strieter *et al* 1989). Chemokines possess proteoglycan-binding sites, and IL-8 can induce transendothelial neutrophil migration, rapid shedding of L-selectin, and upregulation of neutrophil integrins, possibly by generation of a chemotactic gradient of immobilized matrix-associated IL-8 (Huber *et al* 1991 and Rot 1992). Various other neutrophil chemotactic agents are also released from the postischaemic myocardium, leukotrienes (Lee *et al* 1993), and PAF (Montrucchio *et al* 1989). Neutrophil chemoattraction and activation after myocardial ischaemia and reperfusion is therefore likely to be the result of amplification by numerous interacting proinflammatory mechanisms, with several of the involved mediators playing the role of initiator and product.

PAF has been shown to be involved in a variety of inflammatory reactions. Local intra-arterial infusion of PAF leads to an increase in both intestinal microvascular permeability and neutrophil adherence in mesenteric venules (Kubes *et al* 1990). These authors have also shown that reperfusion of the ischaemic intestine is

associated with a five fold increase in the mucosal concentration of PAF. Additionally pre-treatment of the cat mesentery with two structurally dissimilar PAF receptor antagonists resulted in a significant reduction in the rate of neutrophil adherence and emigration normally observed after reperfusion of ischaemic mesenteric venules. These observations indicate that PAF plays an important role in the neutrophil adherence and emigration induced by ischaemia and reperfusion. There is a substantial amount of evidence that oxygen-derived radicals result in the activation of LTB_4, PAF or both. Several lines of evidence support this possibility:

- Exposure of cells to oxidants leads to phospholipase A_2 activation (Lewis *et al* 1988).
- Reperfusion of ischaemic intestine leads to phospholipase A_2 activation (Otamiri *et al* 1988).
- Inhibitors of phospholipaes A_2 attenuate reperfusion induced neutrophil infiltration in intestinal mucosa (Otamiri *et al* 1988).
- Oxidants can stimulate the synthesis of PAF by cultured endothelial cells and subsequently promote the adherence of neutrophils to endothelial cell monolayers (Lewis *et al* 1988).

1.8 Neutrophil Activation during Ischaemia and Reperfusion

Evidence suggests that neutrophils entering tissue which has just been reperfused become activated to increase synthesis of oxygen metabolites and proteolytic enzymes. These neutrophils can then induce injury by adhering to the endothelium. The mechanism by which the neutrophils induce injury is via the secretion of proteolytic enzymes such as elastase, collagenase and gelatinase, probably in conjunction with neutrophil-generated oxidation products such as HOCl and H_2O_2, that are capable of damaging the microvasculature (Carden and Korthuis 1990). These result in lysis of essential structural matrix proteins such as elastin, leading to increased microvascular permeability. It has been demonstrated *in vitro* that adherent neutrophils are capable of degrading various structural components of the basement membrane and that this neutrophil mediated degranulation is prevented by elastase inhibitors. Zimmerman and Granger (1990) reported that the reperfusion-induced increase in intestinal mucosal MPO activity is attenuated in animals pre-treated with

elastase inhibitors. Thus proteases released from both stimulated endothelial cells and activated neutrophils may be an important component of ischaemic reperfusion injury. However neutrophils must not only be present but their adherence to the endothelium is a prerequisite for injury. Neutrophil-endothelial adhesion is thought to create a microenvironment which permits high concentrations of injurious agents to collate (Welbourne *et al* 1991). The binding action of neutrophil CD18 to endothelium appears to be the signal required for the neutrophil to release H_2O_2 and possibly proteases into the extracellular environment (Nathan *et al* 1989).

Even though much evidence exists as to the role of the neutrophil in ischaemic/reperfusion induced microvascular injury the precise mechanisms by which reactive oxygen metabolites produce chemotactic activity is not understood. Furthermore how neutrophil adhesion initiates endothelial and subendothelial damage remains to be fully elucidated.

1.10 Aims of this study

Although there is more to ischaemic/reperfusion injury than hypoxia and reoxygenation, such as changes in ph, a growing body of evidence suggests that reperfusion-induced injury is largely mediated by reactive oxygen metabolites and activated neutrophils on reoxygenation of the endothelium. Xanthine oxidase-derived oxidants appear to initiate the production and release of inflammatory mediators, which in turn promote the adherence and emigration of neutrophils in postcapillary venules. Moreover, adhesion molecules on the surface of the neutrophil, along with their counter ligands on the endothelial cell membrane, appear to promote endothelial dysfunction in ways that go beyond the adherence of neutrophils on the endothelial surface. Though these interactions remain to be fully elucidated adherent neutrophils may mediate microvascular injury by either release of protease, physical disruption of endothelial barrier during emigration or both.

The aim of this thesis is to produce a reliable and reproducible *in vitro* model which simulates this portion of the *in vivo* situation and examines the neutrophil / endothelial cell interactions following periods of hypoxia. For such a model to be effective it must cover the three apparent facets of the injurious process; the enhanced neutrophil adhesion post ischaemia, the heightened migration towards materials released by the hypoxic / reoxygenated cells and neutrophil activation with release of damaging products. This may allow identification of the several substances involved and the chronology of their release. Furthermore the exact nature of the necessary cell to cell interactions may be revealed.

An *in vitro* model, if it adequately mimics the *in vivo* events, should lead to a fuller understanding of the injurious process and identify more precisely targets for pharmacological intervention to ameliorate damage. It was hoped that an immortalized endothelial cell line would be more manipulable and reduce the wide variation in neutrophil adhesion seen in other *in vitro* studies using primary cultures of umbilical vein endothelial cells.

Finally to extend the scope of previous *in vitro* studies on ischaemia/reperfusion injury an attempt will be made to assess damage in terms of tissue function. Thus materials produced by neutrophils and endothelial cell interactions will be assayed for effects on

smooth muscle contractility. It may be that the phenomenon of "myocardial stunning" and "no-reflow" may reflect alterations of muscular function. "Myocardial stunning" is the impaired contractile function that follows periods of ischaemia and reperfusion and "no-reflow" phenomenon is when normal flow is restored post ischaemia but subsequently diminishes. Such an extension may highlight further points for protective intervention in the injurious process.

2. MATERIALS AND METHODS

The methods below have been employed in an attempt to produce an *in vitro* model of hypoxia reperfusion injury. Thus an endothelial cell line (ECV304) was cultured under hypoxic conditions and then restored to normoxia. The effects of such treatment on the adhesion of normal neutrophils and of the myelocytic cell line HL60 was then monitored. The secretions from the hypoxic reperfused endothelial cells were also assessed for their capacity to enhance neutrophil migration and influence smooth muscle contractility. Similarly we measured the capacity of the treated endothelial cells and their products to induce neutrophil activation and generate reactive oxygen intermediates. Where several methods were used to assay a particular function and define best practice the results have been included.

The addresses of all suppliers of chemicals and other materials are listed alphabetically in Appendix 1.

2.1 Culture of cell lines

2.1.1 ECV304 cells

Transformed human umbilical vein cells (ECV304) (European Cell Culture) were cultured in Medium 199 (M199) (Sigma) supplemented further with the antibiotics Streptomycin (100IU/ml), Penicillin (100μg/ml), L-glutamine (2mM), and 10% foetal calf serum (Sigma). All supplementary solutions were filter sterilised using 0.2μM micropore filters prior to their addition to the medium (Gibco Laboratories). Cultures were normally maintained at 37 °C in a 95% air, 5% CO_2 atmosphere. The cells were grown in 30ml of supplemented medium in 75 ml tissue culture flasks (Gibco) until confluent. To sub culture, the supernatant was removed and replaced with 10ml of calcium- and magnesium-free phosphate buffered saline (PBS) (Gibco). After removing the PBS the cells were detached by the addition of 3ml of cell dissociation solution (Sigma) and incubated at 37°C in 95% air, 5 % CO_2 for 10 minutes. The detached cells were then diluted with 120ml of fresh supplemented medium to maintain the culture or isolated for experimentation.

2.1.2 Seeding of 96 well plates

The ECV304 cells were detached as in 2.1.1 and resuspended in supplemented medium at $1x10^6$ cells per ml. They were then added to 96 well tissue culture plates (Gibco), 200µl per well, and grown to confluency at 37^0C in a 95% air, 5% CO_2 atmosphere. Confluency was generally achieved in 24 hours.

2.1.3 Growing on Cytodex microcarriers

Confluent ECV304 cells from a 75ml tissue culture flask were detached as in 2.1.2. The cells were then transferred to a fresh flask and 25ml of supplemented medium added plus 1 ml of Cytodex 1 microcarriers (Pharmacia Biotech). The dry microcarriers were swollen and hydrated in calcium- and magnesium-free PBS (50ml/g Cytodex 1) for at least three hours at room temperature before use. The supernatant was then decanted off and the microcarriers were washed for a few minutes in fresh PBS. This PBS was then discarded and replaced with 30ml of fresh PBS before the beads were autoclaved and ready for use. The flasks containing the cells, media and Cytodex 1 beads were incubated at 37^0C in a 95% air, 5% CO_2 atmosphere for 24 hours. Regular shaking of the flask ensured that the ECV 304 cells coated the spheres uniformly.

In order to assess the cellularity of the microcarriers a Cyto-Pro 480 Kit (BioOrbit) was used. This assay measures intracellular ATP by bioluminescence. The reaction is catalysed by the enzyme luciferase, obtained from the firefly, and results in the release of light at 562nm according to the following scheme;

$$\text{ATP} + \text{Luciferin} + O_2 \xrightarrow[\text{luciferase}]{} \text{Oxyluciferin} + \text{AMP} + \text{PPi} + CO_2 + \text{LIGHT}$$

When all other components of the reaction remain constant, the intensity of the light emitted is proportional to the amount of ATP taking part in the reaction. The light level, and hence the ATP concentration, is detected in a luminometer (BioOrbit), which via a photo cell and multiplier converts the light emitted to a signal, relative light units (RLU).

Briefly 90µl of ECV304 cells grown on the microcarriers were placed in a cuvette and lysed by the addition of 90µl of somalyze (BioOrbit). After 5 minutes the liberated ATP was measured by the addition of 20µl of ATP monitoring reagent, containing luciferin and the enzyme luciferase. All tests were performed in triplicate and the light emitted was measured in a BioOrbit 1250 luminometer. Figure 2.1 shows that there is an increase in ATP with increasing ECV304 numbers. From this standard graph, produced using known numbers of EVC304, the cellularity of any unknown sample was calculated.

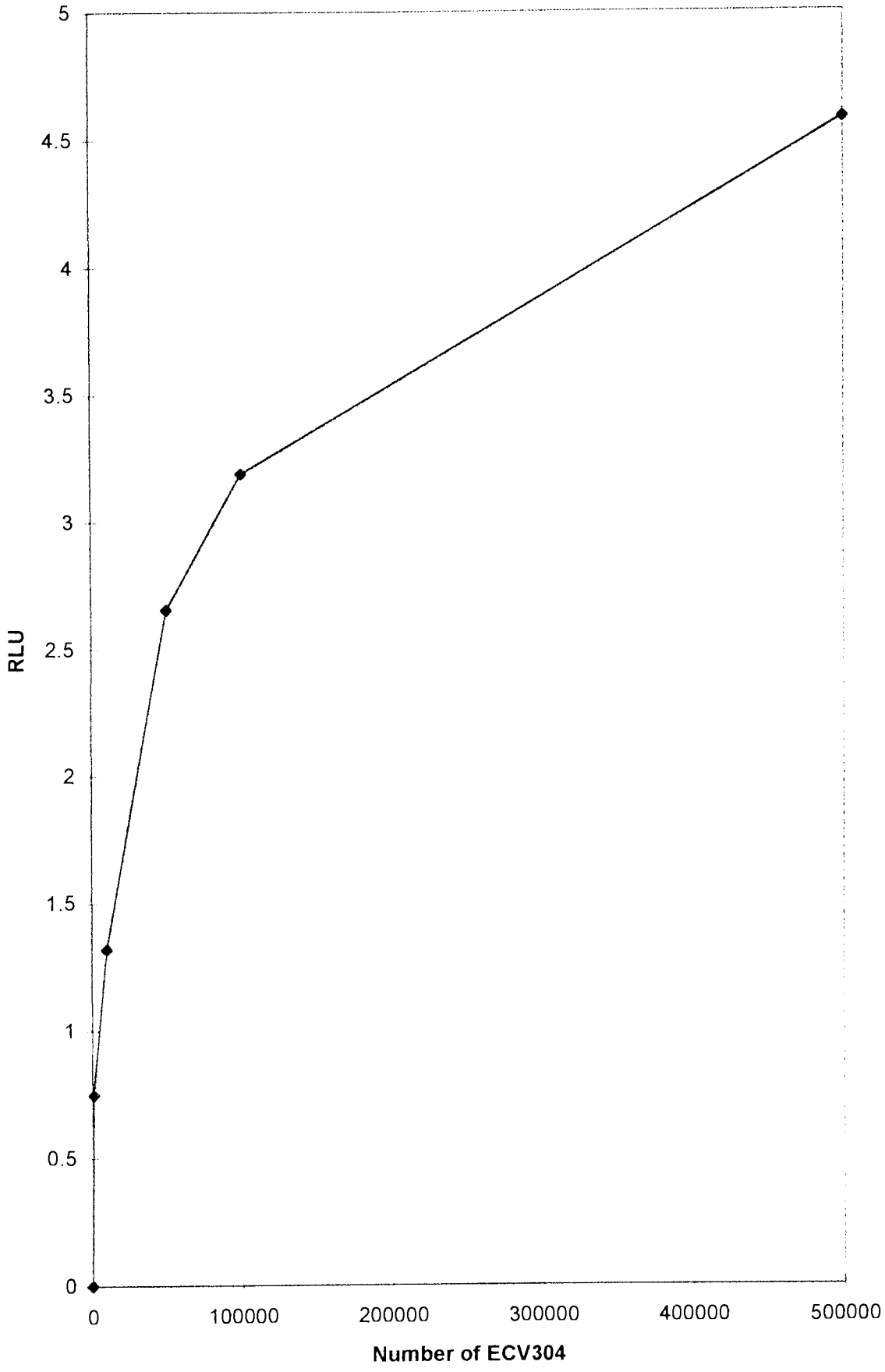
2.1.4 HL60 cells

The human promyelocytic leukaemia cell line HL60 (European Cell Culture) can be differentiated into a neutrophil-like line by the addition of 1.25% DMSO (Harris and Ralph 1985). The cells were, therefore, cultured in 30ml RPMI 1640 (Sigma) supplemented with Streptomycin (100IU/ml), Penicillin (100µg/ml), 2mM L-glutamine, and 10% foetal calf serum. Cultures were normally maintained in 75ml flasks at 37°C, in a 5% CO_2 ,95% air atmosphere up to a density $1x10^6$ cells per ml. The cells were then either diluted 1:5 with fresh media to maintain the culture or differentiated for experimentation by the addition of 1.25% DMSO for 5 days.

2.2 Peripherial blood neutrophil preparation

Human peripheral blood taken from the ante-cubital vein of healthy donors was collected into 10ml dry tubes, uniformly coated with a total of 100 U of Lithium Heparin (L.I.P.,) and gently mixed to avoid clotting. Neutrophils were subsequently isolated by one of four methods so yield, purity and their functional capabilities could be assessed.

Figure 2.1 ATP content of ECV304 cells



Known numbers of ECV304 cells were lysed and their ATP content measured as in Section 2.1.3. Results are mean values where n=3 at each point.

2.2.1 Method 1 (Histopaque 1)

To 3ml of blood 5ml of calcium- and magnesium-free PBS was added. This blood-saline mixture was then carefully layered onto 3ml of Histopaque 1077 (Sigma) and centrifuged at 400g for exactly 30 minutes (IEC Cetra-3C Centrifuge). The resulting opaque leukocyte-rich cell layer was then carefully removed, resuspended in 10ml of PBS and washed twice at 300g for 10 minutes. After the second wash the cells were resuspended in a final volume of 2ml PBS. The 2ml of cell suspension was then layered onto isotonic percoll (Sigma) gradients, consisting of 3ml of 60% percoll on top of 2ml of 80% percoll, and centrifuged at 300g for 20 minutes. Neutrophils were then collected from the interface between the two percoll layers and washed in 10ml of PBS before being resuspended in a final volume of 1ml PBS. Isotonic percoll was prepared by adding 10ml of 10x calcium- and magnesium-free PBS to 90ml of percoll.

2.2.2 Method 2 (Histopaque 2)

Five ml of Histopaque 1077 (Sigma) was carefully layered on top of 3ml Histopaque 1119 (Sigma). To this 3ml of blood was then added and spun at 550g for 30 minutes. The neutrophils were then removed from the interface between the two histopaques and washed twice with 10ml of calcium- and magnesium-free PBS before being resuspended in a final volume of 1ml PBS.

2.2.3 Method 3 (Hespan)

To 10ml of blood 1.5ml of Hespan (AAH Pharmaceuticals. Kingswindford, West Midlands, UK) was added. The resulting mixture was left at room temperature for 45 minutes to allow sedimentation of the red blood cells. The resulting upper leukocyte-rich fraction was then removed and diluted with an equal volume of calcium and magnesium-free PBS. Discontinuous percoll gradients of 3ml 60% percoll and 2ml 80% percoll were then prepared and 2ml of the diluted leukocyte suspension layered on top. The tubes were then centrifuged at 300g for 20 minutes and the neutrophils collected from the percoll interface. The cells were then washed twice in 10 ml of PBS before being resuspended in a final volume of 1ml PBS.

2.2.4 Method 4 (Dextran)

To 10ml of blood 1.5ml of 6% Dextran (MWt 500,000) (Sigma) was added. After 45 minutes neutrophils were isolated from the resulting upper leukocyte rich fraction by the same procedure as 2.2.3.

2.3 Assessment of neutrophil purity

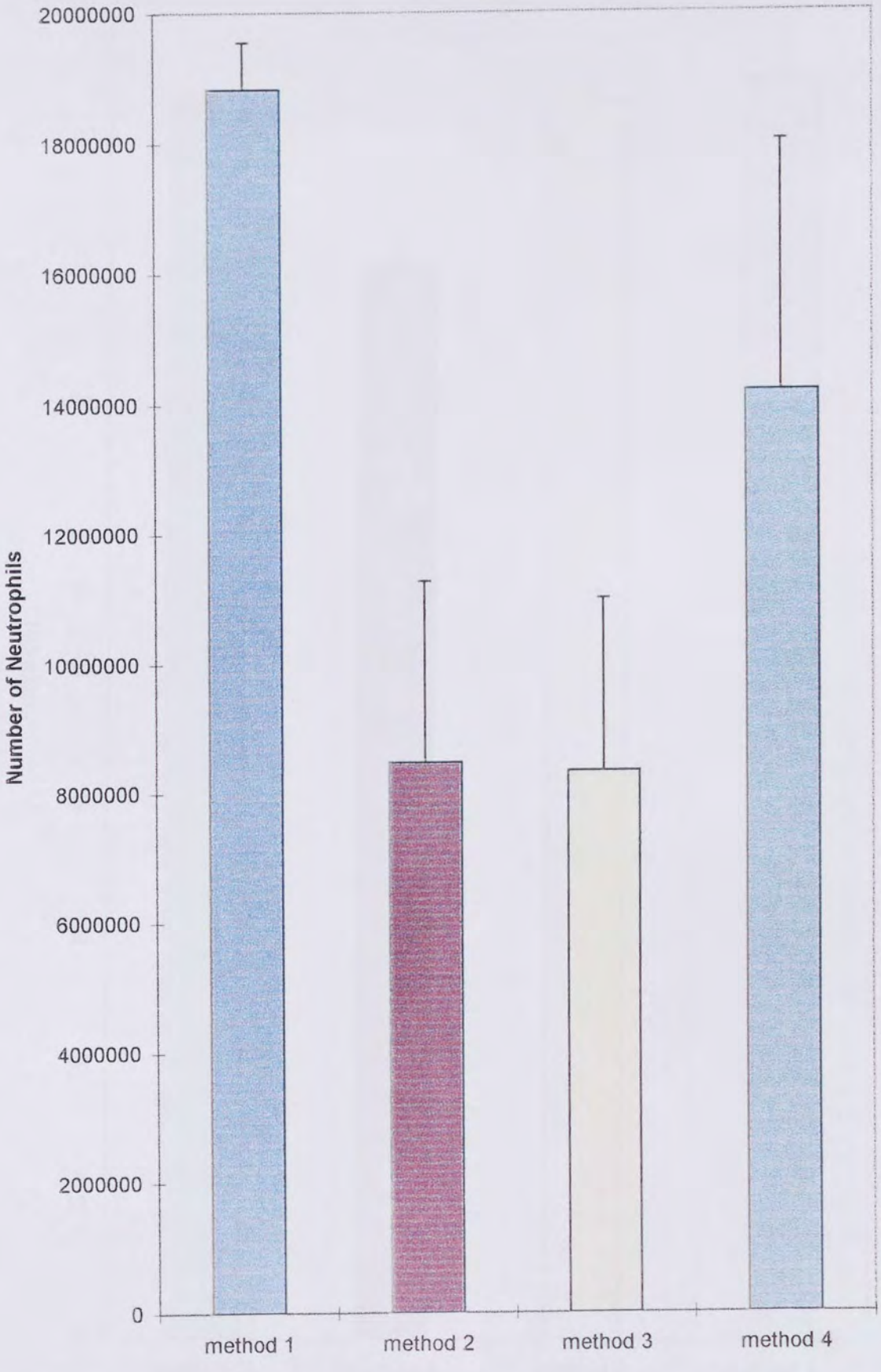
The purity of the samples prepared via the four methods in 2.2 was assessed by a differential white blood cell count. After resuspension of the neutrophils as outlined 50μl samples were spun down onto a glass slide using a cytocentrifuge. After a 3 minute spin at 300rpm the slides were removed and allowed to air dry for 5 minutes. The slides were then fixed in 70% methanol (Shandon) for 8 seconds and subjected to Diff-Quik differential stain 1 for 12 seconds before being counterstained with Diff-Quik differential stain 2 for 20 seconds (Baxters Healthcare Ltd). Total cell counts for the samples were obtained using a haemocytometer.

Although hespan separation did not produce maximum yields of neutrophils they were routinely of >95% purity and on morphological criteria they remained inactive and quiescent (Figure 2.2-2.4) Both histopaque and dextran separation techniques caused cytoplasmic foaming and or nuclear disintegration indicative of premature or unscheduled activation or apoptosis (Hjorth 1981 and Glasser 1990). Therefore hespan separation was superior to the others and has subsequently always been employed.

2.4 Preparation of cytokines

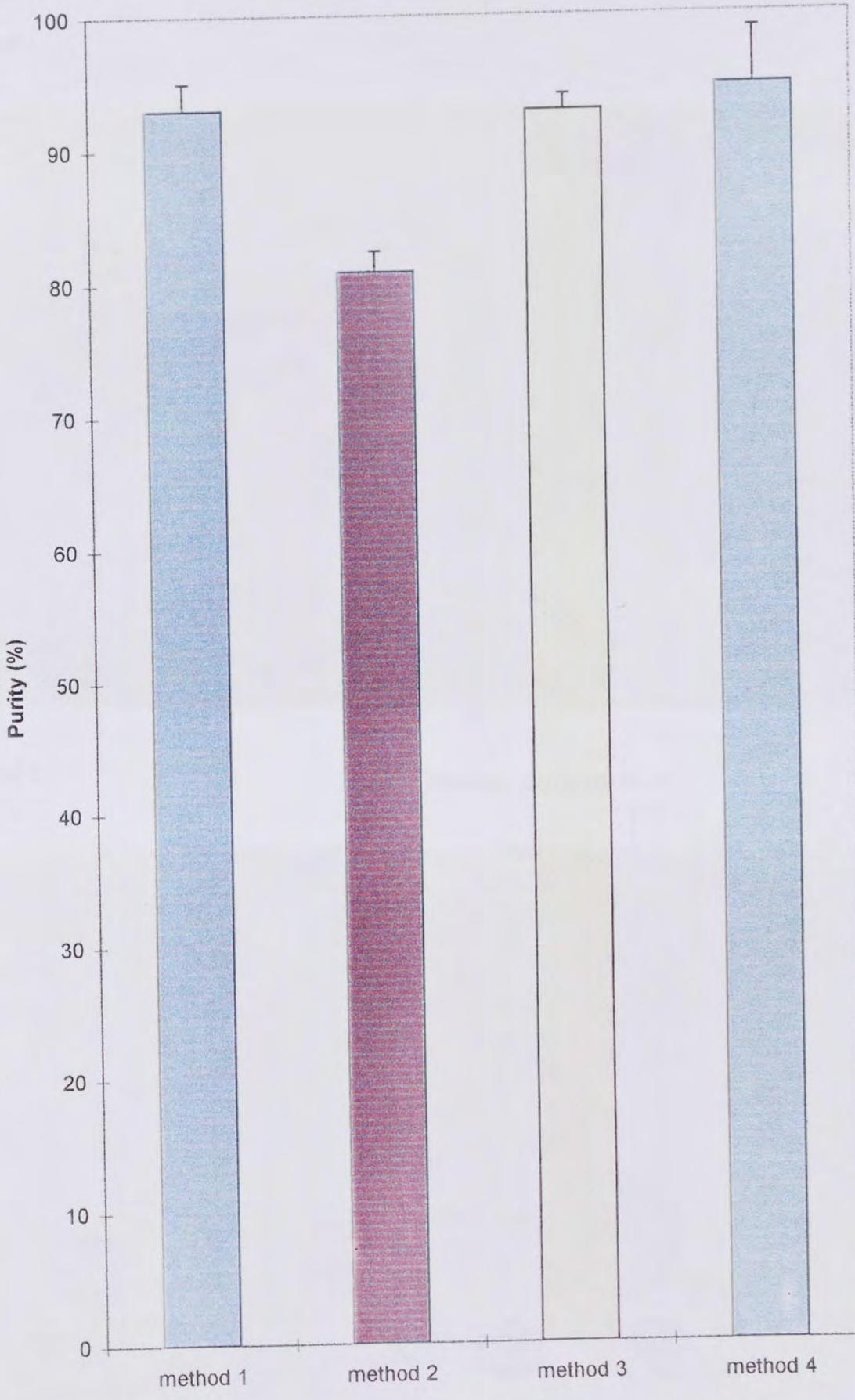
All cytokines used were the generous gifts of Drs. R. Thorpe and M. Wadhwa (NIBSC). Human TNFα and IL-1, were used to upregulate known adhesion molecules on the ECV304 cell surface acting as positive internal controls in all adhesion assays. TNFα and IL-1 were diluted to the recommended concentrations in their vials and filter sterilised as before. Aliquots of 10μl were then placed into.

Figure 2.2 Neutrophil numbers isolated from whole blood



Neutrophils were isolated from 20mls of whole blood using the four different methods in Section 2.2 and counted. The results shown are means +/- SEM derived from between 7 and 19 observations.

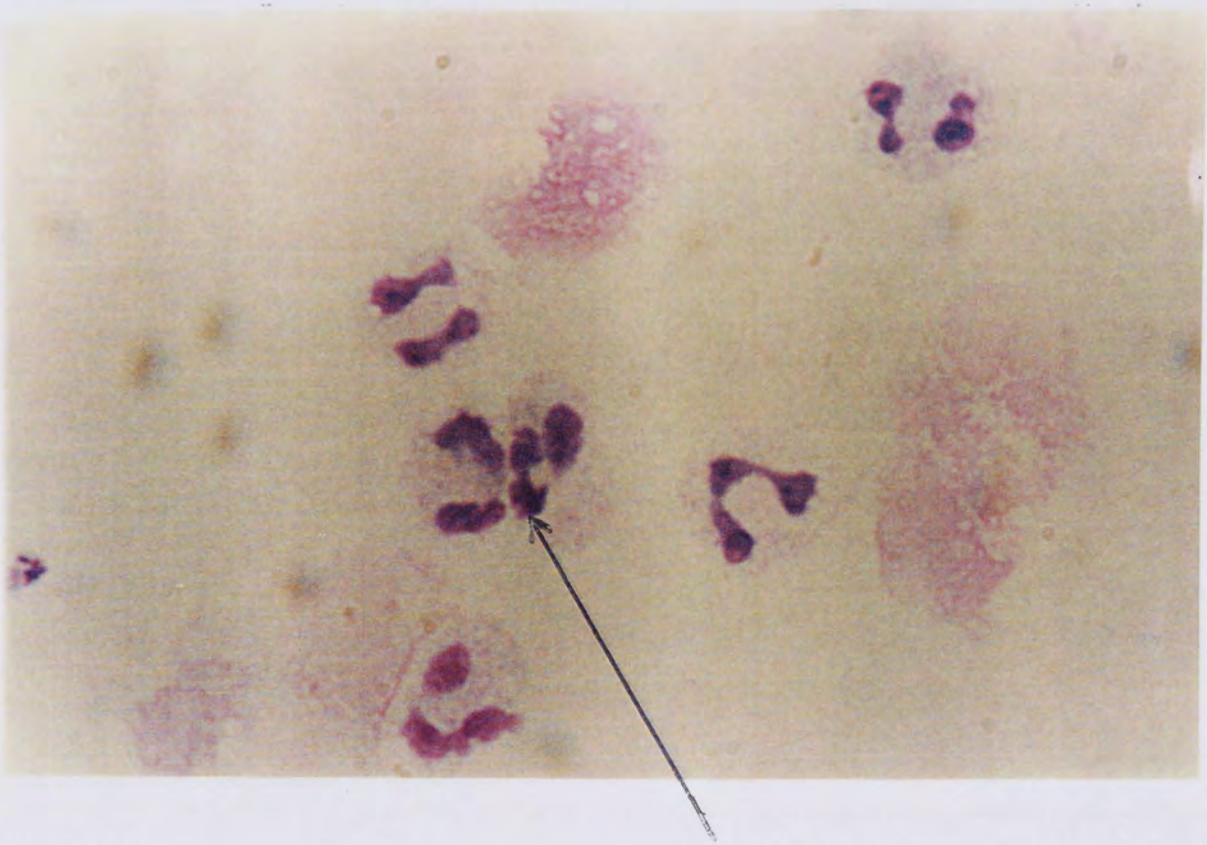
Figure 2.3 Purity of neutrophils isolated from whole blood



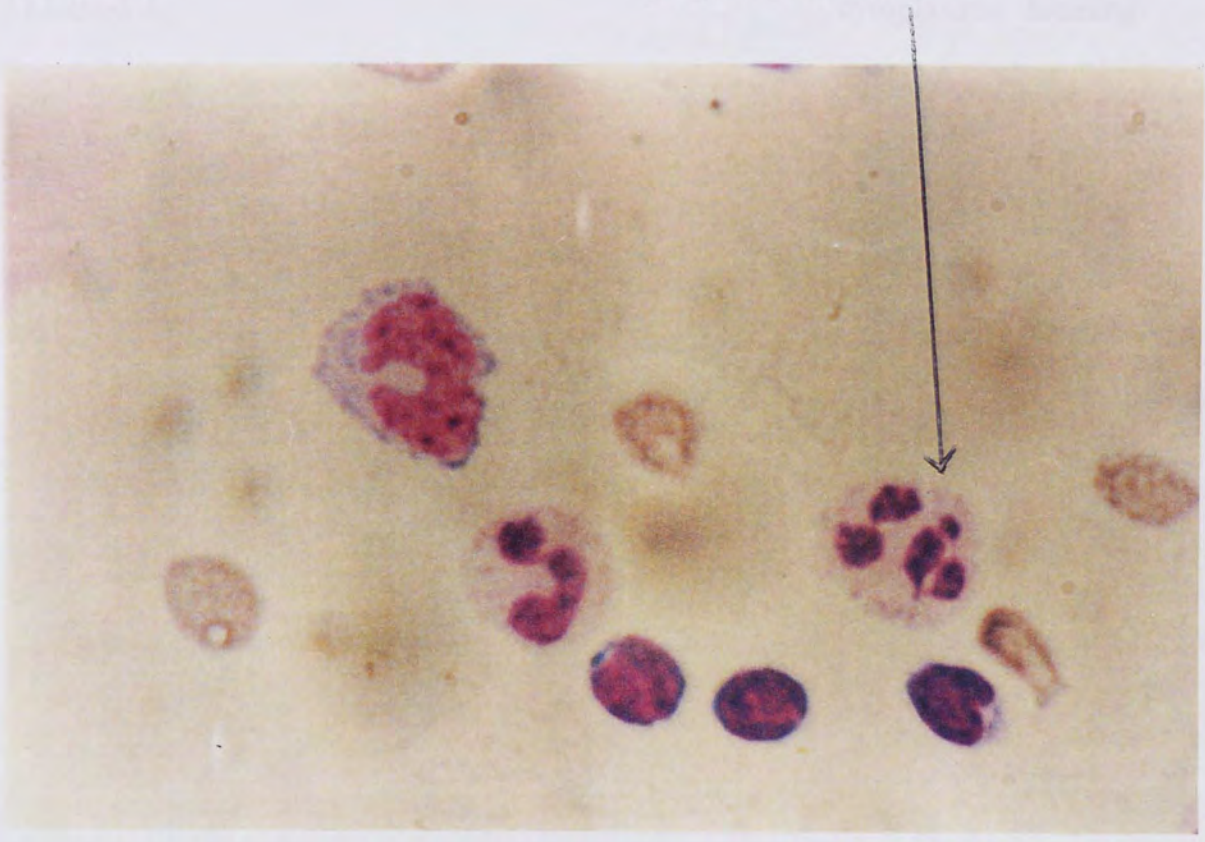
The purity of each of the isolates from Figure 2.2 was assessed by Diff Quick staining and the average of 10 fields of view recorded. The results shown are means +/- SEM derived from between 7 and 19 observations.

Figure 2.4 Photographs of the neutrophils isolated by the four methods in Section 2.2.

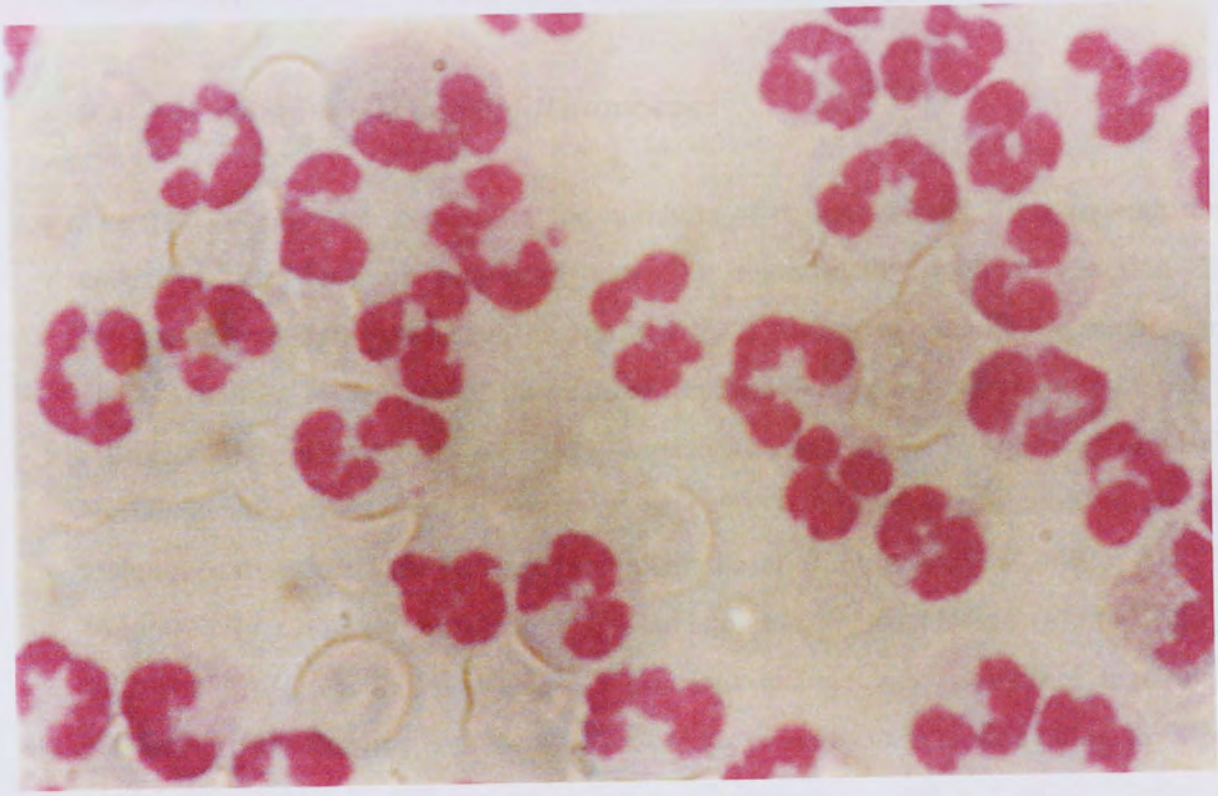
Method 1



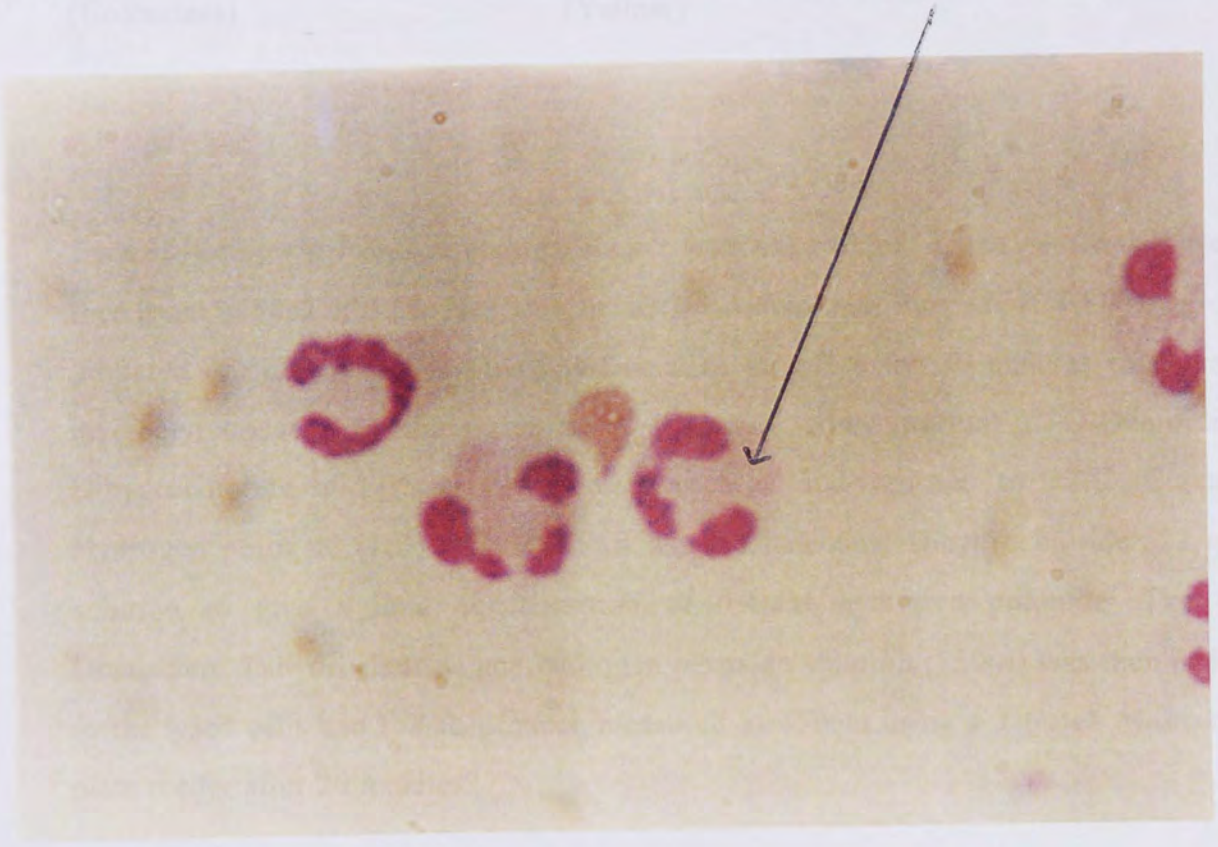
Method 2



Method 3



Method 4



Scale x1500

sterilised plastic micro centrifuge tubes for freezing and storage at -70°C. These were then defrosted as required and working dilutions made in sterile micro centrifuge tubes using unsupplemented culture medium.

2.5 Assessment of neutrophil numbers

The general aim was to measure the numbers of neutrophils adhering to endothelial cells (Section 2.6) or migrating along a chemotactic gradient (Section 2.7). Neutrophil numbers have been determined by a variety of techniques ranging from visual counting to the use of monoclonal antibody/enzyme conjugates (ELISA) and radiolabelling (Bochner *et al* (1991), Luscinska *et al* (1992), Pigott *et al* (1992), Derian *et al* (1995) and Firestein *et al* (1995)). We have chosen to examine techniques that determine neutrophil numbers based upon neutrophil myeloperoxidase content. By using the chromagen O-Dianisidine Dihydrochloride (Sigma) and the substrate hydrogen peroxide (Sigma) a colour change will take place that can be measured, if the enzyme is present. This colour change can then be read using a plate reader (Titertek).

$$\underset{\text{(Colourless)}}{H_2O_2 + ODH} \xrightarrow{\text{MPO}} \underset{\text{(Yellow)}}{H_2O + O_2DH}$$

2.5.1 Method 1

To a 96 well plate 100µl of neutrophils per well were added. These neutrophils were then lysed in 50µl of 0.5% Hexadecyltrimethyl-Ammonium Bromide (CTAB) (Sigma) prepared in calcium- and magnesium-free PBS at pH 6 for 30 minutes to liberate myeloperoxidase. In the meantime a 0.2mg/ml concentration of O-Dianisidine Dihydrochloride in PBS at pH 6 was prepared and warmed to 37°C (Sigma). Hydrogen peroxide (1µl) was added to the O-Dianisidine Dihydrochloride (22 ml) solution to give a final concentration of 0.4mM hydrogen peroxide. The O-Dianisidine Dihydrochloride and hydrogen peroxide solution (250µl) was then added to the lysed cells and the absorbance measured at 450nm using a Titertek Multiskan plate reader after 20 minutes.

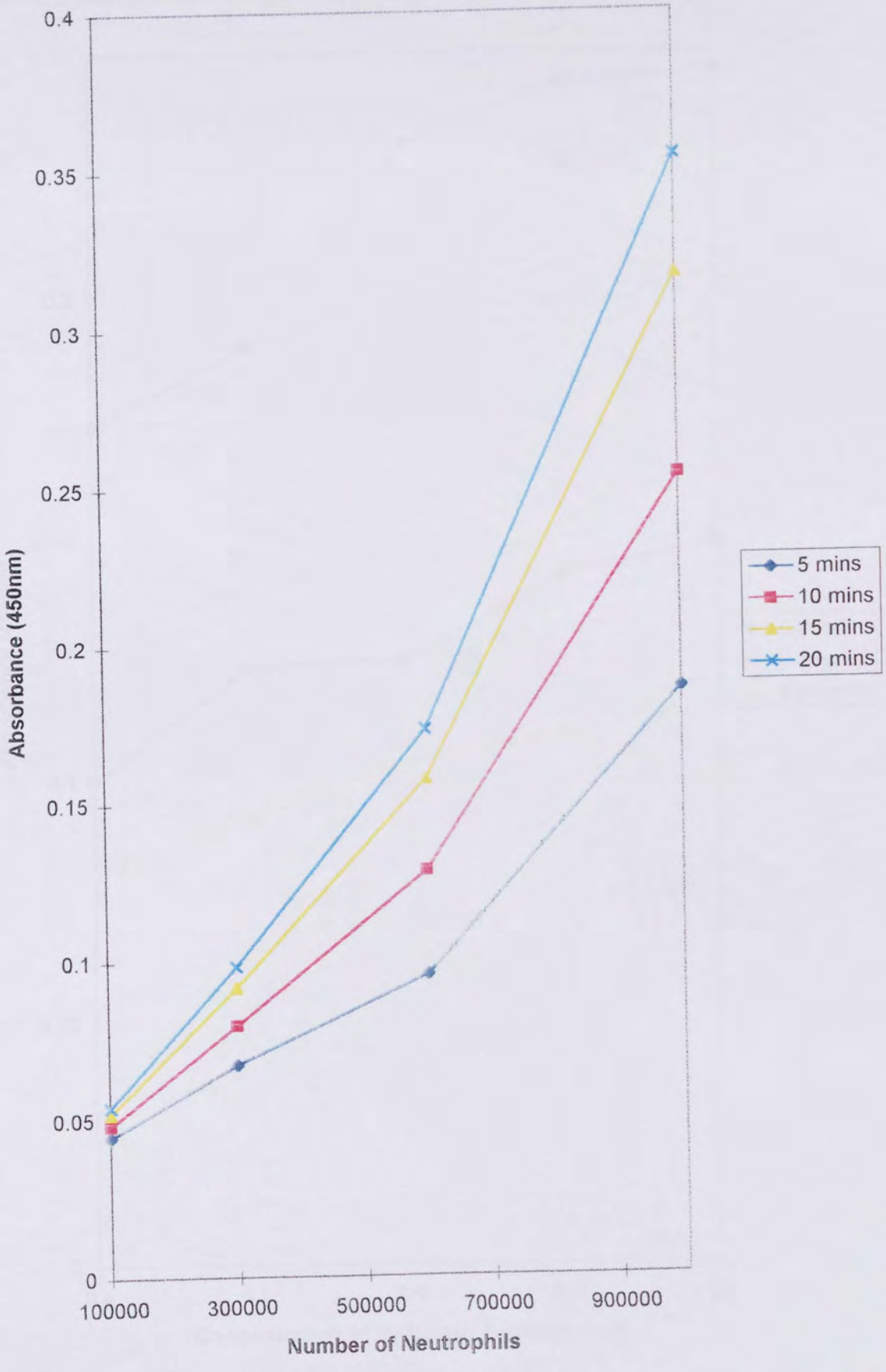
2.5.2 Method 2

The neutrophils (100µl) were lysed by the addition of 40µl of 0.2% v/v Triton (Sigma) in PBS to each well for 5 minutes. Liberated myeloperoxidase was then measured by adding 160µl of a freshly prepared solution of O-Dianisidine Dihydrochloride (0.08mg/ml) in PBS, pH5 containing 0.02% v/v of hydrogen peroxide (6mM). After 5 minutes the absorbance at 405nm was then measured using a Titertek Multiscan Plus plate reader.

Measuring myeloperoxidase concentrations produces a quick, easy and inexpensive method of determining neutrophil numbers. Figure 2.5 shows that for method 1 there is an approximately linear correlation between increasing neutrophil numbers and myeloperoxidase levels, as detected by increasing absorbance. The sensitivity of this method can be increased by varying chromagen and substrate concentrations as well as increasing the incubation time prior to reading the plate (Figure 2.6). Optimum conditions of 0.2mg/ml O-Dianisidine Dihydrochloride, 0.8mM Hydrogen Peroxide and incubation for 20 minutes, as in figure 2.7, make it possible to detect a minimum of $1x10^4$ neutrophils and a maximum of $1x10^6$ neutrophils per well. Figure 2.8, however, shows that by using a modification of this technique employing triton rather than CTAB, a lower concentration of O-Dianisidine Dihydrochloride (0.08 mg/ml) but higher concentration of H_2O_2 (6mM) and measuring absorbance at 405nm, method 2.5.2, as few as 1000 neutrophils per well could be detected. Above $1x10^6$ cells per well either chromagen or substrate was rate limiting as absorbance did not increase linearly after this point. However, between 1000 and $1x10^6$ neutrophils per well, incubated for 5 minutes, absorbance was directly proportional to the number of cells. Myeloperoxidase method 2.5.2 was used to determine neutrophil numbers in all subsequent experiments as it gave us the sensitivity and accuracy required.

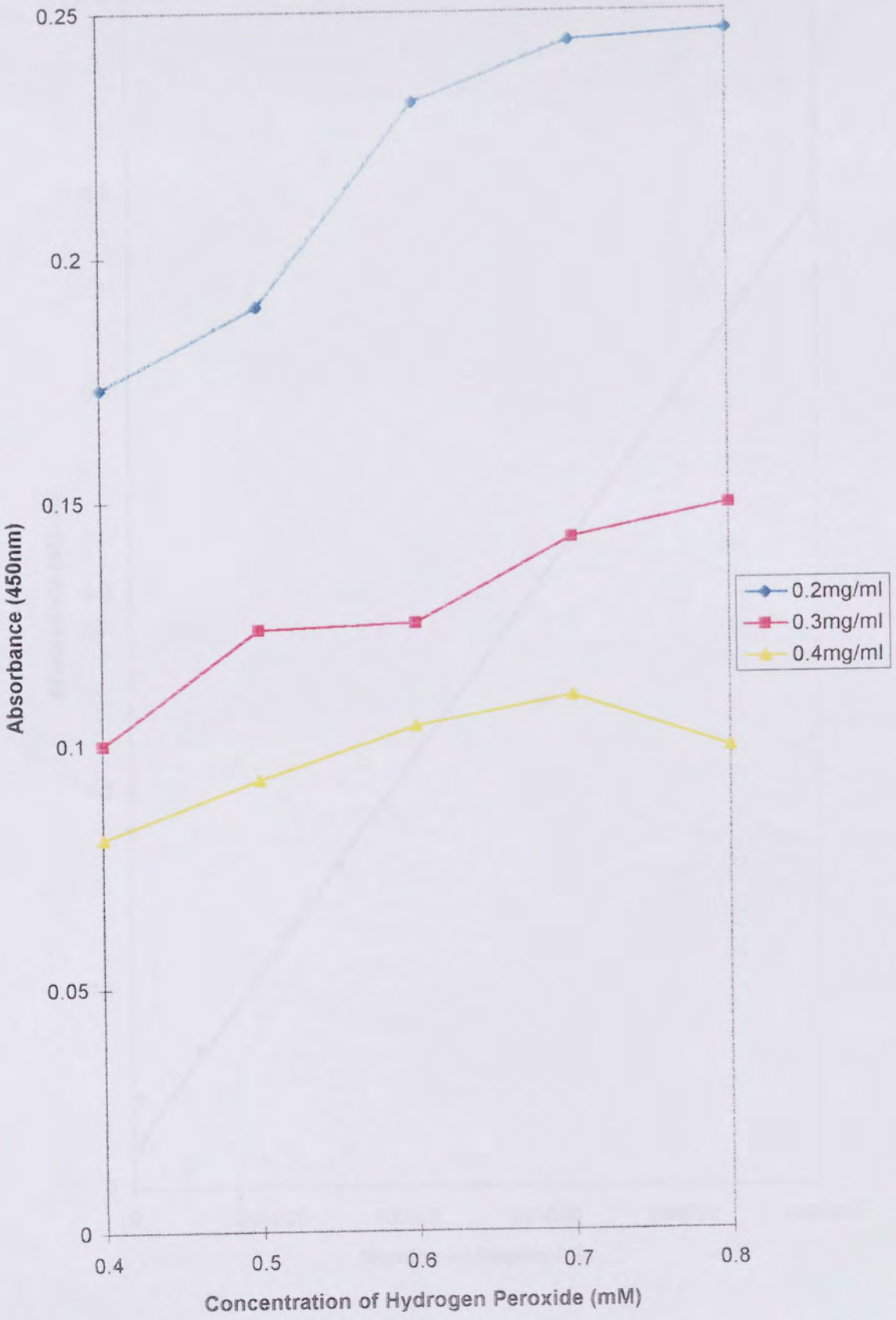
Myeloperoxidase method 2 for determining neutrophil numbers is an indirect method. To ensure we were indeed measuring neutrophil numbers we compared it to the direct visual staining technique given in section 2.5.3.

Figure 2.5 Determining neutrophil numbers



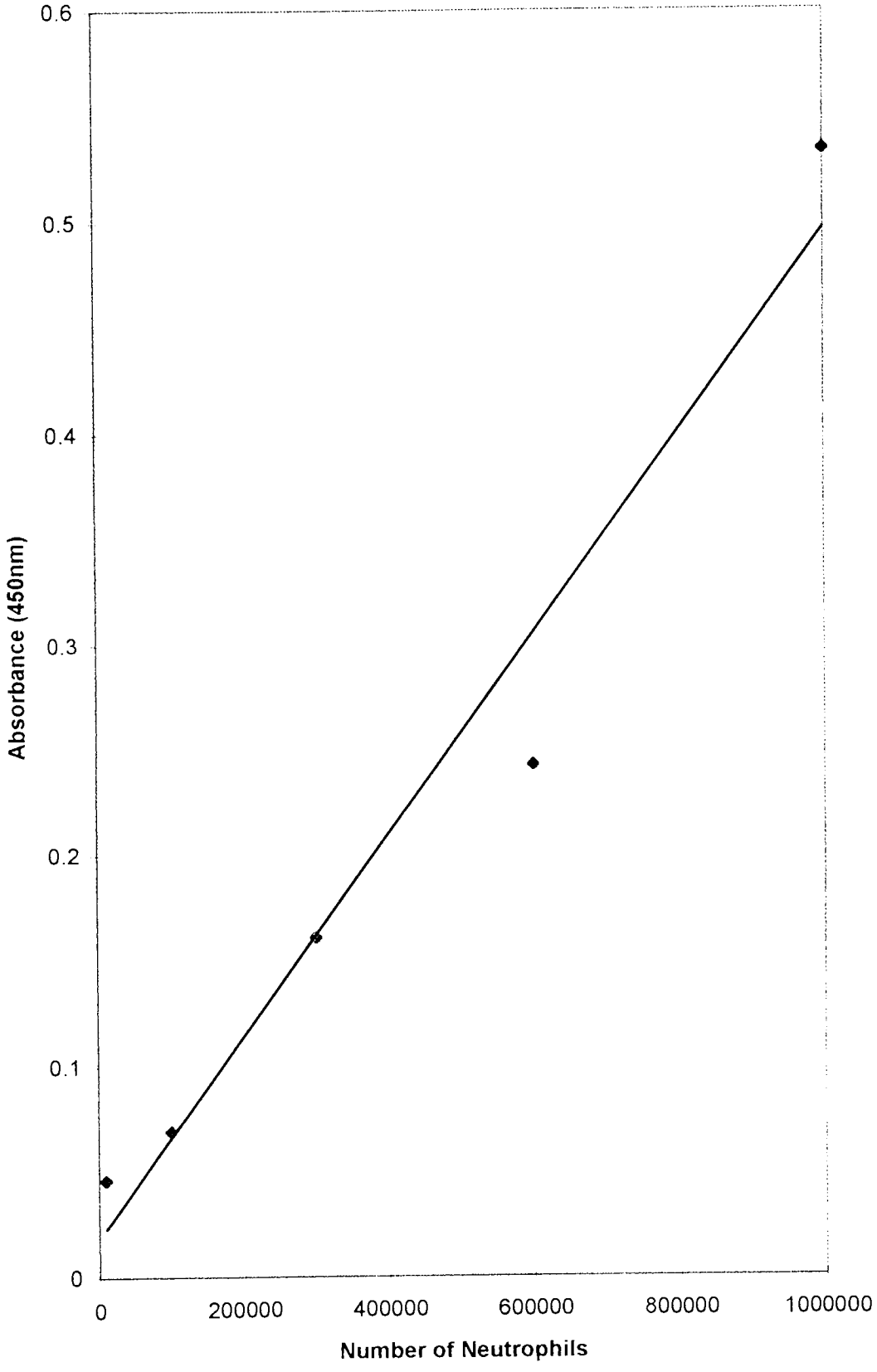
Neutrophil numbers were assessed using method 2.5.1. The absorbance was read at 5, 10, 15 and 20mins. Results are means where n=3 at each point.

Figure 2.6 Effect of varying O-Dianisidine Dihydrochloride and hydrogen peroxide concentrations on determining neutrophil numbers



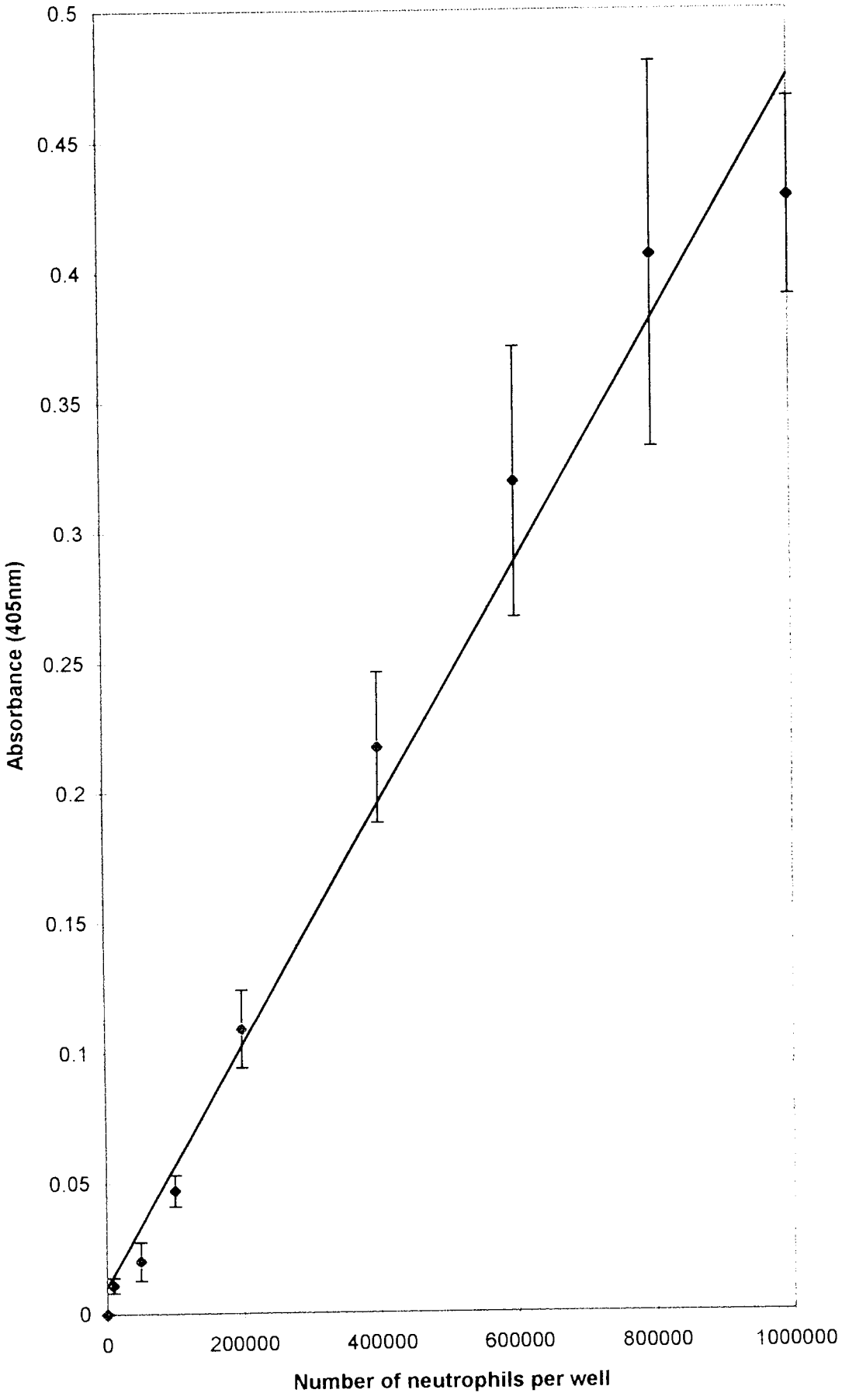
Neutrophil numbers were assessed using method 2.5.1 and varying the O-Dianisidine Dihydrochloride and hydrogen peroxide concentrations. Values given are means where n=3 and the plates are read at 20mins.

Figure 2.7 Optimum conditions for determining neutrophil numbers



Neutrophil numbers were assessed using the optimum conditions found for method 2.5.1. That is 0.2mg/ml O-Dianisidine Dihydrochloride, 0.8mM hydrogen peroxide and reading absorbance after 20mins incubation. Values are means where n=3.

Figure 2.8 Determining neutrophil numbers



Neutrophil numbers were assessed using method 2.5.2. Values given are means +/- SEM where n=4 at each point.

2.5.3 Method 3

The neutrophils were fixed with 70% methanol for 8 seconds. The cells were the visualised by staining with Diff-Quick, as previously described. Excessive stain was removed by washing twice with 200µl of PBS. Each well was then viewed under an inverted light microscope and the total number of adherent neutrophils calculated using the following formula.

Area of well = πr^2 where r = 3.5mm
= 3.142 x 12.25
= 38.5mm^2

Area of field of view = πr^2 where r = 0.35mm
= 3.142 x 0.1225
= 0.385mm^2

Total number of neutrophils = Average number per field of view x $\frac{38.5}{0.385}$

∴ Total number of neutrophils = Average number per field of view x 100

No significant difference was detected between myeloperoxidase method 2 and method 3 visual counting (Figure 2.9). These results support the accuracy of both methods, however, visual counting is extremely time consuming so was not subsequently used.

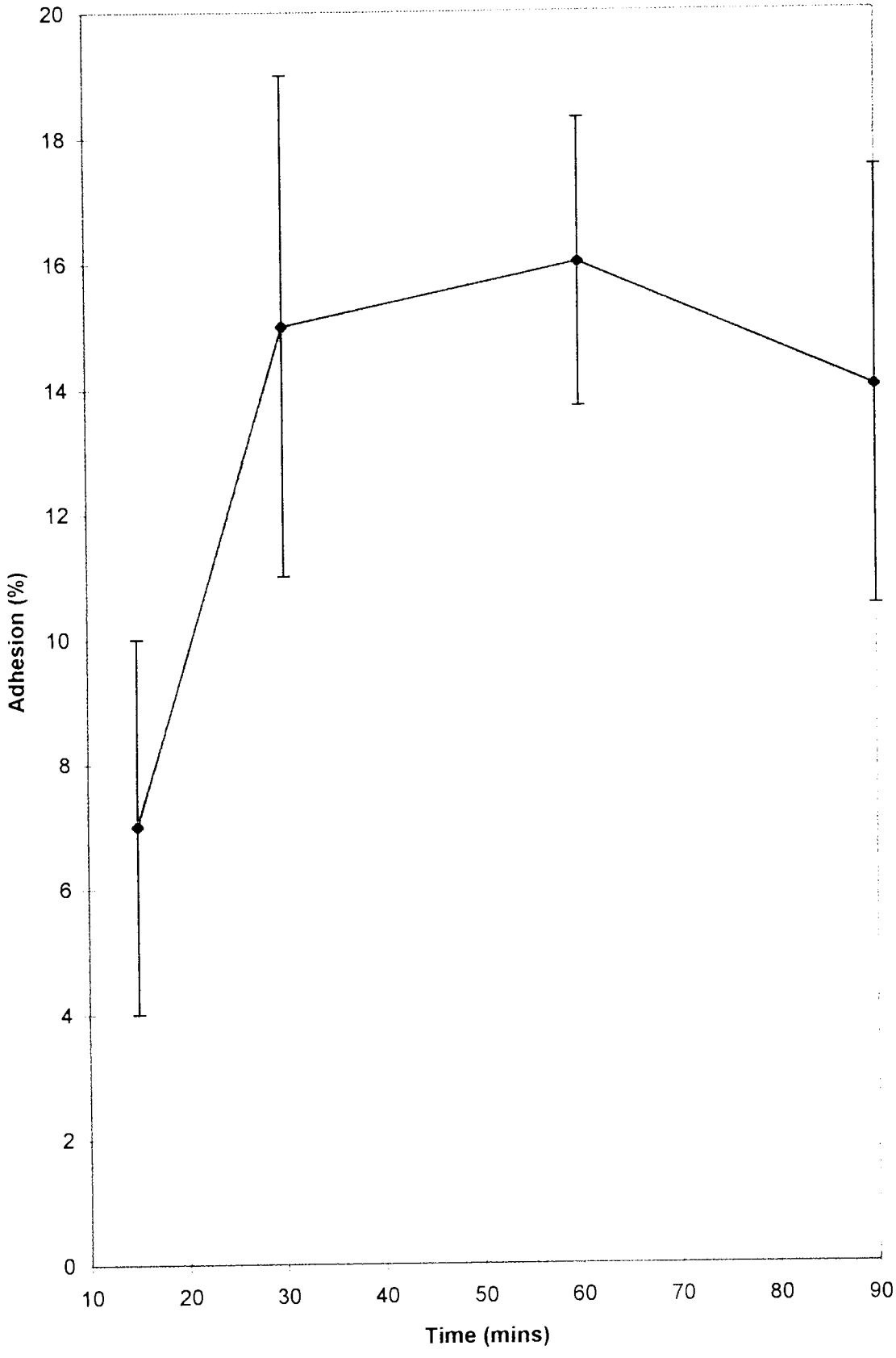
2.6 Detection of neutrophil adhesion

In order to assess neutrophil adhesion ECV304 cells were seeded into 96 well plates as described in section 2.1.2. Once confluent the growth medium was aspirated off and 100µl of 1x10^6 neutrophils per ml added to each well. The plates were then incubated at 37°C in a 95% air, 5% CO_2 atmosphere for varying times (Figure 2.10) After these times non-adherent neutrophils were removed by washing several times with 100µl of calcium- and magnesium-free PBS (Figure 2.11). The remaining adherent neutrophils were assessed using the MPO method outlined in section 2.5.2. and expressed as a percentage of the total cells originally added. All tests were carried out in triplicate and a mean result calculated. Clearly a minimum of 30mins was required for maximum neutrophil adherence and a minimum of 2 washes was required to ensure only adherent cells remained.

Figure 2.9 Number of neutrophils detected using method 2.5.2 and 2.5.3

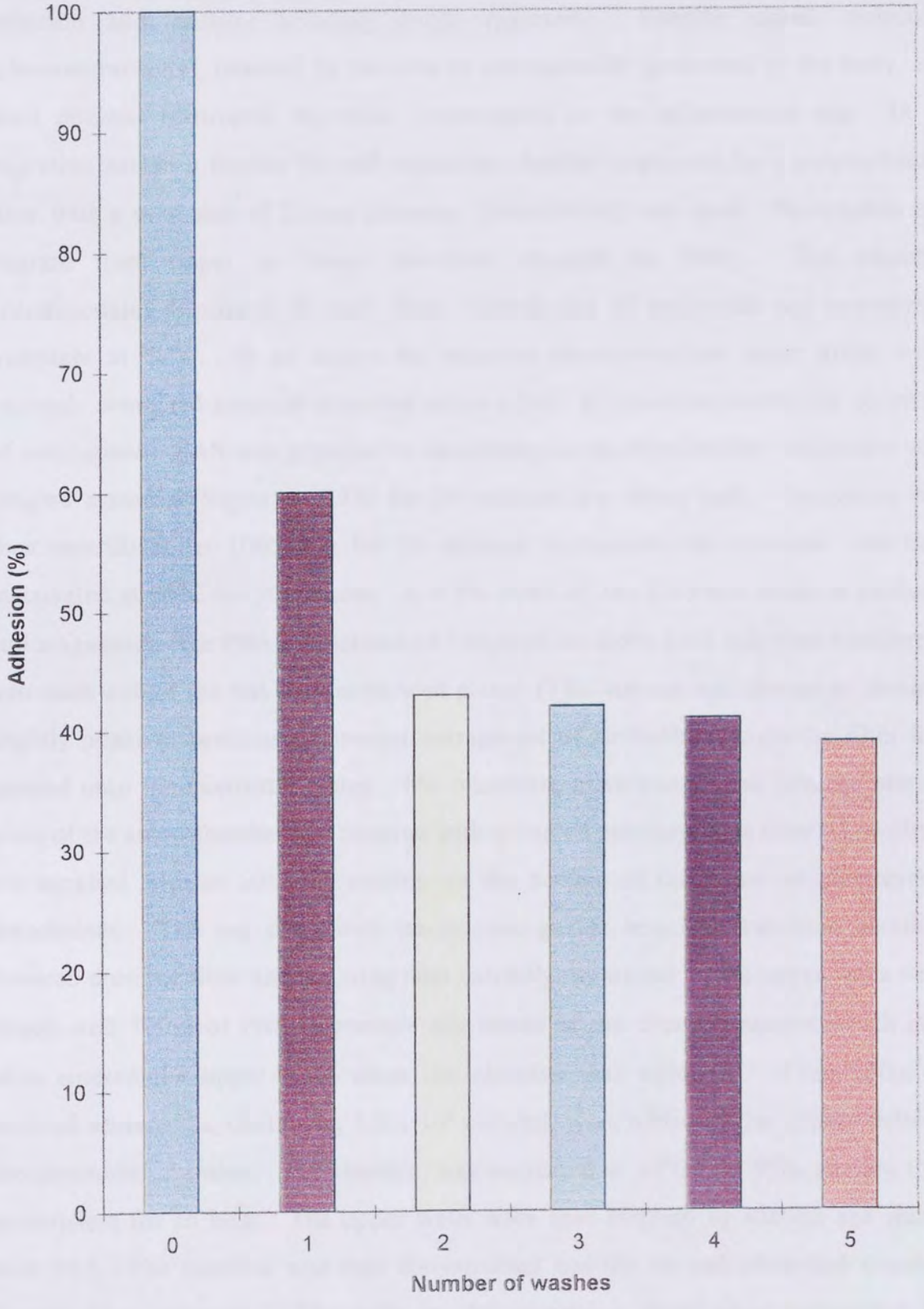
The number of neutrophils present in a well was determined by methods 2.5.2 and 2.5.3. Values given are means where n=3 in each case.

Figure 2.10 Effect of increasing time on neutrophil adhesion



Isolated neutrophils were incubated with ECV304 cells for varying time periods and adherent neutrophils assessed as in method 2.5.2. Results are mean values +/- SEM, where n=4 at each point.

Figure 2.11 Effect of washing on neutrophil adhesion



Neutrophils were allowed to adhere to ECV304 cells as in Section 2.6. The cells were then washed to see how many washes were required to remove non-adherent neutrophils. Values given are means where n=3.

2.7 Detection of neutrophil migration

Neutrophils play a major role in host defense because they rapidly migrate to sites of infection and destroy invading micro-organisms. Specific signal molecules (chemoattractants), released by bacteria or endogenously generated by the body, can elicit directed neutrophil migration (chemotaxis) to the inflammation site. In all migration assays a double 96-well migration chamber separated by a polycarbonate filter with a pore size of 2.0μm diameter (NeuroProbe) was used. Neutrophils can migrate from upper to lower chambers through the filter. The chamber accommodates disposable 96 well plates (Gibco) and all equipment was prewarmed overnight at 37°C. In all assays for putative chemoattractant some lower wells routinely contained zymosan activated serum (ZAS) as a positive control for purposes of comparison. ZAS was prepared by incubating serum from healthy volunteers with 5mg/ml zymosan (Sigma) at 37°C for 30 minutes in a water bath. The serum was then centrifuged at 1000 x g for 20 minutes, to remove the zymosan, and heat inactivated at 56°C for 30 minutes. A 0.5% (v/v) of the ZAS was made in calcium- and magnesium-free PBS. A volume of 340μl of the 0.5% ZAS was then transferred into each well of the flat bottom 96-well plate. (This volume was chosen to obtain a slightly positive meniscus to prevent entrapment of air bubbles when the filter was layered onto the microtitre plate). The microtitre plate was placed into the bottom plate of the assay chamber and covered with a framed polycarbonate filter. The filters are supplied with an adhesive coating on the bottom of the frame which prevents detachment. The top plate with its silicons gasket attached was then carefully lowered onto the filter and the wing nuts carefully tightened. The upper wells were rinsed with 100μl of PBS to remove any traces of the chemoattractant which may have entered the upper wells when the chamber was tightened. Then 200μl of isolated neutrophils, containing 2.5 x 10^6 cells/ml, were added to the upper wells of the assembled chamber. The chamber was incubated at 37°C in a 95% air, 5% CO_2 atmosphere for an hour. The upper wells were then emptied by suction and rinsed with PBS. The chamber was then disassembled and the 96-well plate and attached framed filter removed. The cells in the microtitre plate were sedimented by centrifugation at 250 x g for 15 minutes. After this time the filter was removed and 200μl of supernatant discarded to make room for the myeloperoxidase reagents. The number of cells which had migrated were assessed as in section 2.5.2. Again all test

conditions were performed in triplicate. Control sedimentation through filters due to gravity was assessed using PBS alone in the lower wells.

2.8 Detection of neutrophil oxidative burst

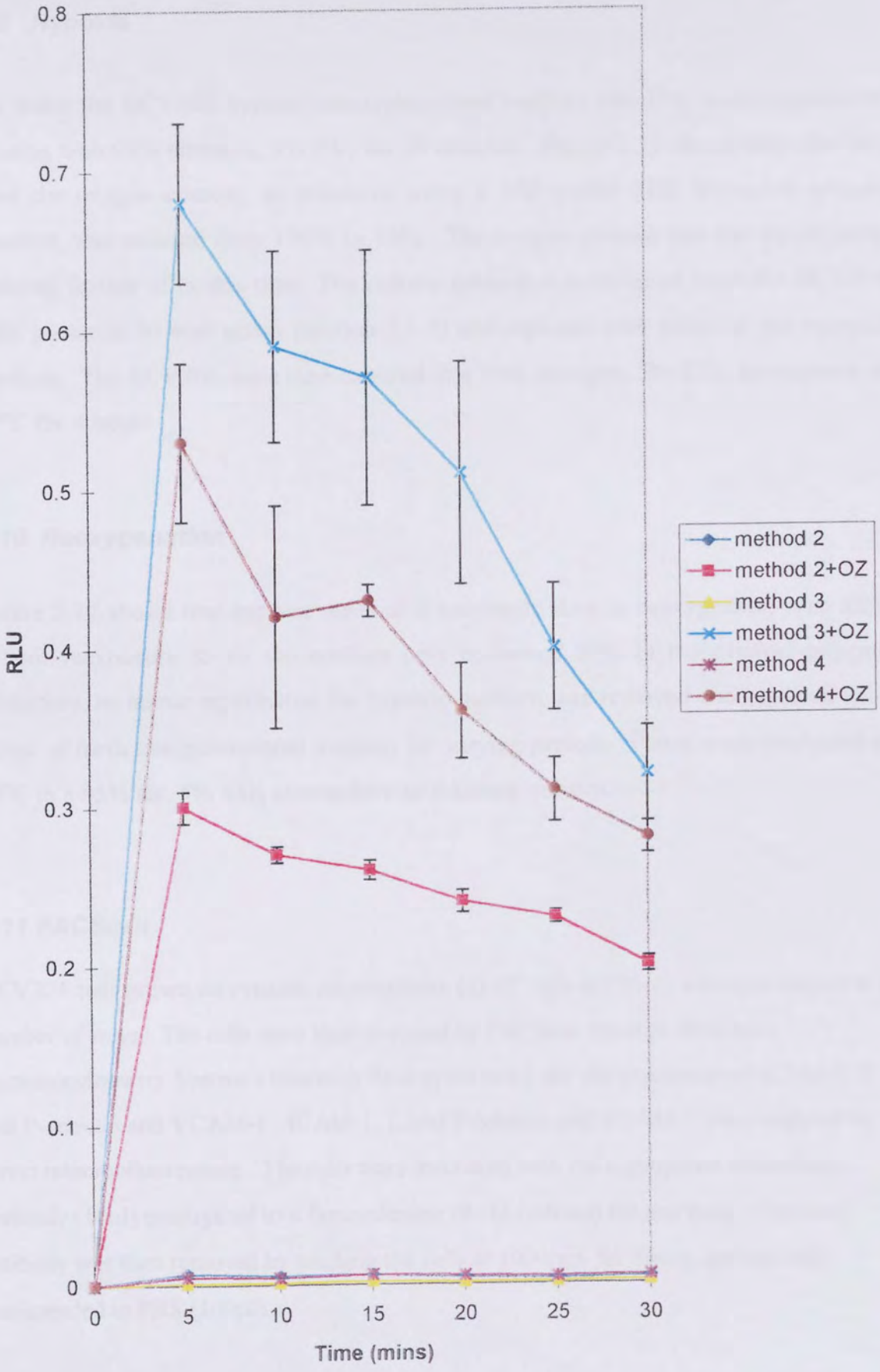
Neutrophil activation and the generation of an oxidative burst can be monitored luminometrically (Bio-Orbit) using enhanced chemiluminesence assays. A generalized luminescent reaction can be expressed as follows.

$$\underset{\text{(lucigenin)}}{\text{Reduced luminescent reactant}} + H_2O_2 \longrightarrow \underset{\text{(425nm)}}{\text{Oxidised luminescent reactant + Light}}$$

The light is detected and converted to RLU's as described in section 2.1.3. Prior to use the luminometer was switched on and allowed to warm up for approximately 15 minutes. In some experiments neutrophil activation was monitored using whole blood while in others purified neutrophils prepared by the methods outlined in 2.2 were used. Generally in a cuvette 100µl of whole blood or neutrophils at $5x10^6$ per ml were mixed with 100µl lucigenin ($5x10^{-4}$M) (Sigma) and 300µl test material, e.g. opsonised zymosan. The cuvette was then placed in a darkened waterbath at 37°C and RLU readings taken at 5 minute intervals. Each test was performed in triplicate and the average reading recorded. Opsonized zymosan was prepared by incubating 1ml of guinea pig complement (Sigma) with 5mg of Zymosan for 20 mins. After this time the zymosan was spun down using a microcentrifuge, the supernatant removed, and resuspended in 1ml of unsupplemented medium.

The neutrophils isolated by Hespan isolation method 2.2.3 were very pure. Analysis of the supernatant suggested minimal MPO release which could have been an artefact of the preparative technique indicative of damage. Furthermore the neutrophils isolated by 2.2.3 showed little spontaneous activity in terms of ROS and maximum activity when challenged with OZ (Figure 2.12). These results concur with the results obtained by cellular staining (Section 2.3), suggesting that neutrophils isolated by this procedure are largely inactive supporting our decision to routinely use method 2.2.3 to isolate all neutrophils from healthy donors.

Figure 2.12 Effect of isolation on the oxidative burst produced by neutrophils



The oxidative burst of neutrophils isolated as in Section 2.2 were measured to no stimuli and OZ (5mg/ml). Values given are means +/- SEM where n=4 at each point.

2.9 Hypoxia

To make the ECV304 hypoxic unsupplemented medium was first made hypoxic by gassing with 95% nitrogen, 5% CO_2 for 30 minutes. Figure 2.13 shows that after this time the oxygen content, as measured using a YSI model 5300 biological oxygen monitor, was reduced from 100% to 15%. The oxygen content was not significantly reduced further after this time. The culture medium was removed from the ECV304 cells grown in 96 well plates (section 2.1.2) and replaced with 100µl of this hypoxic medium. The ECV304 were then cultured in a 95% nitrogen, 5% CO_2 atmosphere at 37°C for 4 hours.

2.10 Reoxygenation

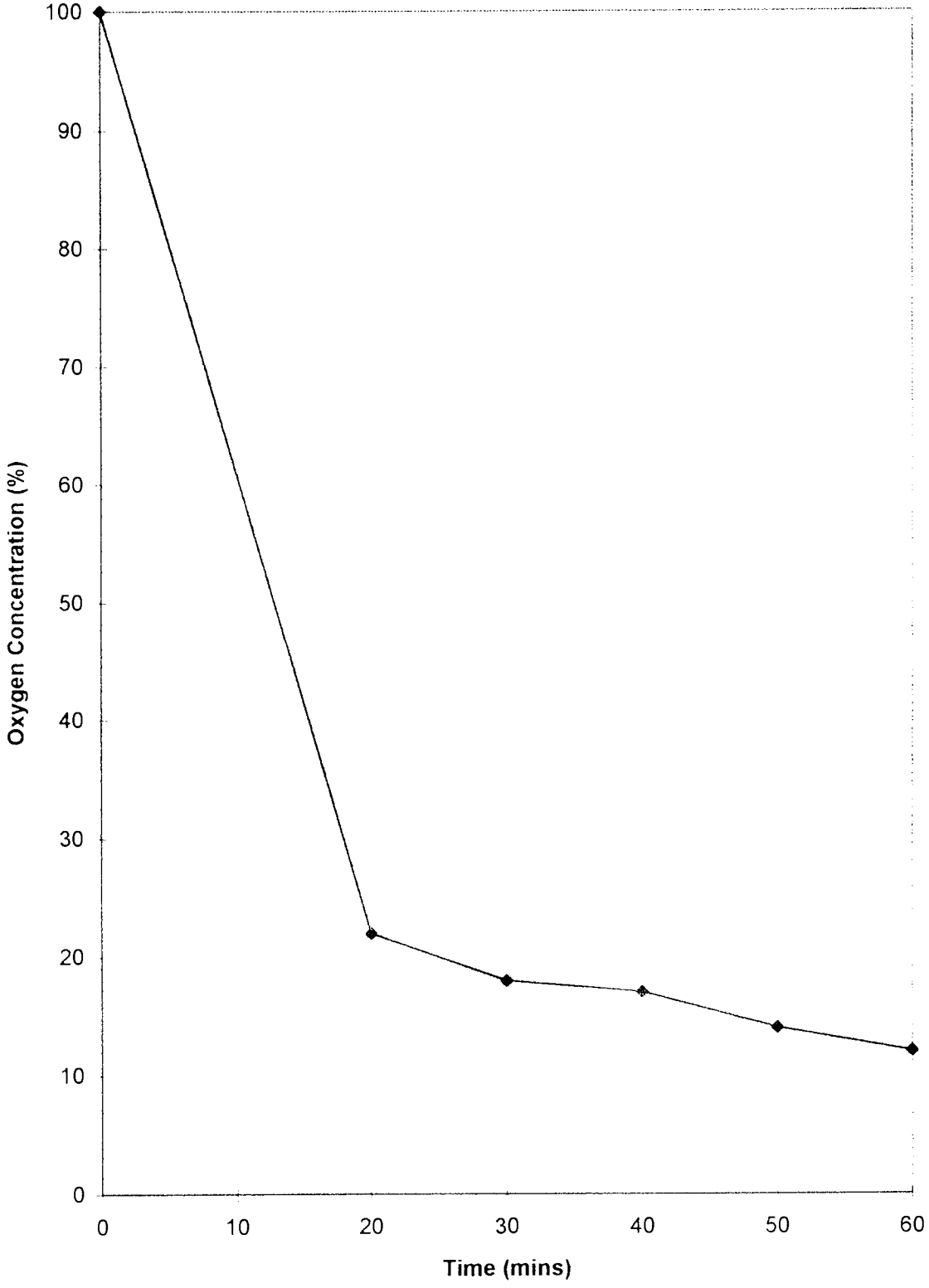
Figure 2.14 shows that hypoxic medium is extremely slow to reoxygenate; even after 1 hour reexposure to air the medium only contained 25% of the original oxygen. Therefore, to mimic reperfusion the hypoxic medium was removed and replaced with 100µl of fresh unsupplemented medium for varying periods. Plates were incubated at 37°C in a 95% air, 5% CO_2 atmosphere to maintain normoxia.

2.11 FACScan

ECV304 cells grown on cytodex microcarriers ($1x10^6$ cells in 100µl) were pre-treated in a number of ways. The cells were then analysed by FACScan (Becton Dickinson Immunocytometry System's benchtop flow cytometer), for the expression of ICAM-1, E and P-selectin and VCAM-1. ICAM-1, E and P-selectin and VCAM-1 were analysed by direct immunofluoresence. The cells were incubated with the appropriate monoclonal antibody (10µl) conjugated to a fluorochrome (R+D systems) for one hour. Unbound antibody was then removed by washing the cells at 1000rpm for 5mins, and the cells resuspended in PBS (100µl).

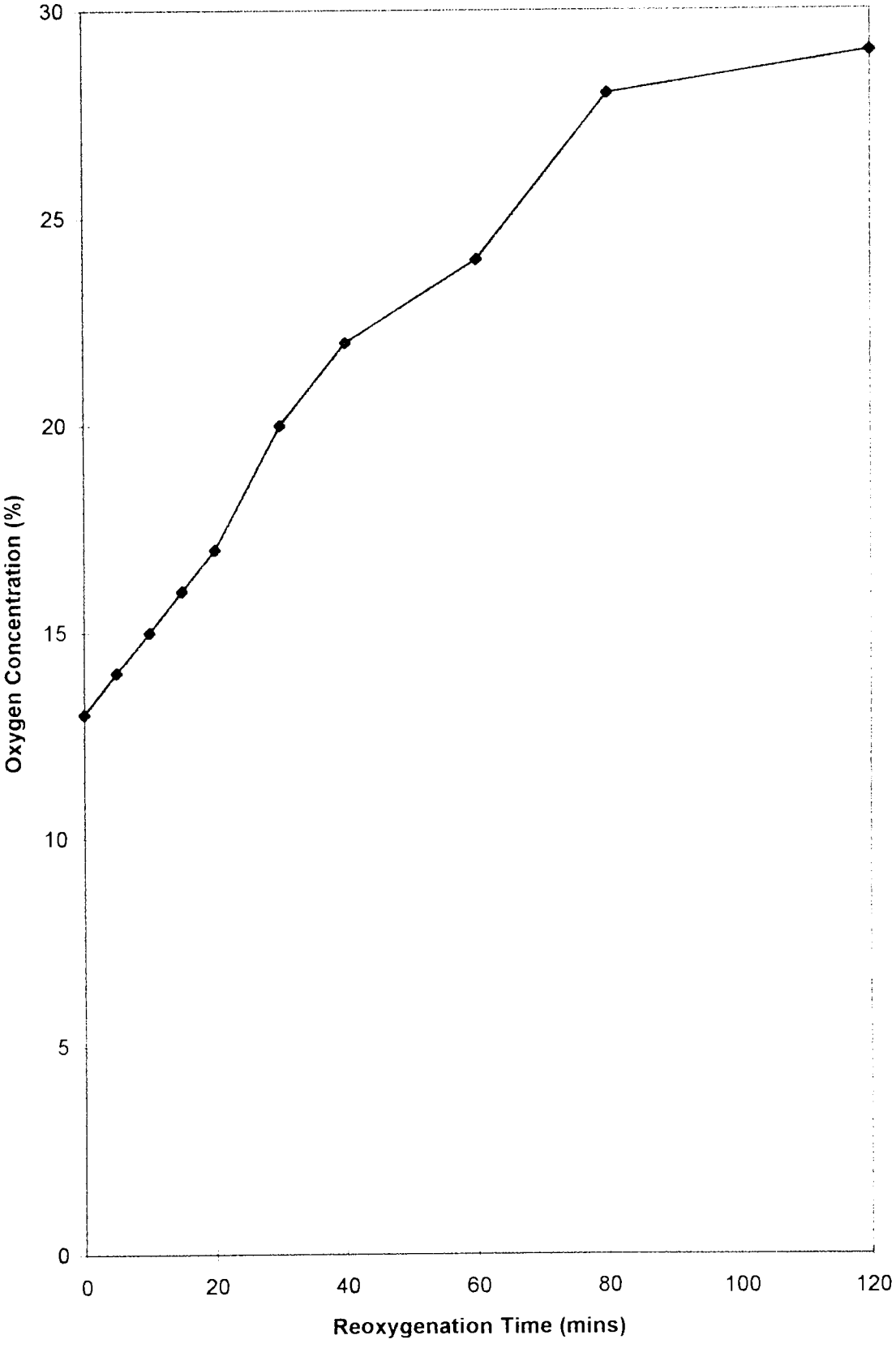
FACScan analyses cells as they travel in a moving fluid stream past a fixed laser beam. As a cell passes in front of the laser, several measurements are made based on the physical

Figure 2.13 Effect of time on degree of hypoxia



Unsupplemented medium was made hypoxic by gassing with 95% nitrogen and 5% CO2 for up to 60mins and the oxygen concentration measured using a YSI model 5300 biological oxygen meter.

Figure 2.14 Effect of time on degree of reoxygention



Unsupplemented medium was made hypoxic for 30mins and allowed to naturally reoxygenate. The oxygen concentration was then measured up to 120 mins later.

characteristics of the cell. These characteristics, which pertain to how the cells scatters the laser light and emits fluorescence, provides information about the cell's size, internal complexity and relative florescence intensity.

2.12 Cytokine analysis

The supernatant from ECV304 cells pre-treated in varying ways were analysed for IL-1, IL-8, LTB_4, and TNFα using ELISAs obtained from R+D systems. The assays employed fall into two categories. IL-1, IL-8 and TNFα use a sandwich enzyme immunoassay technique while LTB_4 uses a competitive enzyme immunoassay.

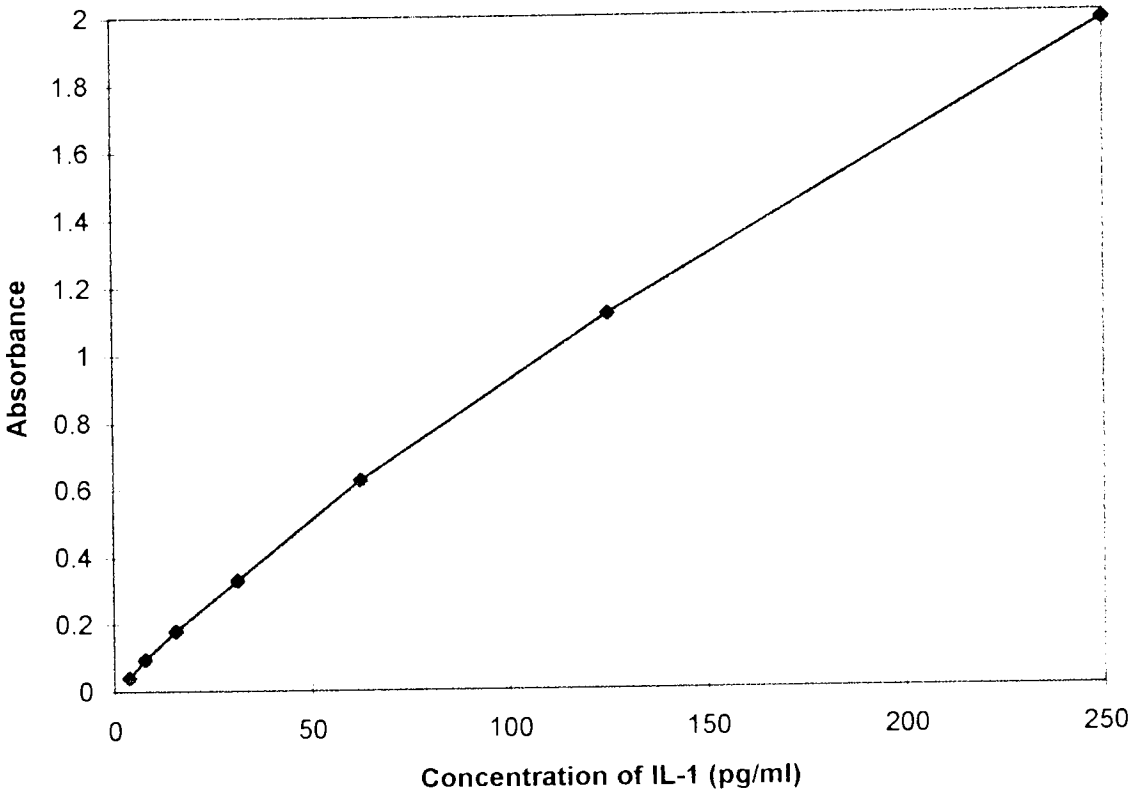
2.12.1 IL-1, IL-8 and TNFα

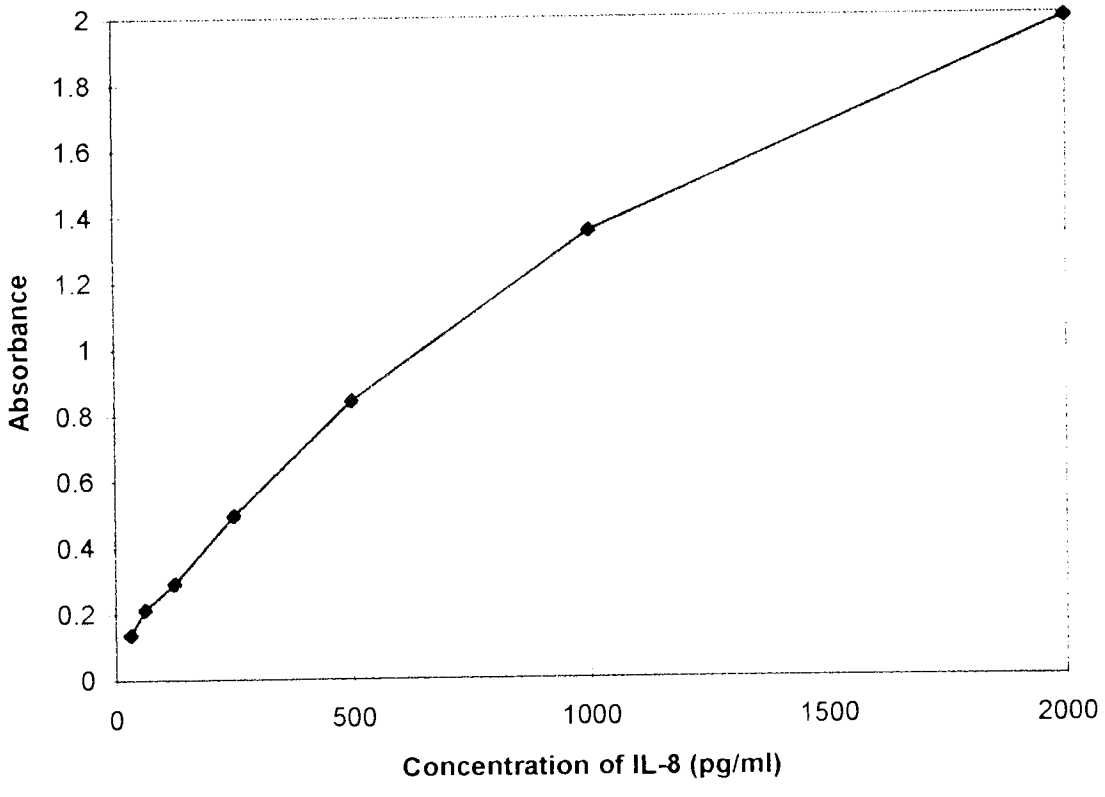
Although each of these assays employs different reagents and samples the principle of each assay is the same. A monoclonal antibody specific for the cytokine being analysed is pre-coated onto a microtitre plate. Standards, samples, and conjugate (an enzyme-linked polyclonal antibody specific for the cytokine being assayed conjugated to horseradish peroxidase) are then pipetted into the wells and any cytokine present is sandwiched by the immobilized antibody and conjugate. Following a wash to remove any unbound substances and/or antibody-enzyme reagent, a colourless substrate solution, containing hydrogen peroxide and the chromagen tetramethylbenzidine, is added to the wells and colour develops in proportion to the amount of bound cytokine. The colour development is then stopped by the addition of 2N sulphuric acid and the intensity of the blue colour is measured at 450nm. The amount of cytokine present can then be calculated by directly comparing the results to a standard curve, as produced in Figure 2.15.

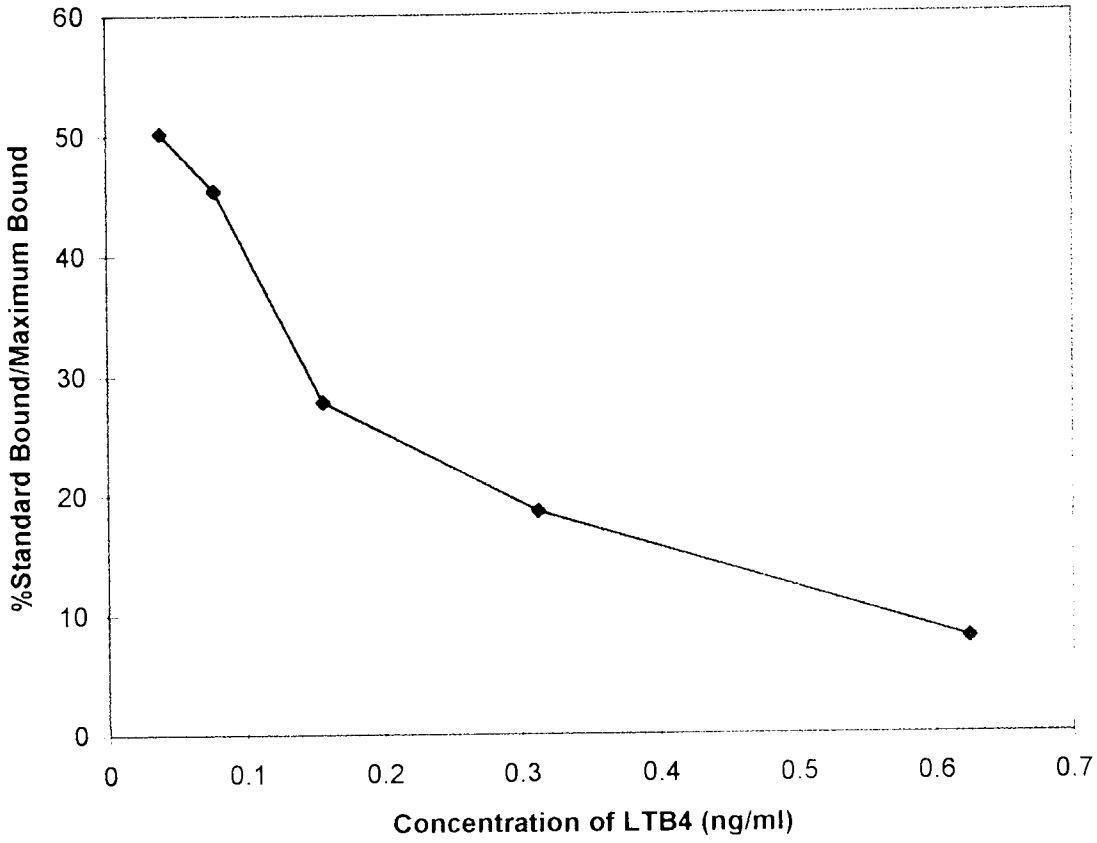
2.12.2 LTB_4

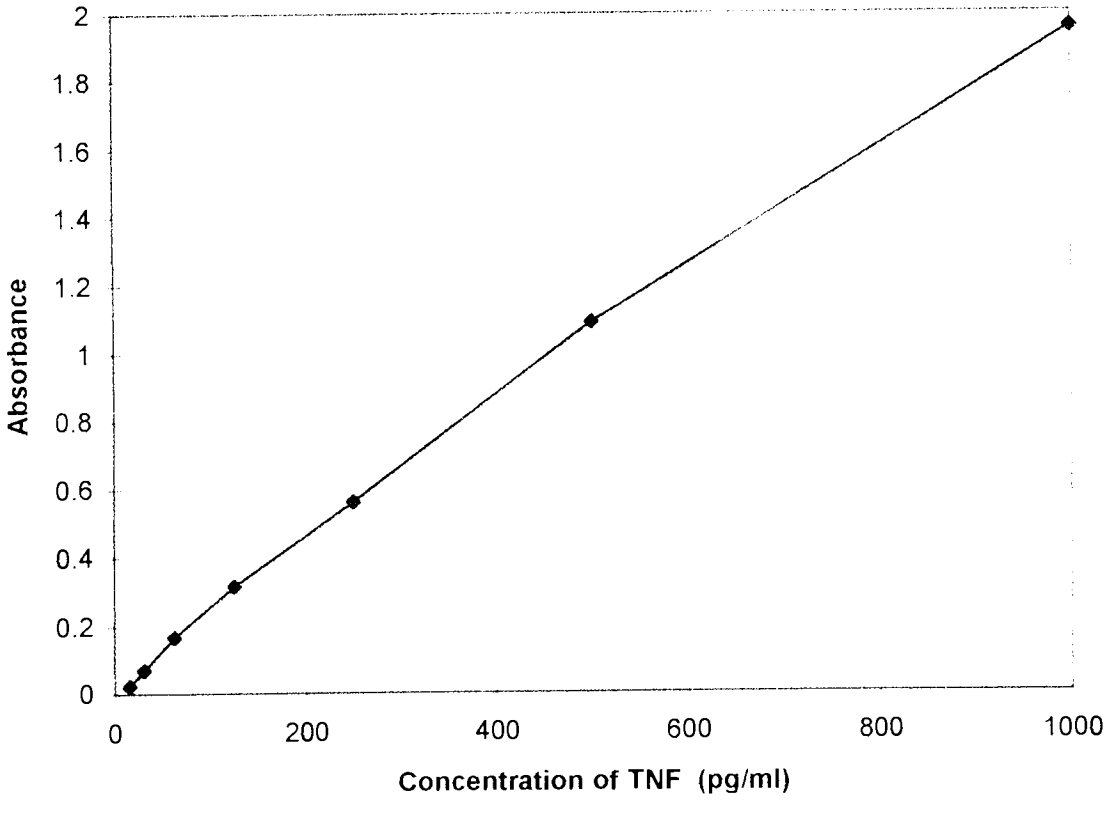
This assay is based on the competition between free LTB_4 and a LTB_4 tracer (LTB_4 linked to a acetylcholinesterase molecule) for a limited number of LTB_4 specific antiserum binding sites. The concentration of the LTB_4 tracer is held constant while the concentration of free LTB_4 , in the sample or standard, varies. Thus the amount of LTB_4 tracer that is able to bind to the antiserum will be inversely proportional to the concentration of free LTB_4 in the well. This antiserum-LTB_4 (either free or tracer) complex binds to the monoclonal antibody that has been previously attached to the well.

Figure 2.15 Standard curves for IL-1, IL-8, LTB4, and TNFα ELISAs.






60
50
40
30
20
10
0
%Standard Bound/Maximum Bound
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Concentration of LTB4 (ng/ml)


2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Absorbance
0
200
400
600
800
1000
Concentration of TNF (pg/ml)

The plate is then washed to remove any unbound reagents and then Elleman's Reagent (which contains the substrate to acetylcholinesterase) is added to the well. The product of this enzymatic reaction has a distinct yellow colour and absorbs strongly at 412nm. The intensity of this colour, determined spectrophotometrically, is proportional to the amount of LTB_4 tracer bound to the well, which is inversely proportional to the amount of free LTB_4 present in the well during the incubation. The amount of cytokine present can then be calculated by directly comparing the results to the standard curve shown in Figure 2.15.

2.1.3 Tissue Contractility

2.1.3.1 Tissue preparation

The aorta was dissected from male Wistar rats (200-250g) killed by cervical dislocation, just prior (within half an hour) to commencement of the experiment. A length of the thoracic aorta was removed and cleared of its surrounding connective tissue using forceps and scissors. This was then cut into four equal segments, 6-8mm long, mounted and mechanically rubbed on a metal triangle, to remove the endothelial layer.

2.1.3.2 Apparatus

Another metal triangle enabled the tissue to translate any contraction or relaxation to an electrical signal by attaching the bottom of the framework to the organ-bath, and the top to a Pioden isometric transducer (UFI) via a length of wire. The transducer then relayed the message, via a Lectromed pre-amplifier, to a BBC flat-bed recorder to produce a trace.

Each organ bath held 10mls of Krebs solution, warmed to 37°C by a hot-water jacket, and could be emptied and refilled with Krebs' solution quickly, so as not to damage the tissue. The physiological solution were gassed with 95% O_2, 5% CO_2 within the organ-bath prior to filling to ensure good buffer control.

2.1.3.3 Tension

The tissue was placed under a tension of 2g (measured by twenty units on the trace) whilst resting, by finely adjusting the height of the transducer. The tissues were then allowed to equilibrate for 45 mins.

2.1.3.4 Contractility Experiments

The rings were suspended in either 0.6mM or 2.5mM Ca^{2+} Krebs solution containing propranolol (10^{-6} M), ascorbic acid ($5x10^{-5}$ M) and EDTA (10^{-5} M). After 1 hour equilibration cumulative KCl concentration-response curves were constructed and the Krebs then replaced with normoxic, hypoxic or hypoxic/reoxygenated supernatant from the ECV304 cells. A second concentration-response curve to KCl was then made either immediately after applying the supernatant (0hrs) or 1hr after it had been removed from the cells. Finally a third concentration-response curve was constructed in Krebs containing either 0.6 or 2.5mM Ca^{2+}.

2.1.3.5 Analysis of Results

The results were converted to g tension, a simple enough process as 10mm on the chart = 1g. These were then plotted as tension vs KCl for each treatment applied at 0hr or 1hr in the two different calcium Krebs solutions and normalised by plotting % response vs. KCl. Finally the results were plotted as histograms of Emax (the maximum response to KCl in g) for each treatment and EC50 (the concentration of KCl, in mM, producing 50% of the maximum response) for each treatment. Only the EC50 have been shown.

2.1.4 Statistical analysis

The data was analysed using the EXCEL package for Windows on the PC. Where the data followed a normal distribution a Student's paired T-test was performed. If more than one condition was to be analysed an extension of the T-test , Two Way Anova , was carried out. All data was expressed as means +/- SEM. Statistical significance was

considered if p values were less than 0.05. Statistical evaluation was only carried out if sample groups were atleast n = 4.

3. NEUTROPHIL ADHESION

3.1 Introduction

Evidence suggests that neutrophils entering tissue which has been reperfused become activated to increase synthesis of oxygen metabolites and proteolytic enzymes, by soluble factors produced by the endothelium (Smedley *et al* 1986, Anderson and Harken 1990, Milhoan *et al* 1992 and Anderson 1995). Once adherent to the endothelium activated neutrophils can then cause injury (Welbourn *et al* 1991 and Kurose and Granger 1994). Moreover neutrophils must not only be present but their adherence to endothelium appears to be a prerequisite for microvascular injury (Bokoch 1995, Crockett *et al* 1995 Farhood *et al* 1995 and Liles *et al* 1995). Adhesion molecules on the surface of the neutrophil, along with their counter ligands on the endothelial cell membrane, appear to promote endothelial dysfunction in ways that go beyond the simple adherence of neutrophils on the endothelial surface (Downey *et al* 1996 and Lefer and Lefer 1993). In fact neutrophil-endothelial adhesion is thought to create a microenvironment which permits high concentrations of injurious agents to develop (Ward and Varani 1990 and Welbourne *et al* 1991).

Neutrophil adhesion to the endothelium, extravasation from the blood vessel and migration into the underlying tissue is an active, dynamic process involving a variety of cell-cell adhesion molecules (Lewis *et al* 1988 and Smith *et al* 1989). These events must be accurately regulated so that adhesion normally occurs only at sites of infection or inflammation (Hynes and Lander 1992, McEver 1992, Adams and Shaw 1994 and Springer 1994).

E and P selectin are expressed in small amounts on the endothelial surface (Kubes *et al* 1995). These selectins recognize sialylated carbohydrates on the neutrophil permitting it to roll along the endothelium in the direction of blood flow (Garcia-Crido *et al* 1995). Tethering allows time for firm adhesion to occur (Springer *et al* 1987). Firm adhesion is believed to be mediated by endothelial ICAM-1 and its counter ligands LFA-1 and MAC-1 (Milhoan *et al* 1992 and Pigott *et al* 1992). PAF is involved in the activation of integrins and firm adhesion (Milhoan *et al* 1992, Arnould *et al* 1993 and Kurose and Granger 1994). Once firm adhesion has

occurred neutrophils then appear to probe for the path of least resistance at interendothelial junctions before inserting a cytoplasmic process into the junction and crawling through (Harlan 1985).

Monoclonal antibodies (Mabs) directed against various neutrophil and endothelial cell adhesion molecules have been used to define the molecular determinants of the neutrophil adhesion induced by ischaemia/reperfusion (Yoshida *et al* 1992). Pre-treatment with CD18 specific MABs completely prevents the neutrophil adhesion and emigration normally observed in feline mesentery exposed to ischaemia and reperfusion (Oliver *et al* 1991). This finding is supported further by Mileski *et al* (1990), Yoshida *et al* (1992), Arnould *et al* (1993), Hartman *et al* (1995), Nakano *et al* (1995), Oshiro *et al* 1995 and Tamiya *et al* 1995). These results indicate that neutrophil adherence to ischaemic/reperfused endothelial cells is mediated by both LFA-1 and Mac-1 on neutrophils and that ICAM-1 on endothelial cells acts as the ligand for both heterodimers of CD11/CD18. Furthermore, the *in vivo* data indicates that endothelial cells exposed to hypoxia/reoxygenation release a soluble factor that results in the expression and/or activation of CD11b/CD18 on neutrophils (Springer *et al* 1986).

There appear to be two basic pathways by which neutrophils are recruited from the circulation following ischaemia/reperfusion injury. The first is immediate and does not require protein synthesis. As discussed above it is due to an increase in neutrophil adhesiveness for the endothelium by fast conformational changes in LFA-1 and MAC-1 (Smith *et al* 1988 and Yoshida *et al* 1992). ICAM-the counter ligand for LFA-1 and MAC-1 is basally expressed (Pigott *et al* 1992). The second pathway, however, requires protein synthesis by the endothelium and , therefore, takes 2-4 hours. This results in the up-regulation of a host of adhesion molecules which bind to resting and activated neutrophils (Pigott *et al* 1992). Such adhesion molecules may be ICAM-1 and E-selectin. Studies by Bruce *et al* 1991 have shown that the cytokines IL-1 and TNFα are both produced by ischaemic/reperfused tissue. Both molecules have autocrine actions on the endothelium and can cause the up-regulation of the adhesion molecules ICAM-1 and E selectin within 2-4 hours.

In order to produce a reliable and reproducible *in vitro* model of ischaemic/reperfusion injury it was first essential to determine whether neutropil

adherence to endothelial cells is significantly increased following hypoxia and reoxygenation, in line with some *in vivo* studies. This chapter details the enhanced adhesion and dissects out the role of other factors involved in ischaemic/reperfusion injury. A monoclonal antibody to ICAM-1 was used to confirm the involvement of ICAM-1 and its counter ligands on the neutrophil surface. In addition to this the effect of IL-1, IL-8, PAF, TNFα and supernatants from normoxic, hypoxic and hypoxic/reoxygenated endothelial cells were assessed for their ability to increase neutrophil adhesion to naive and hypoxic/reoxygenated endothelium.

3.2 Results

3.2.1 IL-1 treatment of ECV304 cells increases neutrophil adhesion

On average 34.2 +/- 0.8% of neutrophils adhered to unstimulated ECV304 cells. This can be maximally elevated to 60.4 +/- 7.0% after the ECV304 cells were pre-treated for 4 hours with 1000pg/ml IL-1 (Figure 3.1). This IL-1-mediated increase in neutrophil adhesion was actually significantly elevated ($p<0.05$) after pre-treatment with levels as low as 10 pg/ml IL-1.

3.2.2 TNFα treatment of ECV304 cells increases neutrophil adhesion

When ECV304 were pre-treated with varying concentrations of TNFα for 4 hours and neutrophils ($1x10^6$/ml) allowed to adhere for 30 mins the basal adhesion of 23.1+/-4.3% was increased maximally to 57.3 +/- 3.4% with 5 IU/ml (Figure 3.2) . Further increasing the concentration of TNFα caused a reduction in neutrophil adhesion to levels as low as 39.3 +/- 6.7%. Clearly in this *in vitro* models the cytokines IL-1 and TNFα can enhance adhesion mimicking the physiological effects of these molecules *in vivo*. TNFα (5IU/ml) was therefore used in all subsequent assays as a positive control.

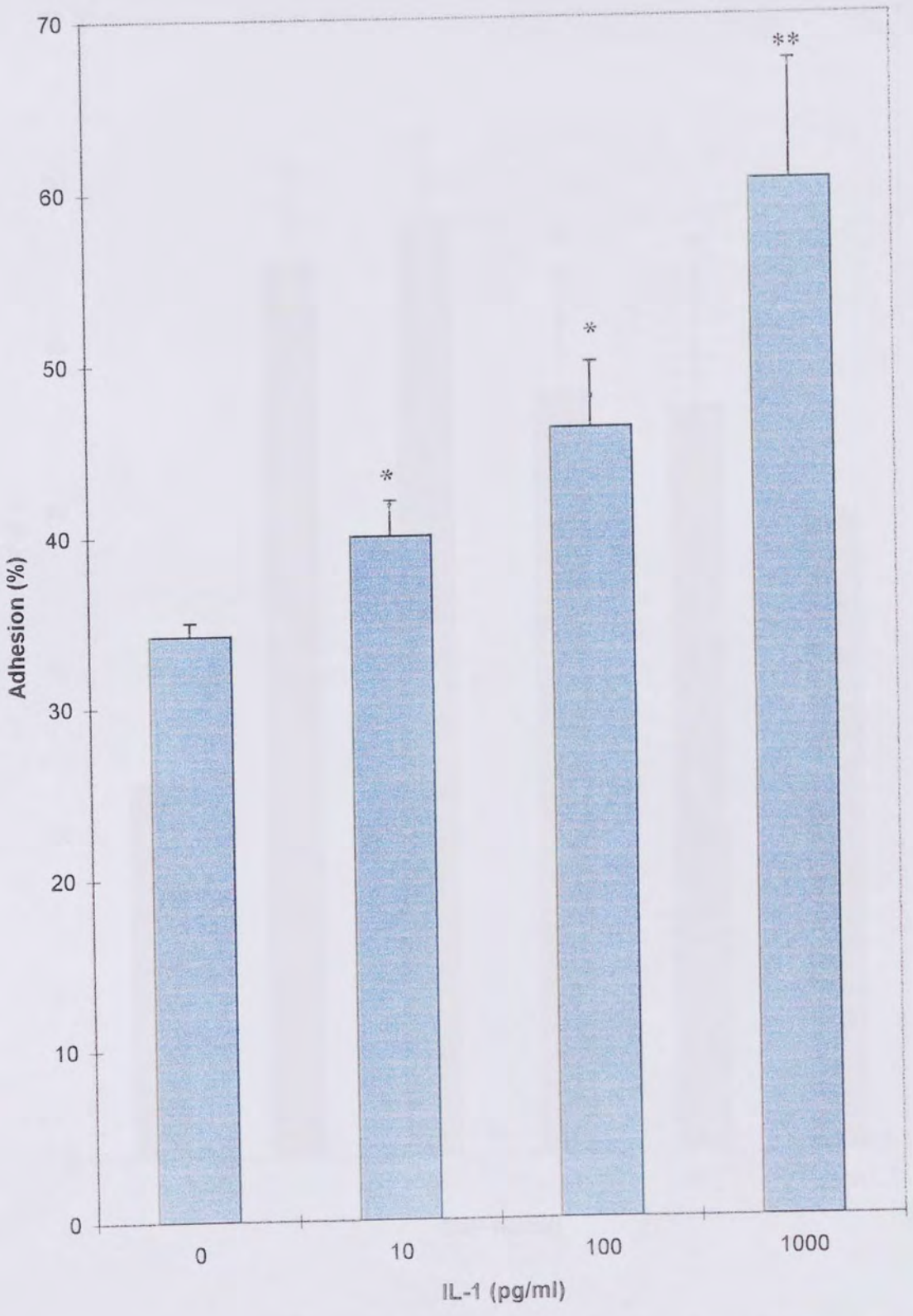
3.2.3 Hypoxia and reoxygenation causes increased neutrophil adhesion to ECV304 cells

Hypoxia for 4 hours in medium containing calcium and magnesium followed by periods of reperfusion in PBS for 1/2, 1, 2, or 4 hours without these ions shows a significant increase in neutrophil adhesion ($p<0.05$) following 30 mins reoxygenation (Figure 3.3). This effect was transient as it was no longer evident at 1, 2 and 4 hours

3.2.4 Involvement of divalent cations

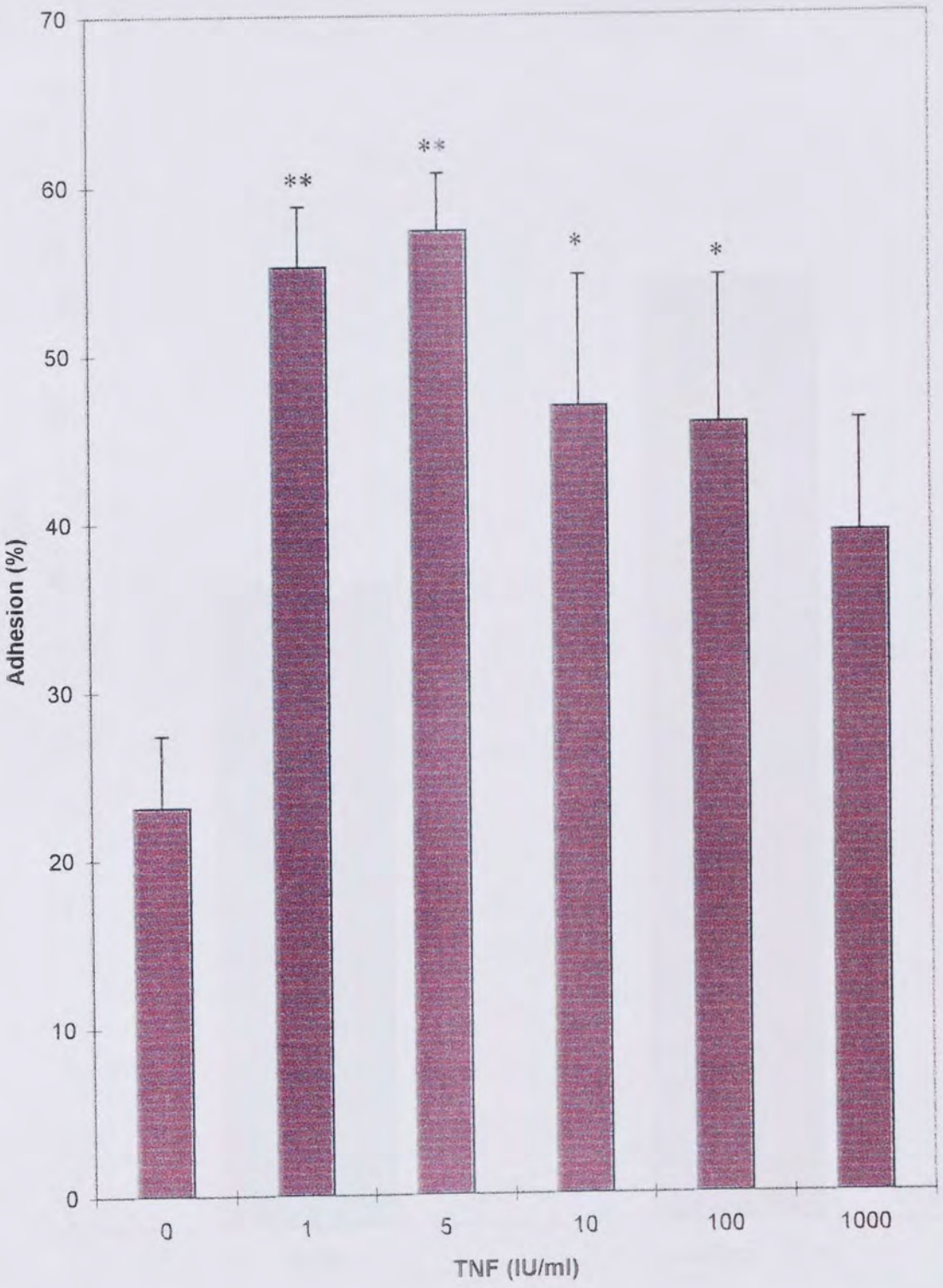
When the endothelial reperfusion was conducted in PBS containing calcium and magnesium, at 0.6mM and 1mM respectively, neutrophil adhesion was still significantly increased ($p<0.05$) from 23.1 +/- 3.9% to 31.9 +/- 8.9% (Figure 3.4).

Figure 3.1 Effect of IL-1 on neutrophil adhesion to ECV304 cells.



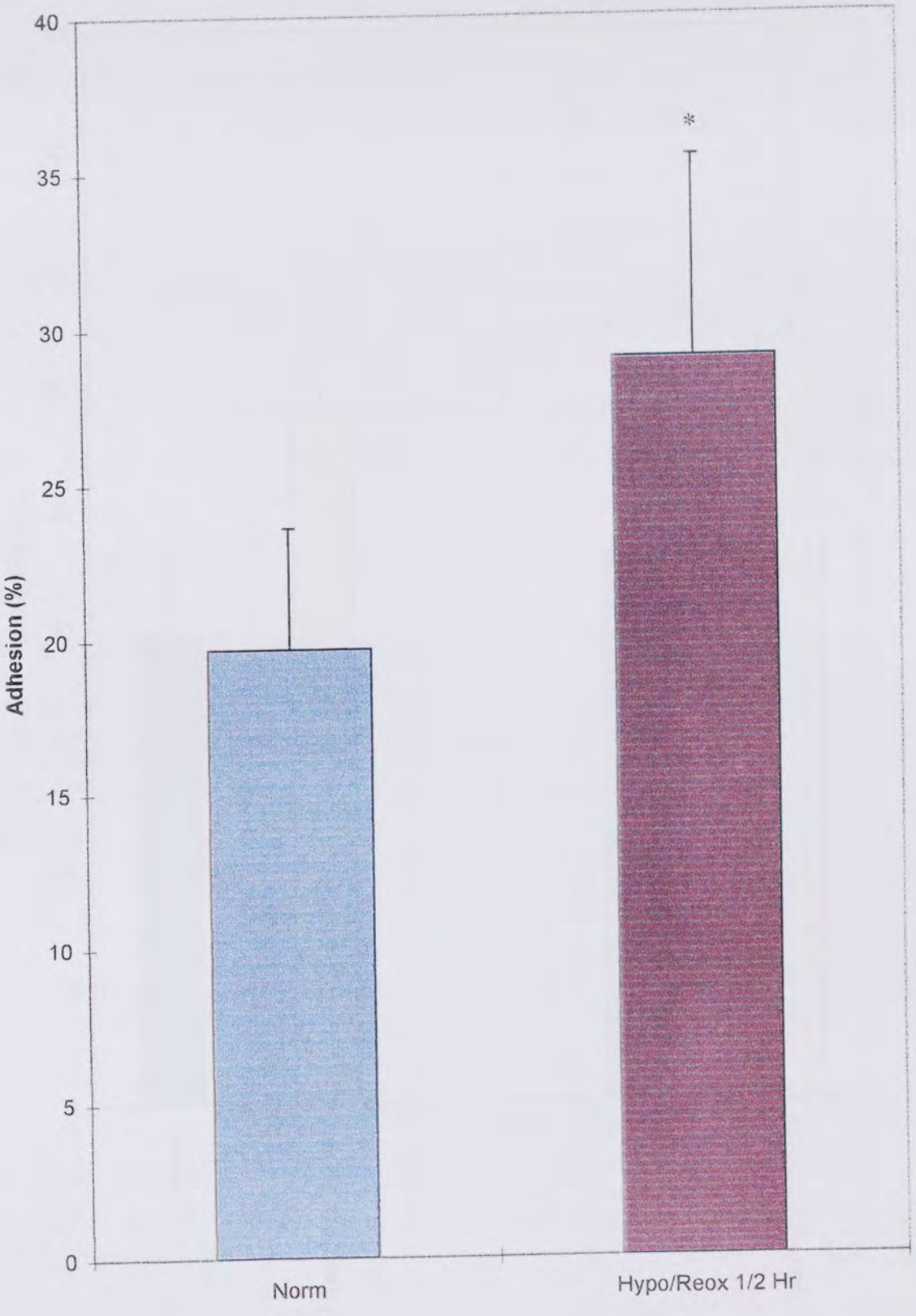
Neutrophil adhesion ($1x10^6$ per ml) to ECV304 cells that have been cultured in unsupplemented medium containing varying concentrations of human IL-1 for 4 hours. Values given are means +/- SEM, where n=4, at each point. * indicates a significant difference ($p<0.05$) ** ($p<0.01$) from control using a Two Way Anova.

Figure 3.2 Effect of TNFα on neutrophil adhesion to ECV304 cells.



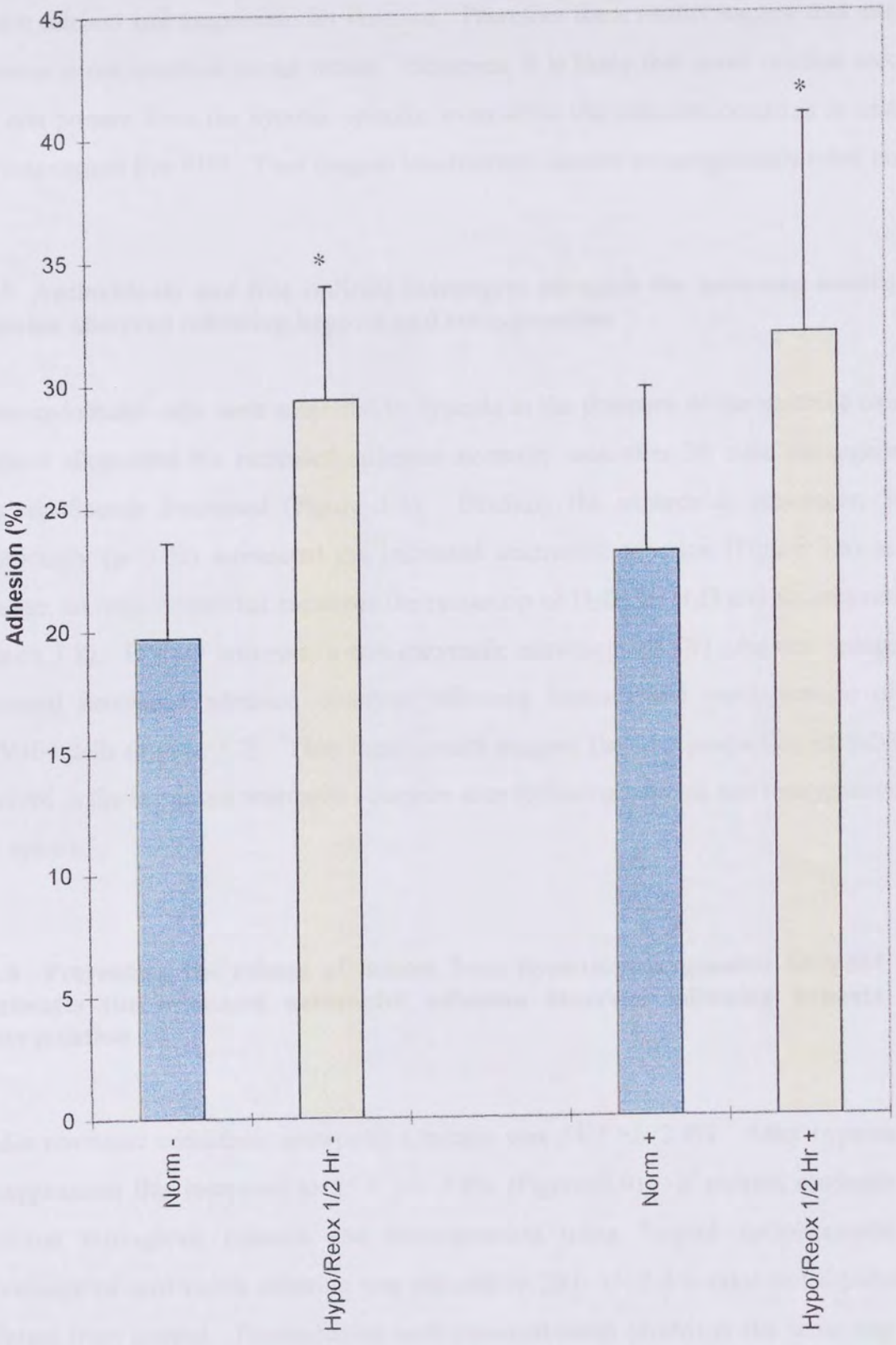
Neutrophil adhesion ($1x10^6$ per ml) to ECV304 cells that have been cultured in unsupplemented medium containing varying concentrations of human TNFα for 4 hours. Values given are means +/- SEM, where n=4, at each point. * indicates a significant difference ($p<0.05$) ** ($p<0.01$) from control using a Two Way Anova.

Figure 3.3 Effect of hypoxia and reoxygenation of endothelial cells on neutrophil adhesion.



Neutrophil adhesion ($1x10^6$ cells per ml) to ECV304 cells made hypoxic for 4 hours and reoxygenated for 30 mins. Values are means +/- SEM where n=13. * indicates a significant difference ($p<0.05$) from normoxic using a Two Way Anova. If the reoxygenation time was increased to 1, 2 or 4 hours there was no significant difference from normoxia.

Figure 3.4 Effect of hypoxia and reoxygenation of endothelial cells on neutrophil adhesion in the presence or absence of calcium and magnesium.



Neutrophil adhesion ($1x10^6$ cells per ml) to ECV304 cells made hypoxic for 4 hours and reoxygenated for 30 mins in the presence (+) or absence (-) of calcium and magnesium. Values given are means +/- SEM where n=4. * indicates a significant difference ($p<0.05$) from normoxic using a Two Way Anova.

No significant difference was found between normoxic or hypoxic/reoxygenated adhesion in the presence or absence of calcium and magnesium. Integrin subunits require calcium and magnesium for function. Therefore these results suggest that integrin adhesion is not involved in our model. However, it is likely that some residual calcium was still present from the hypoxic episode, even when the adhesion occurred in calcium and magnesium free PBS. Thus integrin involvement can not be categorically ruled out.

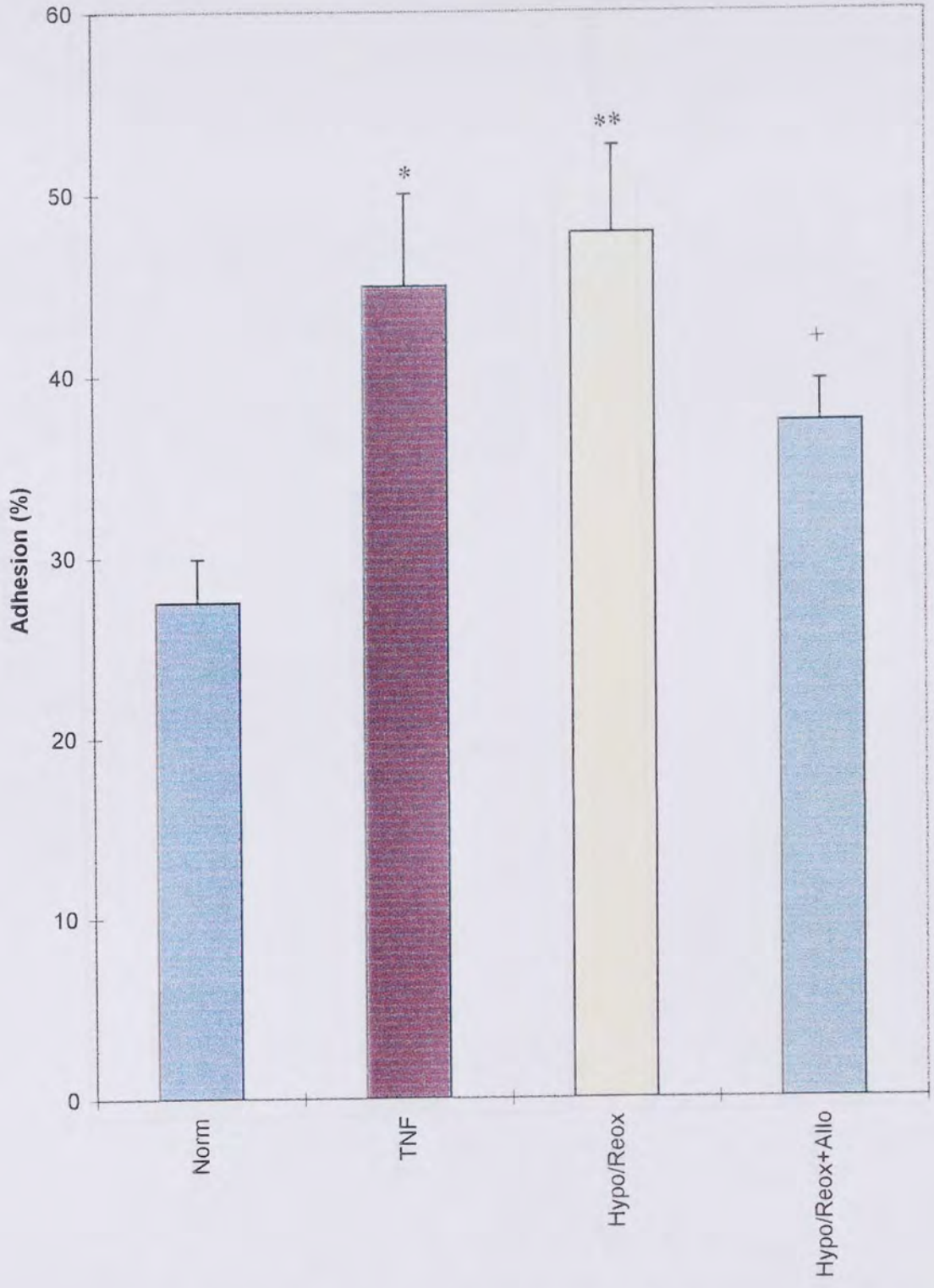
3.2.5 Antioxidants and free radicals scavengers abrogate the increased neutrophil adhesion observed following hypoxia and reoxygenation

When endothelial cells were subjected to hypoxia in the presence of the xanthine oxidase inhibitor allopurinol the increased adhesion normally seen after 30 mins reoxygenation was significantly decreased (Figure 3.5). Similarly the superoxide scavenger, SOD, significantly ($p<0.05$) attenuated the increased neutrophil adhesion (Figure 3.6) as did catalase, an antioxidant that catalyzes the reduction of H_2O_2 to H_2O and O_2 respectively (Figure 3.8). DMSO however, a non-enzymatic scavenger of OH^-, did not reduce the increased neutrophil adhesion observed following hypoxia and reoxygenation of the ECV304 cells (Figure 3.7). Thus these results suggest that the production of ROS are involved in the increased neutrophil adhesion seen following hypoxia and reoxygenation in our system.

3.2.6 Preventing the release of factors from hypoxic/reoxygenated ECV304 cells attenuates the increased neutrophil adhesion observed following hypoxia and reoxygenation

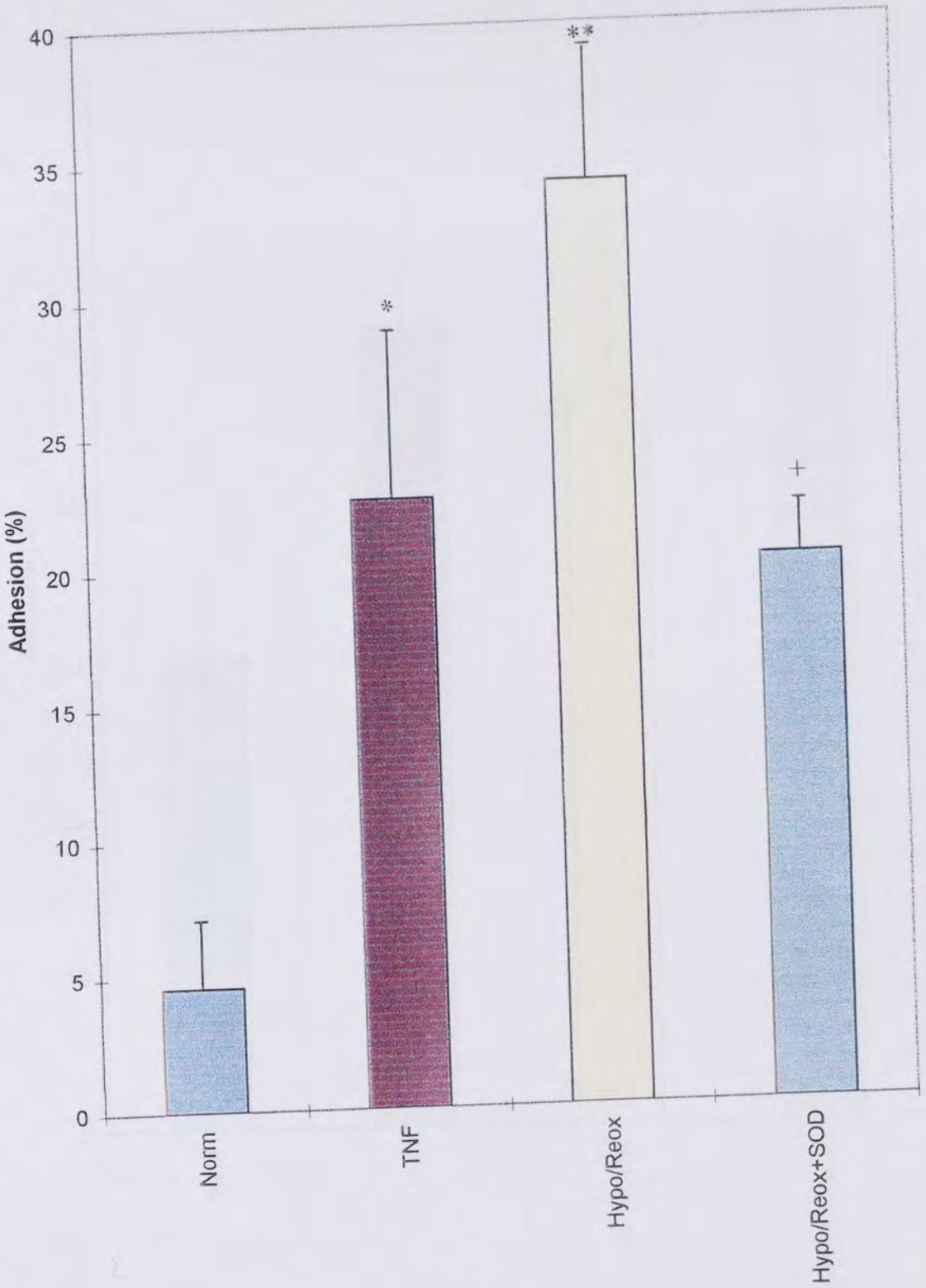
Under normoxic conditions neutrophil adhesion was 24.7 +/- 2.4%. After hypoxia and reoxygenation this increased to 55.8 +/- 3.4% (Figure 3.9). If protein synthesis was inhibited throughout hypoxia and reoxygenation using 5μg/ml cycloheximide the percentage of neutrophils adhering was reduced to 26.6 +/- 2.3 a value not significantly different from control. Preincubation with dexamethasone (5mM) in the same way also attenuated the increased neutrophil adhesion following hypoxia and reoxygenation (Figure 3.10). More specific blocking of factors possibly released by the ECV304 cells following hypoxia and reoxygenation was carried out by using a PAF secretion antagonist and a matrix metalloproteinase inhibitor (MMPI), which can block TNFα release. Figure 3.11

Figure 3.5 Effect of allopurinol on neutrophil adhesion to endothelial cells after hypoxia and reoxygenation.



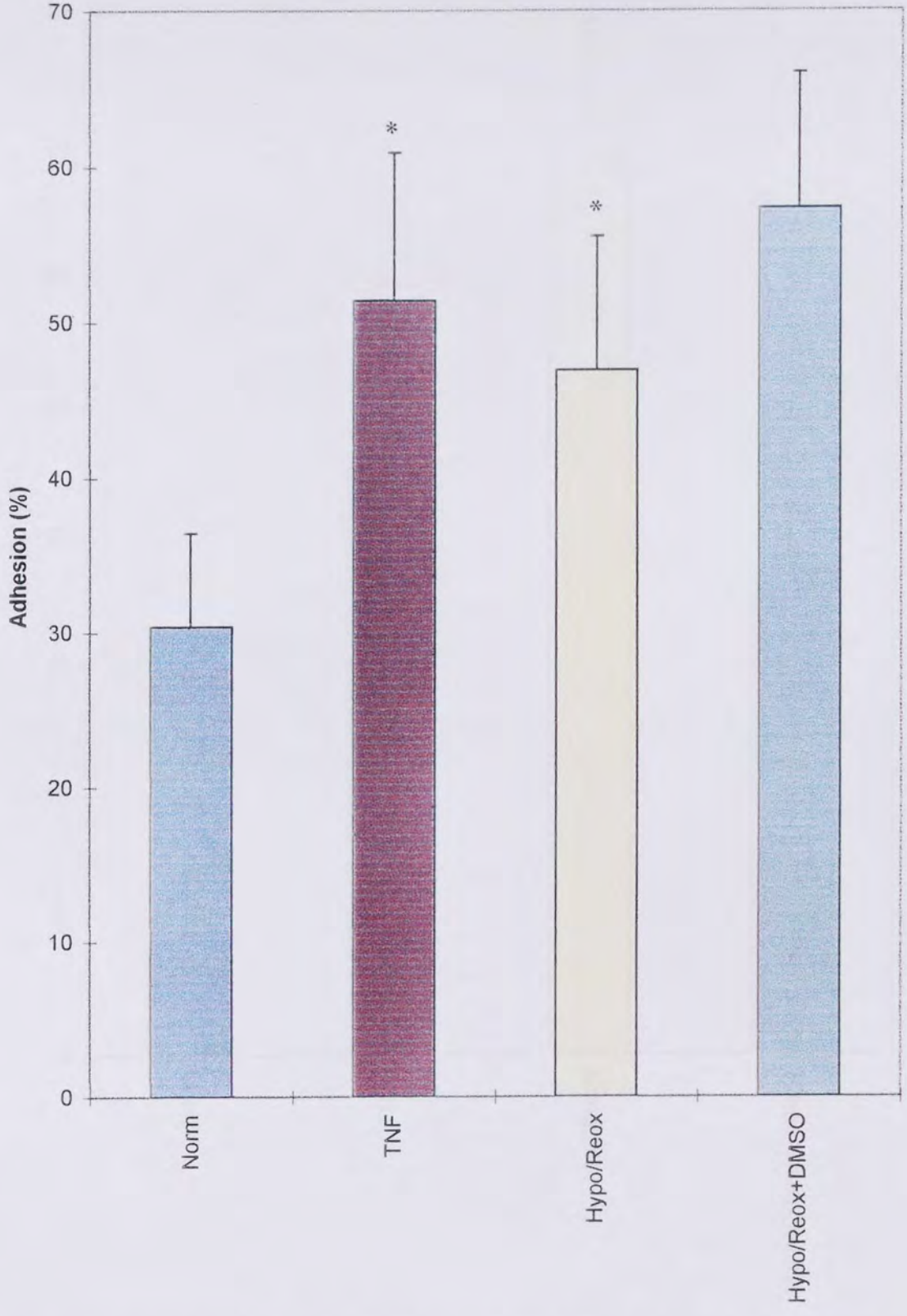
Neutrophil adhesion ($1x10^6$ cells per ml) to ECV304 made hypoxic for 4 hours and reoxygenated for 30 mins in the presence or absence of allopurinol. Allopurinol (20μM) treatment consisted of 1 hour pre-treatment before hypoxia and presence throughout the hypoxic period. Treatment of the ECV304 for 4 hours with TNFα was used as a positive control. Values given are means +/- SEM where n=4 at each point. * indicates a significant difference ($p<0.05$) ** ($P<0.01$) from normoxia while + indicates a significant difference ($p<0.05$) from hypoxia/reperfusion using a Two Way Anova.

Figure 3.6 Effect of SOD on neutrophil adhesion to endothelial cells after hypoxia and reoxygenation.



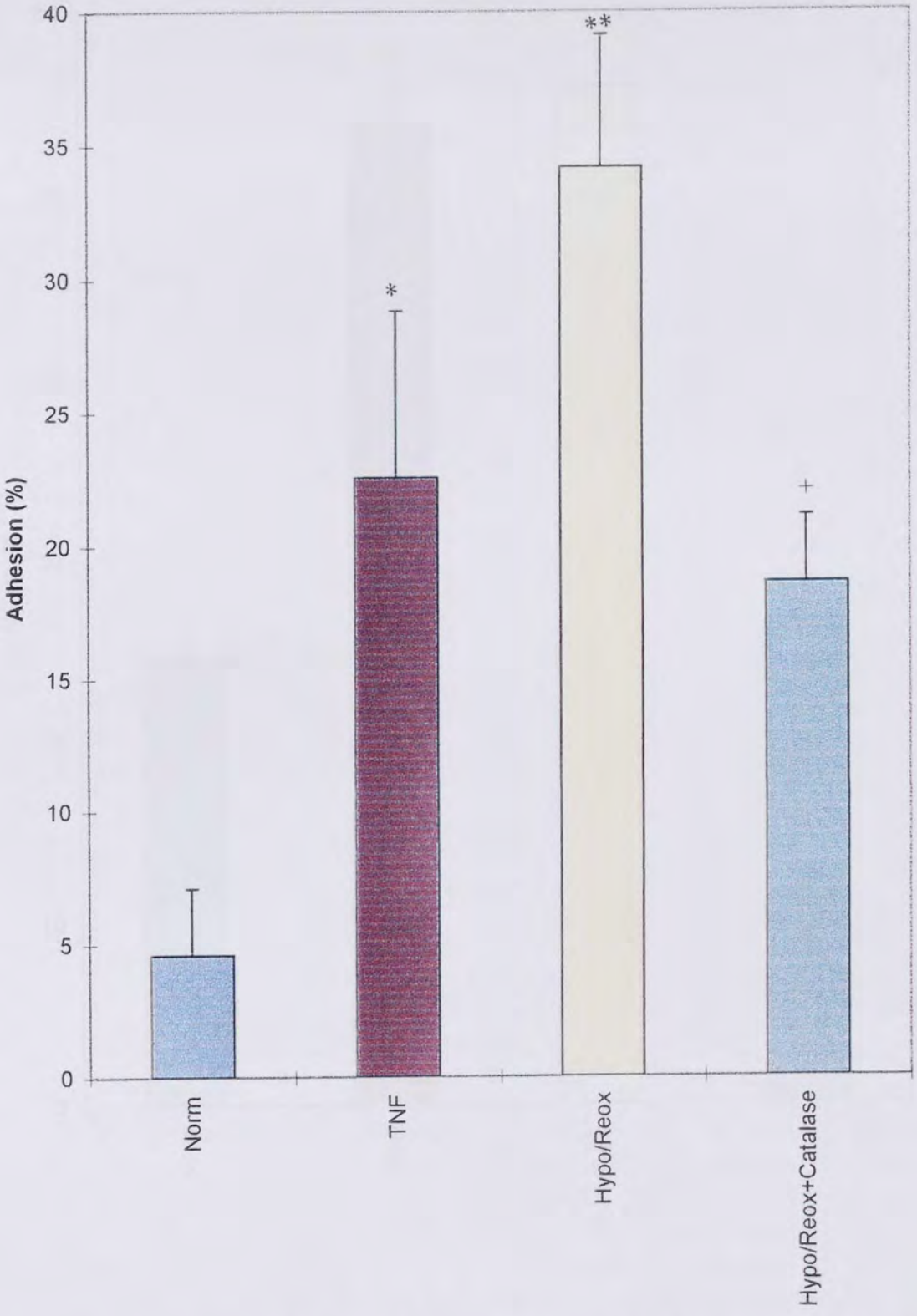
Neutrophil adhesion ($1x10^6$ cells per ml) to ECV304 made hypoxic for 4 hours and reoxygenated for 30 mins in the presence or absence of 1 unit per ml superoxide dismutase (SOD). SOD was present throughout the hypoxic and reoxygenation period. Treatment of the ECV304 for 4 hours with TNFα was used as a positive control. Values given are means +/- SEM where n=4 at each point. * indicates a significant difference ($p<0.05$) ** ($P<0.01$) from normoxia while + indicates a significant difference ($p<0.05$) from hypoxia/reperfusion using a Two Way Anova.

Figure 3.7 Effect of DMSO on neutrophil adhesion to endothelial cells after hypoxia and reoxygenation.



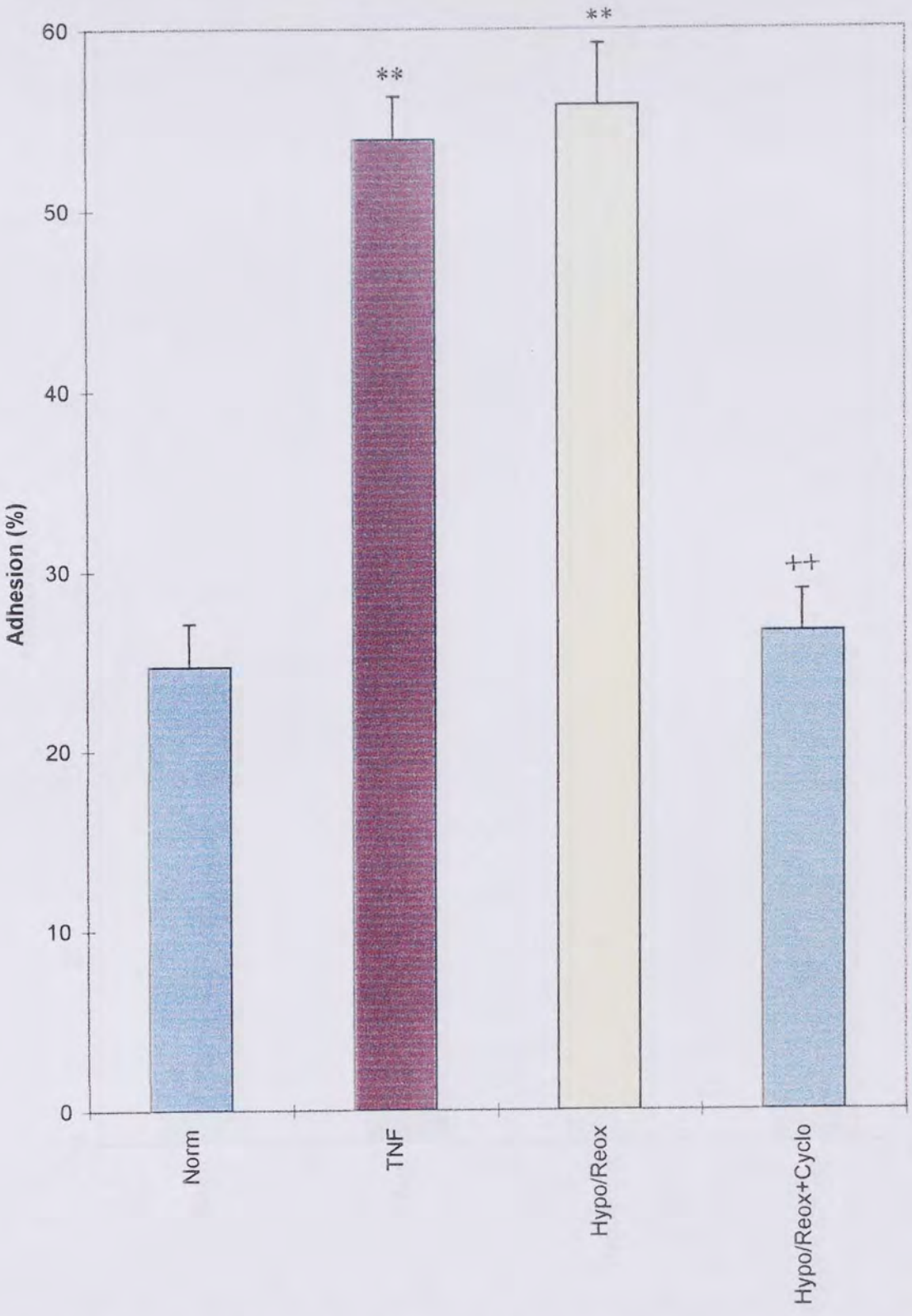
Neutrophil adhesion ($1x10^6$ cells per ml) to ECV304 made hypoxic for 4 hours and reoxygenated for 30 mins in the presence or absence of 10% DMSO. DMSO was present throughout the hypoxic and reoxygenation period. Treatment of the ECV304 for 4 hours with TNFα was used as a positive control. Values given are means +/- SEM where n=4 at each point. * indicates a significant difference ($p<0.05$) from normoxia using a Two Way Anova. There was no significant change following DMSO treatment.

Figure 3.8 Effect of catalase on neutrophil adhesion to endothelial cells after hypoxia and reoxygenation.



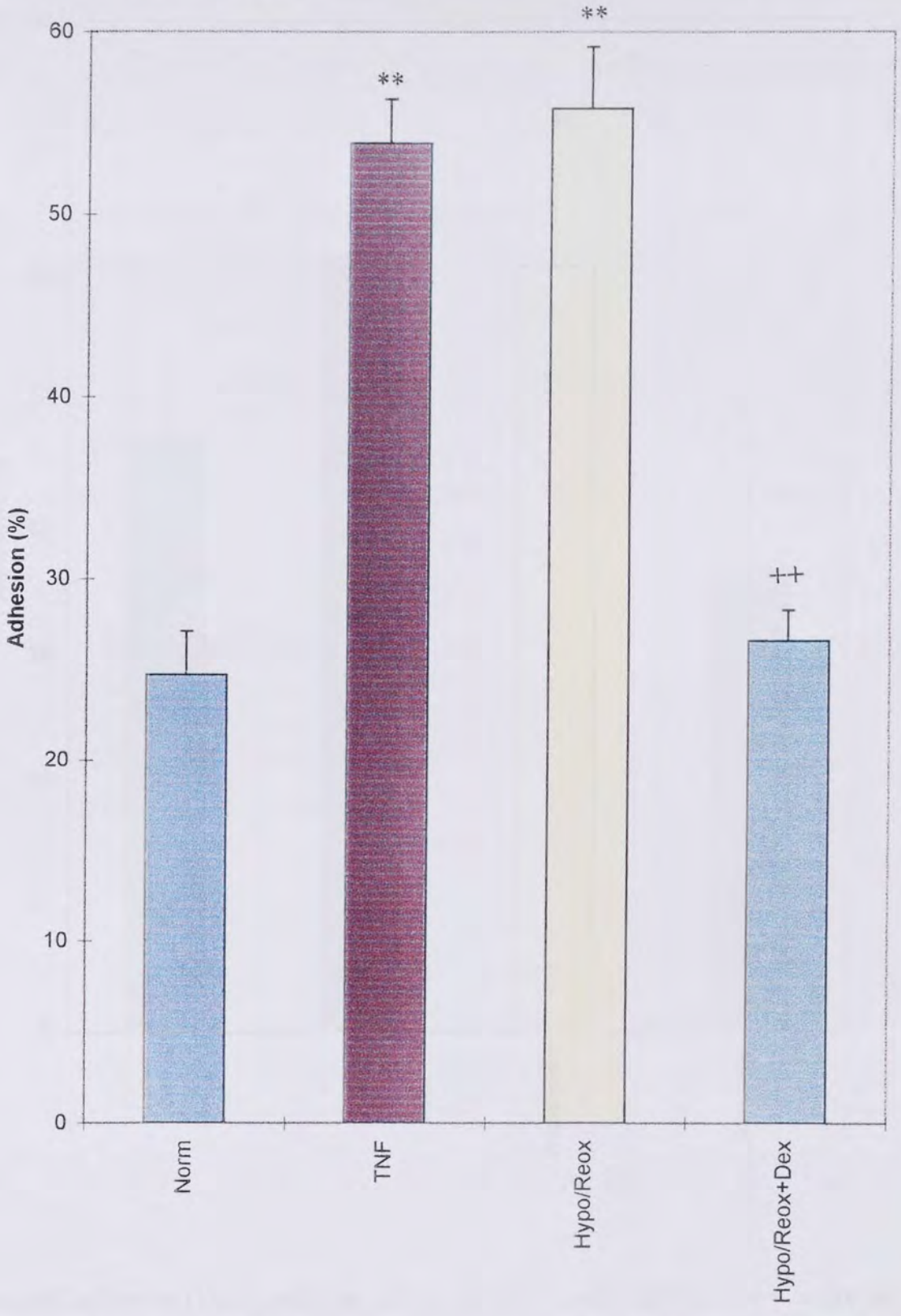
Neutrophil adhesion ($1x10^6$ cells per ml) to ECV304 made hypoxic for 4 hours and reoxygenated for 30 mins in the presence or absence of 1 unit per ml of catalase. Catalase was present throughout the hypoxic and reoxygenation period. Treatment of the ECV304 for 4 hours with TNFα was used as a positive control. Values given are means +/- SEM where n=4 at each point. * indicates a significant difference ($p<0.05$) ** ($P<0.01$) from normoxia while + indicates a significant difference ($p<0.05$) from hypoxia/reperfusion using a Two Way Anova.

Figure 3.9 Effect of cycloheximide on the ability of hypoxia and reoxygenation to induce neutrophil adhesion to ECV304 cells.



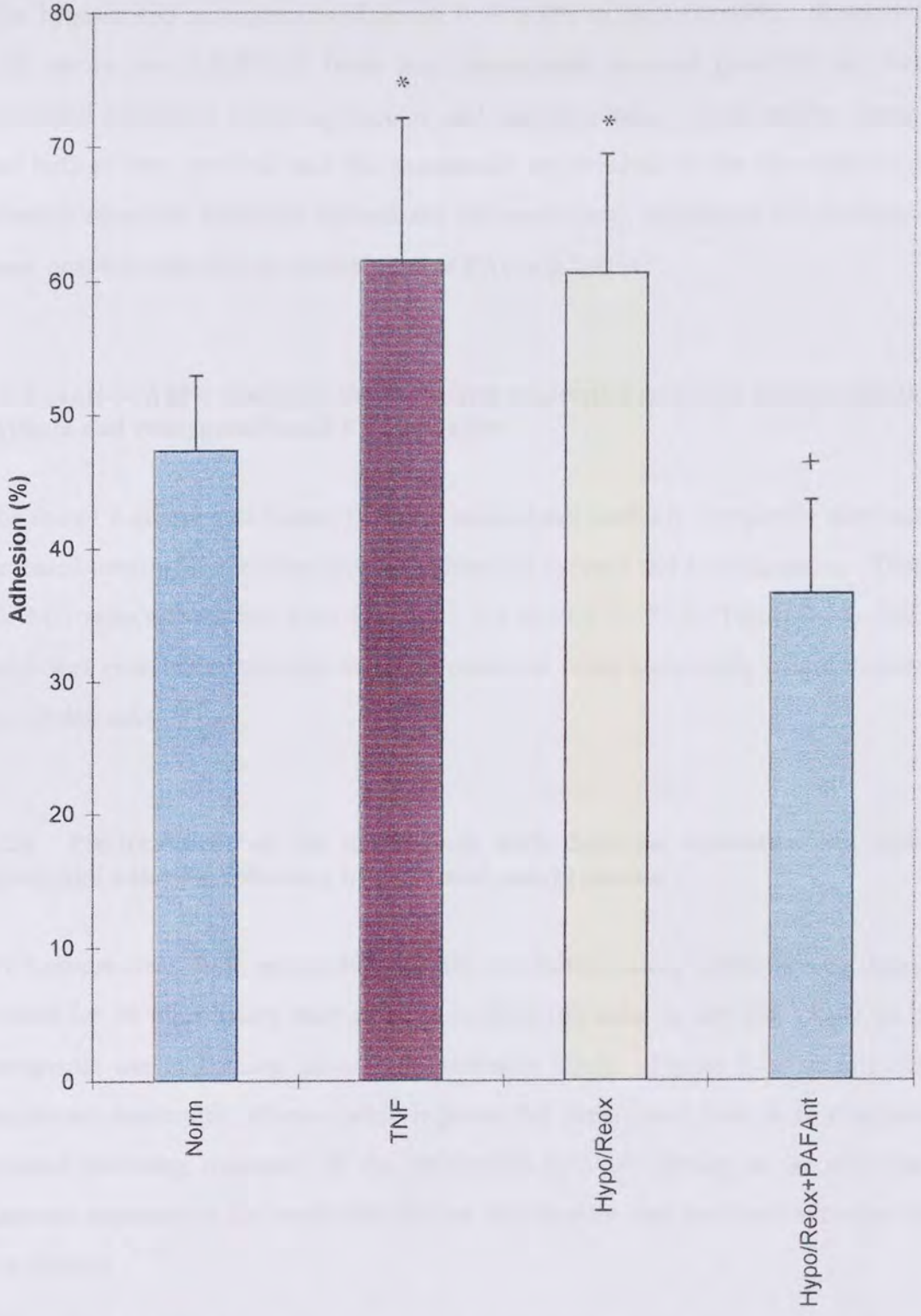
Neutrophil adhesion ($1x10^6$ cells per ml) to ECV304 made hypoxic for 4 hours and reoxygenated for 30 mins in the presence or absence of 5mg/ml cycloheximide. Cycloheximide was present throughout the hypoxic and reoxygenation period. Treatment of the ECV304 for 4 hours with TNFα was used as a positive control. Values given are means +/- SEM where n=4 at each point. ** indicates a significant difference ($P<0.01$) from normoxia while ++ indicates a significant difference ($p<0.01$) from hypoxia/reperfusion using a Two Way Anova.

Figure 3.10 Effect of dexamethasone on the ability of hypoxia and reoxygenation to induce neutrophil adhesion to ECV304 cells.



Neutrophil adhesion ($1x10^6$ cells per ml) to ECV304 made hypoxic for 4 hours and reoxygenated for 30 mins in the presence or absence of 5mM dexamethasone. Dexamethasone was present throughout the hypoxic and reoxygenation period. Treatment of the ECV304 for 4 hours with TNFα was used as a positive control. Values given are means +/- SEM where n=4 at each point. ** indicates a significant difference ($P<0.01$) from normoxia while ++ indicates a significant difference ($p<0.01$) from hypoxia/reperfusion using a Two Way Anova.

Figure 3.11 Effect of a PAF secretion antagonist on neutrophil adhesion to endothelial cells after hypoxia and reoxygenation.



Neutrophil adhesion ($1x10^6$ cells per ml) to ECV304 made hypoxic for 4 hours and reoxygenated for 30 mins in the presence or absence of a PAF secretion antagonist (10^{-8}M)(1-0-HEXADECYL-2-ACETYL-*sn*-GLYCERO-3-PHOSPHO-(N,N,N-TRIMETHYL)-HEXANOLAMINE). PAF antagonist was present throughout the hypoxic and reoxygenation period. Treatment of the ECV304 for 4 hours with TNFα was used as a positive control. Values given are means +/- SEM where n=4 at each point. * indicates a significant difference ($P<0.05$) from normoxia while + indicates a significant difference ($p<0.05$) from hypoxia/reperfusion using a Two Way Anova.

demonstrates that the PAF secretion antagonist at 10^{-8} M decreased neutrophil adhesion after hypoxia and reoxygenation from 60.5 +/- 8.8% to 36.5 +/- 6.9%. Similarly Figure 3.12 shows that MMPI at 1mM also significantly lowered ($p<0.05$) the increased neutrophil adherence following hypoxia and reoxygenation. These results demonstrate that both protein synthesis and the eicosanoids are involved in the increased neutrophil adhesion observed following hypoxia and reoxygenation , suggesting the involvement of many possible contributing factors, such as PAF and TNF∝.

3.2.7 Anti-ICAM-1 abolishes the increased neutrophil adhesion witnessed following hypoxia and reoxygenation of EVC304 cells

The use of a mouse anti-human ICAM-1 monoclonal antibody completely eliminated the increased neutrophil adhesion observed following hypoxia and reoxygenation. Thus anti-ICAM-1 reduced adhesion from 57.3 +/- 1.1% to 19.0 +/- 7.1% (Figure 3.13).This latter value was even lower than the adhesion observed when neutrophils bound to normoxic endothelial cells.

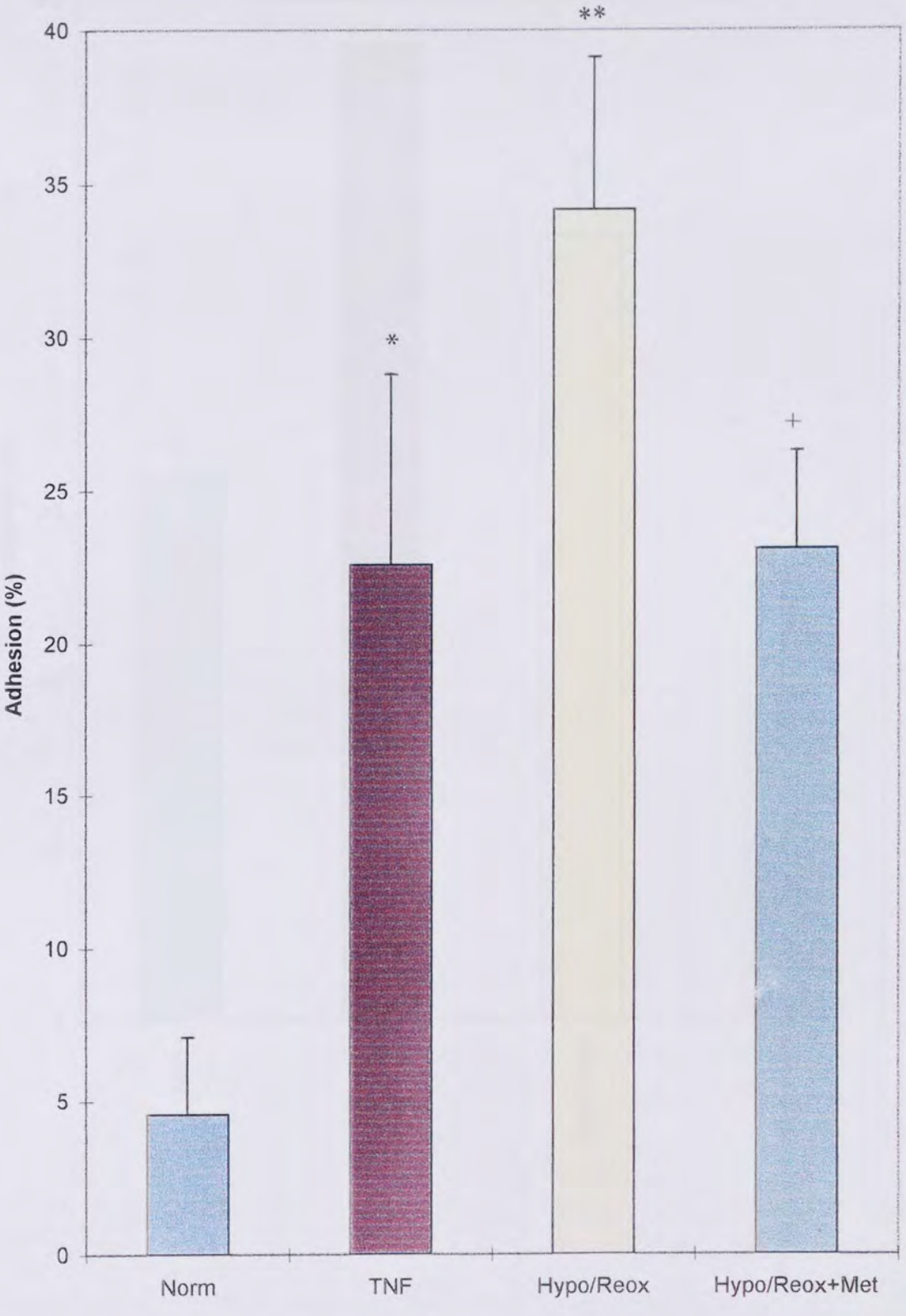
3.2.8 Pre-treatment of the neutrophils with dapsone attenuates the increased neutrophil adhesion following hypoxia and reoxygenation

We then pre-treated the neutrophils with the anti-inflammatory sulphone drug dapsone at 0.1mM for 30 mins before their addition to ECV304 cells, to see if it would be of any therapeutic use in limiting ischaemic/reperfusion injury. Figure 3.14 reveals that the heightened neutrophil adhesion after hypoxia and reperfusion was in fact significantly lowered following treatment of the neutrophils ($p<0.05$) hinting at its potential use. Dapsone treatment of the neutrophils did not significantly alter normoxic adhesion (results not shown).

3.2.9 A soluble factor produced by ECV304 cells following hypoxia and reoxygenation augments neutrophil adhesion

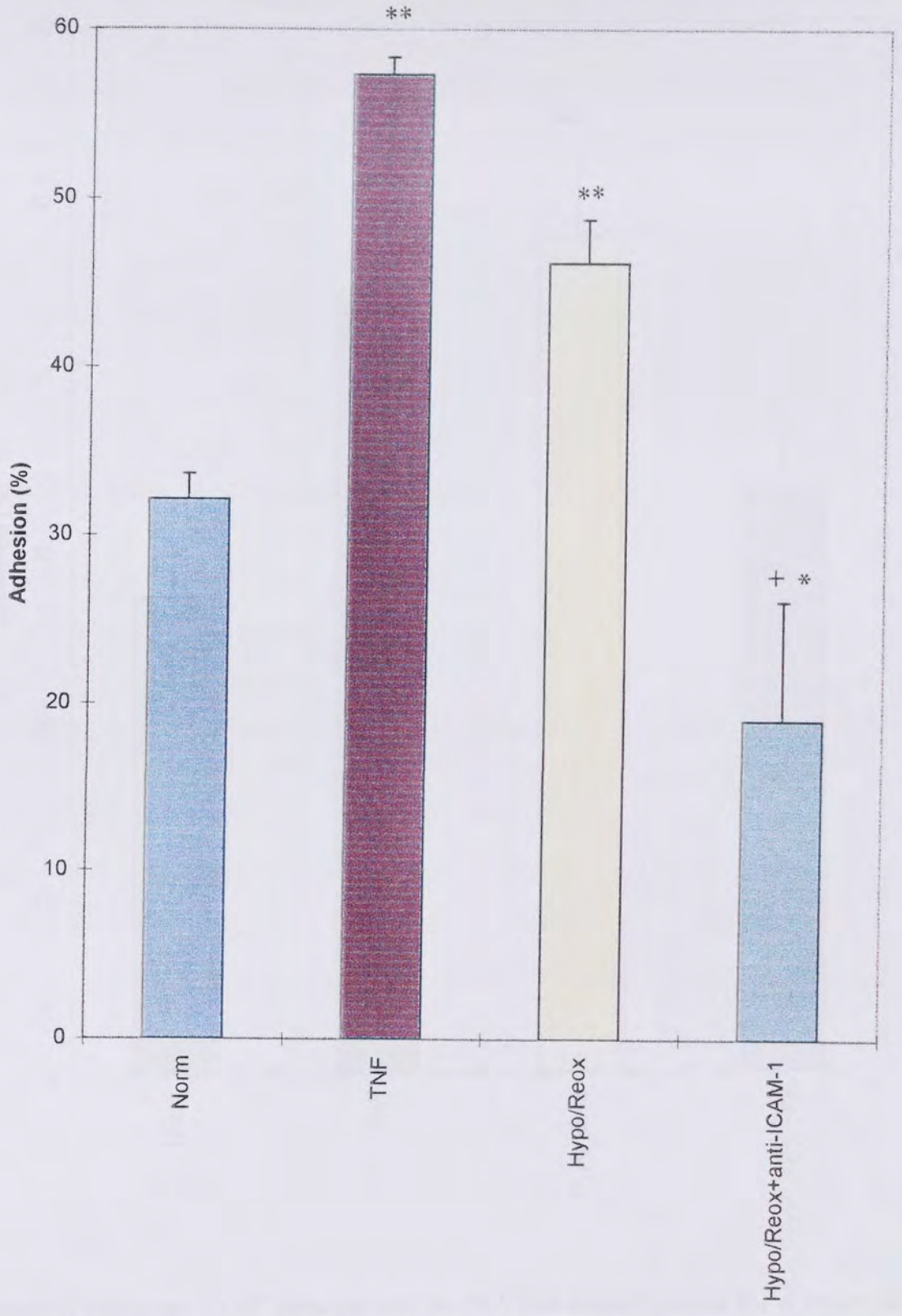
Isolated neutrophils ($1x10^6$/ml) were resuspended in the supernatant from ECV304 cells that were kept normoxic for four hours, hypoxic for four hours or hypoxic for four hours and reoxygenated for 30 mins. These neutrophils were then allowed to adhere to naive

Figure 3.12 Effect of MMPI on neutrophil adhesion to endothelial cells after hypoxia and reoxygenation.



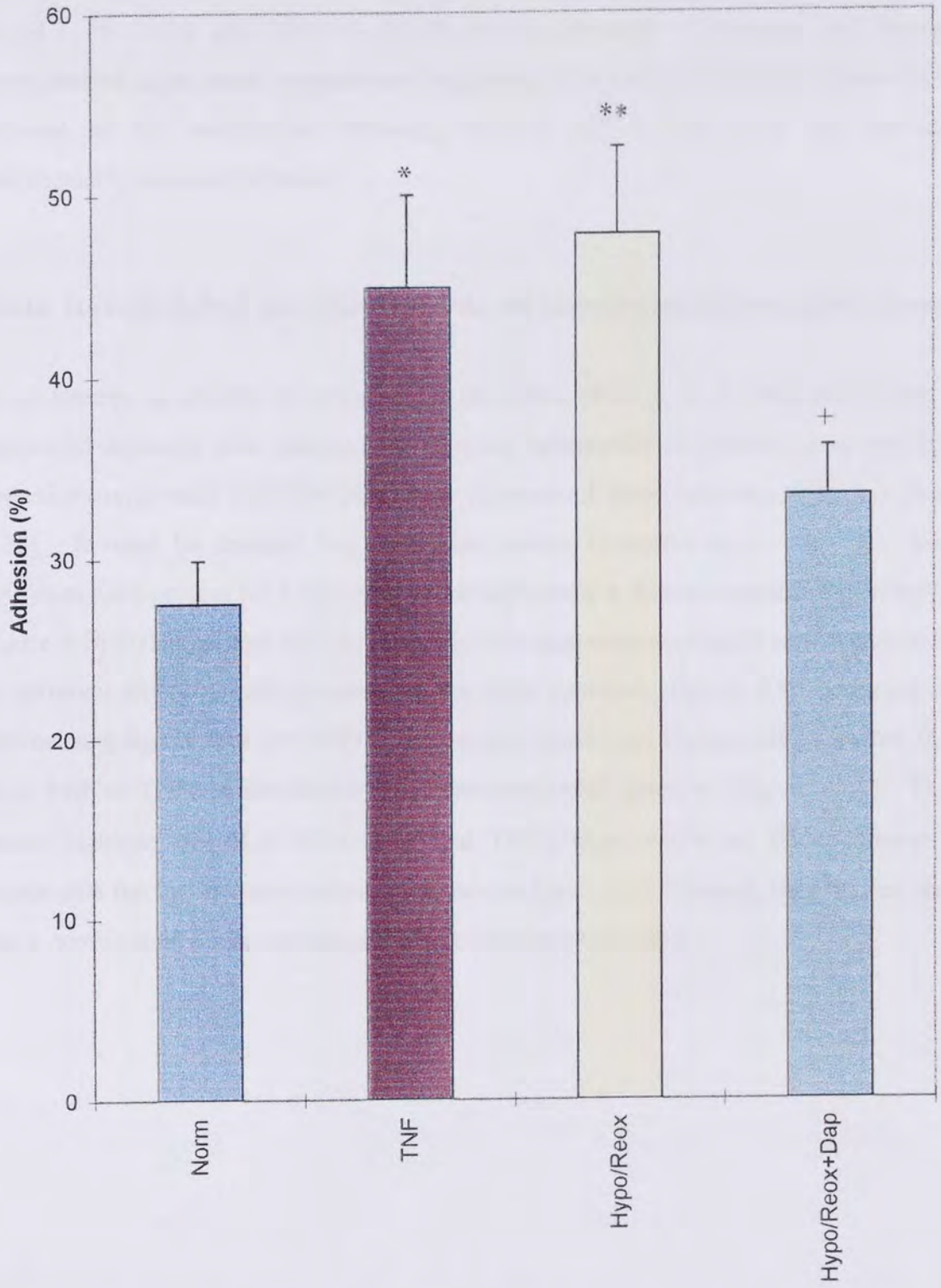
Neutrophil adhesion ($1x10^6$ cells per ml) to ECV304 made hypoxic for 4 hours and reoxygenated for 30 mins in the presence or absence of 1mM MMPI. MMPI was present throughout the hypoxic and reoxygenation period. Treatment of the ECV304 for 4 hours with TNFα was used as a positive control. Values given are means +/- SEM where n=4 at each point. * indicates a significant difference ($p<0.05$) ** ($p<0.01$) from normoxia while + indicates a significant difference ($p<0.05$) from hypoxia/reperfusion using a Two Way Anova.

Figure 3.13 Effect of anti-human-ICAM-1 on neutrophil adhesion to endothelial cells after hypoxia and reoxygenation.



Neutrophil adhesion ($1x10^6$ cells per ml) to ECV304 made hypoxic for 4 hours and reoxygenated for 30 mins in the presence or absence of 10mg/ml anti-ICAM-1. Anti-ICAM-1 was present throughout the hypoxic period. Treatment of the ECV304 for 4 hours with TNFα was used as a positive control. Values given are means +/- SEM where n=4 at each point. ** indicates a significant difference ($p<0.01$) from normoxia while + indicates a significant difference ($p<0.05$) from hypoxia/reperfusion using a Two Way Anova.

Figure 3.14 Effect of dapsone on the ability of hypoxia and reoxygenation to induce neutrophil adhesion to ECV304 cells.



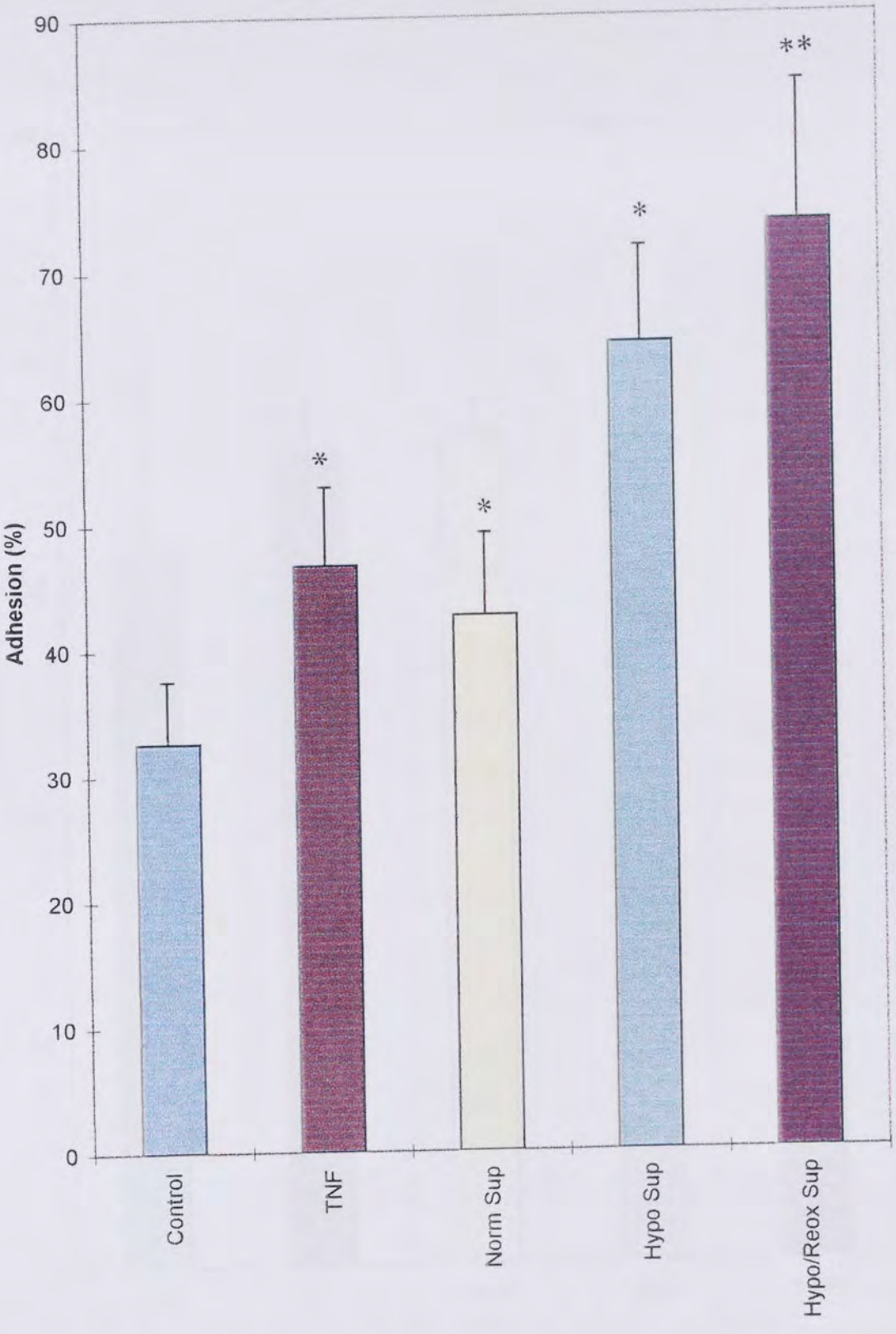
Neutrophil adhesion ($1x10^6$ cells per ml) to ECV304 made hypoxic for 4 hours and reoxygenated for 30 mins in the presence or absence of 0.1mM Dapsone. The neutrophils were pre-treated with dapsone for 30 mins before being added to the ECV304 cells. Treatment of the ECV304 for 4 hours with TNFα was used as a positive control. Values given are means +/- SEM where n=4 at each point. * indicates a significant difference ($p<0.05$) ** ($p<0.01$) from normoxia while + indicates a significant difference ($p<0.05$) from hypoxia/reperfusion using a Two Way Anova.

normoxic ECV304 cells. Figure 3.15 infact shows that normoxic cells secrete a modest amount of adhesion promoting material ($p<0.05$). However adhesion is increased further to 64.1 +/- 7.5% and 73.6 +/- 11.1% in the presence of hypoxic and hypoxic-reoxygenated supernatant respectively suggesting that additional soluble factor (s) are released by the endothelium following hypoxia and reoxygenation that act upon neutrophils to augment adhesion.

3.2.10 IL-1, IL-8, PAF and TNFα alone do not cause increased neutrophil adhesion

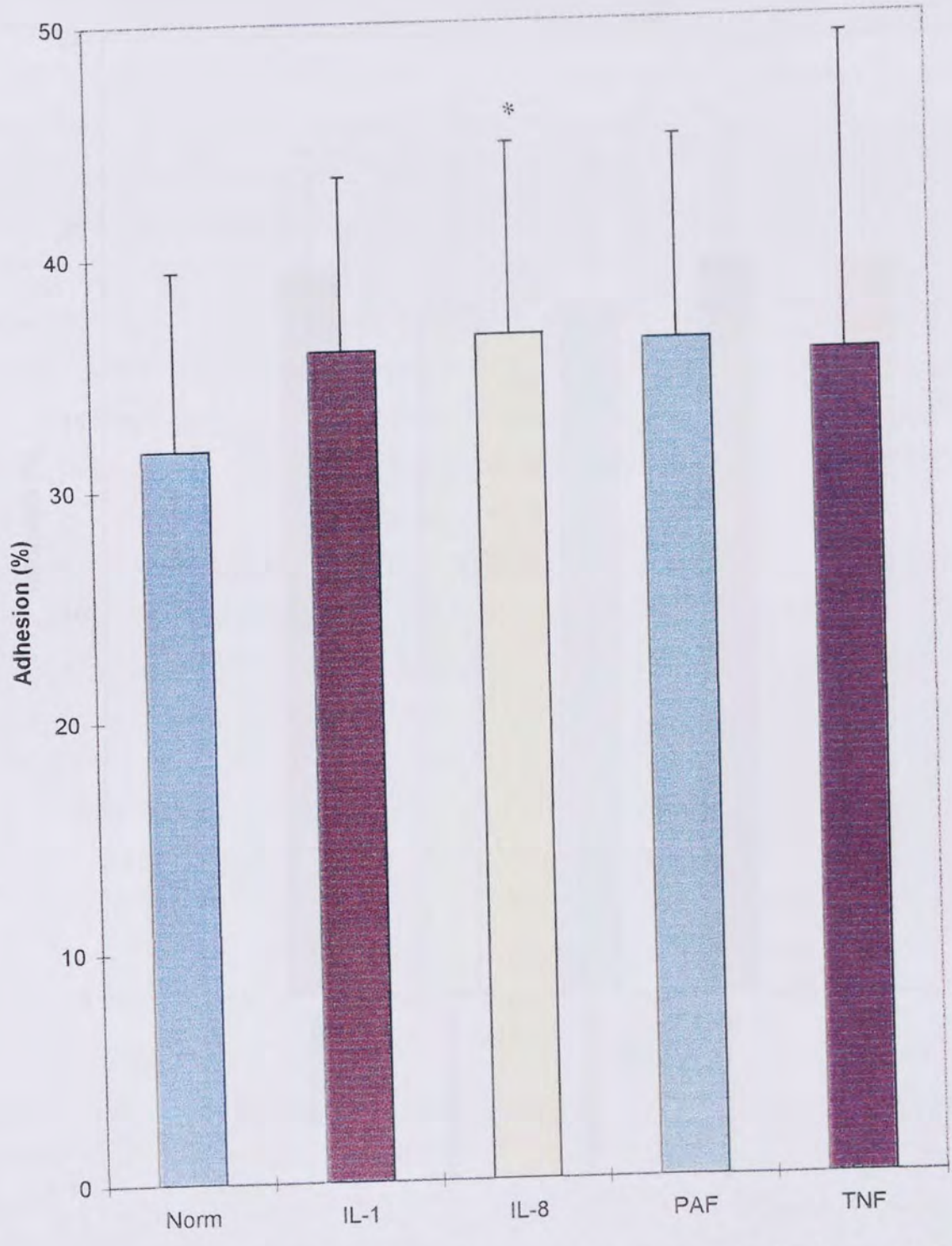
In an attempt to identify these factor (s) the effect of IL-1, IL-8, PAF and TNFα on neutrophil adhesion was assessed by allowing neutrophils to adhere to normoxic or hypoxic/reoxygenated ECV304 cells in the presence of these cytokines (Figure 3.16 and 3.17). It must be stressed that unlike the results illustrated in 3.1 and 3.2, where cytokines were present for 4 hrs, in this experiment only a 30mins exposure was involved. Figure 3.16 highlights that only IL-8 significantly augments neutrophil adhesion ($p<0.05$) to normoxic ECV304 cells compared to the other cytokines, though PAF treatment was approaching significance ($p<0.059$). Following hypoxia and reoxygenation neither IL-1, IL-8, PAF or TNFα could further potentiate neutrophil adhesion (Figure 3.17). These results highlight that IL-1, IL-8, PAF and TNFα alone could not within 30mins be responsible for the increased adhesion shown in figure 3.15 although they do not show that a combination of the cytokines could not produce this effect.

Figure 3.15 Effect of normoxic, hypoxic and hypoxic/reoxygenated supernatant from ECV304 cells on neutrophil adhesion to naive ECV304 cells.



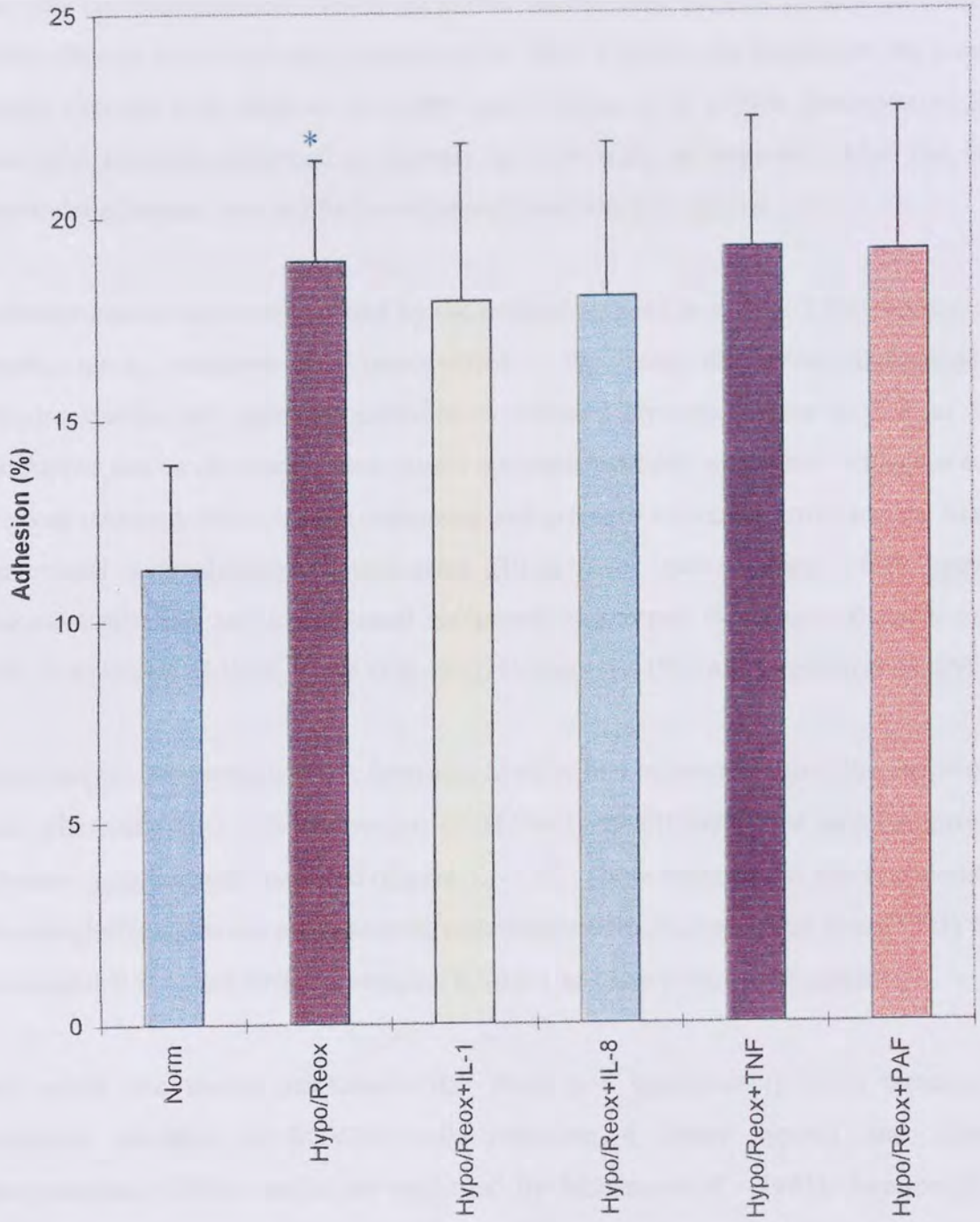
Neutrophil adhesion ($1x10^6$ cells per ml) to normoxic ECV304 cells in the presence of supernatant from ECV304 that have been exposed to either normoxia for 4 hours, made hypoxic for 4 hours or made hypoxic for 4 hours and reoxygenated for 30 mins. Treatment of the ECV304 for 4 hours with TNFα was used as a positive control. Values given are means +/- SEM where n=4. * indicates a significant difference ($p<0.05$) ** ($p<0.01$) from control adhesion using a Two Way Anova.

Figure 3.16 Effect of IL-1, IL-8, PAF and TNFα on neutrophil adhesion to naive ECV304 cells.



Neutrophil adhesion ($1x10^6$ cells per ml) to naive ECV304 cells in the presence of either IL-1 (100pg/ml), IL-8 (100pg/ml), PAF (2x10-8M) or TNFα (100IU/ml). Not shown here treatment of the ECV304 for 4 hours with TNFα was used as a positive control and gave a significant difference from normoxic ($p<0.05$). Values given are means +/- SEM where n=4. * indicates a significant difference ($p<0.05$) from normoxia using a Two Way Anova.

Figure 3.17 Effect of IL-1, IL-8, PAF and TNFα on neutrophil adhesion to endothelial cells that have been made hypoxic and reoxygenated.



Neutrophil adhesion ($1x10^6$ cells per ml) to ECV304 cells that have been made hypoxic for 4 hours and reoxygenated for 30 mins in the presence of either IL-1 (100pg/ml), IL-8 (100pg/ml), PAF (2x10-8M) or TNFα (100IU/ml). Not shown here treatment of the ECV304 for 4 hours with TNFα was used as a positive control and gave a significant difference from normoxic ($p<0.01$). Values given are means +/- SEM where n=4. ** indicates a significant difference ($p<0.01$) from normoxic using a Two Way Anova..

3.3 Discussion

In summary we developed an *in vitro* model of ischaemia/reperfusion injury by exposing endothelial cells cultured in 96 well plates to 4 hours hypoxia and adding neutrophils to the cells on reoxygenation. Although earlier studies used hypoxic periods as short as 30min (Brown *et al* 1990 and Yasuhara *et al* 1991) 4 hours was chosen for the present studies because both Budd *et al* (1989) and Walden *et al* (1990) demonstrated that neutrophil adhesion continued to increase up to 4 hours of hypoxia. After this time neutrophil adherence was not further enhanced by additional hypoxia.

Adherent neutrophils were assessed by the method outlined in section 2.5.2 because it is simple, quick, objective and reproducible. By using the triton, O-Dianisidine Dihydrochloride and hydrogen peroxide to measure myeloperoxidase as few as 600 neutrophils can be detected. These results compare favorably with other techniques such as visual counting, which is time consuming and prone to subjective error, and the use of monoclonal antibody/enzyme conjugates (ELISA) or radiolabelling which require expensive materials and sophisticated equipment to interpret the results (Bochner *et al* 1991, Lucinska *et al* 1992, Pigott *et al* 1992, Derian *et al* 1995 and Firestein *et al* 1995).

Using this *in vitro* model we have been able to show that by pre-incubating the endothelial cells with either IL-1 (10-1000pm/ml) or TNFα (1-1000IU/ml) for 4 hours neutrophil adhesion is significantly increased (Figure 3.1-3.2). These results show that our model is physiologically viable and are consistent with other studies such as Bruce *et al* (1991) that conclude that IL-1 and TNFα upregulate ICAM-1 and hence neutrophil adhesion.

Our model also shows conclusively that there is a significant ($p<0.05$) increase in neutrophil adhesion to ECV304 cells following 4 hours hypoxia and 30mins reoxygenation. These results are supported by Mullane *et al* (1983), Romson *et al* (1983), Jolly *et al* (1986), Linas *et al* (1988), Budd *et al* (1989), Yoshida *et al* (1992) and Walden *et al* (1990) who have all been able, either *in vitro* or *in vivo*, to show that neutrophil adhesion is enhanced following episodes of hypoxia and reoxygenation. Looking at the differences in normoxic adhesion in the several experiments in this chapter and neutrophil migration in Chapter 4 it is easy to see that a large variability exists. This problem is an inherent one not restricted to this thesis. For example Yoshida *et al* (1992) found that mean basal adhesion varied from 8-25% in different experiments using 4

subjects on each occasion. Variability observed between results from different authors of course could be due to the use of different neutrophil isolation techniques and/or degree and duration of hypoxia. Nevertheless using the same dextran sedimentation isolation technique and the same endothelial cell monolayers Budd *et al* (1989) reported a 20% neutrophil adherence whereas Yoshida *et al* (1992) found only 8% of neutrophils would adhere. Clearly these differences in normoxic adhesion and the day to day variations in our own studies must be attributed to individual variation. In these cases the differences in adhesion are simply due to individual variability and the fact that the same person's blood can behave differently on different days simply due to the presence of unsymptomatic illness. Thus because there is inevitably a large coefficient of variation a paired t-test or two-way anova was used to analysis any results. Like experiments were also carried out on the same day using 4 different individual's blood , and preferably on the same plate of cultured endothelial cells.

When the endothelial cells were subjected to hypoxia in the presence of the xanthine oxidase inhibitor allopurinol the increased adhesion normally seen after 30 mins reoxygenation was significantly decreased (Figure 3.5). Similarly the superoxide scavenger, SOD, significantly ($p<0.05$) attenuated the increased neutrophil adhesion (Figure 3.6) as did catalase, an antioxidant that catalyzes the reduction of H_2O_2 to H_2O and O_2 respectively (Figure 3.8). The use of antioxidants and free radicals scavengers to abrogate the increased neutrophil adhesion observed following hypoxia and reoxygenation illustrates conclusively that the production of ROS are involved in the increased neutrophil adhesion observed following hypoxia and reoxygenation of the ECV304 cells. Many other investigators have been able to support these findings (Simpson *et al* 1987, Flaherty *et al* 1994, Pollak *et al* 1993, Coghlan *et al* 1994 and Deune *et al* 1996) as summarized by Kurose and Granger (1994). DMSO however, a non-enzymatic scavenger of $OH^{\cdot}$, in our hands did not reduce the enhanced neutrophil adhesion following hypoxia and reoxygenation(Figure 3.7). The reason for this may be the very low concentrations employed (1%). We deliberately examined this concentration because several of our agonists were prepared in DMSO and then diluted to 1% for the ensuing culture. As this concentration of DMSO did not affect adhesion at we can safely say that any effect seen was not the consequence of the vehicle used.

Although ROS may directly affect neutrophil adhesion the effect observed in Figure 3.3 was still present at 30mins, long after the generation of ROS by the endothelial cells

would have ceased (Lefer and Lefer 1993). These results suggest that ROS are capable of inducing the release of a labile material (s) which enhances neutrophil adhesion, either through upregulation of adhesion molecules on the endothelial surface or by its direct actions on the neutrophil. To identify what these factors were likely to be cycloheximide, a protein synthesis inhibitor (Obrig *et al* 1971), an eicosanoid inhibitor, a PAF secretion antagonist (Grigoriadis and Stewart 1991) and a MMPI, that prevents secretion of TNFα (Gearing *et al* 1994), were used. The results from this set of experiments (Figure 3.8-3.12) demonstrate that both protein synthesis and eicosanoids may be implicated in the increased neutrophil adhesion observed following hypoxia and reoxygenation. This suggests many contributory soluble factor (s) maybe released by the endothelium within 30 mins of reoxygenation to augment neutrophil adhesion.

In an attempt to identify these factor more precisely (s) the effect of IL-1, IL-8, PAF and TNFα on neutrophil adhesion was assessed by allowing neutrophils to adhere to normoxic or hypoxic/reoxygenated ECV304 cells in the presence of these cytokines (Figure 3.16 and 3.17). Figure 3.16 highlights that only IL-8 significantly increased neutrophil adhesion ($p<0.05$) to normoxic ECV304 cells compared to the other cytokines, though PAF treatment was approaching significance ($p<0.059$). Following hypoxia and reoxygenation neither IL-1, IL-8, PAF or TNFα could potentiate further neutrophil adhesion during these short 30 mins incubations (Figure 3.17). These results highlight that IL-1, IL-8, PAF and TNFα alone could not be responsible for the increased adhesion shown in figure 3.15. Combinations of these materials might of course be capable of producing the desired affect.

To analyze the adhesion molecules involved in our *in vitro* model an anti-ICAM monoclonal antibody was used. Incubating the ECV cells with this throughout the hypoxic period completely abolished the heightened adhesion. These results indicate that the neutrophil adherence and emigration observed in our system is mediated by ICAM-1 on the endothelial cells (Oliver *et al* 1991). However, as already demonstrated , Figure 3.1-3.2, upregulation of ICAM-1 takes four hours. Since our enhanced adhesion is visible within 30mins ICAM-1 must be the counterligand for adhesion molecules that are quickly modified on the neutrophil to allow enhanced adhesion. The most likely candidates are the neutrophil integrins. Several authors have demonstrated a requirement for calcium and magnesium in integrin action. We

found enhanced adhesion after hypoxia even in the absence of these ions. Indeed adhesion to both normoxic and hypoxic/reoxygenated endothelial cells seemed unaffected by the presence or absence of divalent cations. It may be that small yet adequate levels of calcium and magnesium remained when the culture medium was reoxygenated by PBS. An integrin/ICAM involvement in the enhanced adhesion found after hypoxic episodes has also been confirmed by Mileski *et al* (1990), Yoshida *et al* (1992), Arnould *et al* (1993), Hartman *et al* (1995), Nakano *et al* (1995), Oshiro *et al* (1995) and Tamiya *et al* (1995). Their results indicate that neutrophil adherence to ischaemic/reperfused endothelial cells is mediated by both LFA-1 and Mac-1 on neutrophils and that ICAM-1 on endothelial cells acts as the ligand for both heterodimers of CD11/CD18.

Confirmation from our own studies is shown in figure 3.15 where the supernatants from normoxic, hypoxic or hypoxic/reoxygenated ECV were able to increase adhesion to naive ECV cells. The supernatants were only present during the adhesion period so time would not have allowed up regulation of adhesion molecules on the endothelial surface. Some soluble factor (s) released by hypoxia/reoxygenated endothelial cells can clearly enhance adhesion of neutrophil to naive normoxic endothelial cells during a very short incubation. A rapid conformational change in neutrophil integrins seems most likely. These results suggest that by blocking ICAM-1 on the endothelium or LFA-1/MAC-1 on the neutrophil it may be possible to prevent ischaemic/reperfusion injury.

Our observation that the anti-inflammatory drug dapsone can attenuate the increased neutrophil seen following hypoxia/reoxygenation suggests an additional therapeutic avenue (Figure 3.14). Dapsone is frequently effective in cutaneous disease characterized by immune complex deposition and accumulation of neutrophils. Thuong-Nguyen (1993) certainly demonstrated reduced neutrophil adhesion after pre-treatment with dapsone.

In conclusion this chapter has illustrated that neutrophil adhesion to ECV cells following hypoxia and reoxygenation is significantly increased. This is a direct consequence of the hypoxic period and generation of ROS. Furthermore the generation of ROS promotes the release of transient labile material (s) from the endothelial cells that probably cause rapid conformational changes in MAC-1 and

LFA-1. This may allow for increased adhesion to the ICAM-1 that is constitutively expressed on the endothelial surface. The nature of the material still needs to be elicited though it is unlikely to be any single factor but a highly complex mixture of known, TNFα, PAF and IL-8, and maybe other unknown factors. Potential therapies for limiting ischaemic/reperfusion injury therefore include inhibiting the production of ROS, blocking the release of factors from the endothelial cells, preventing neutrophil adhesion by Mabs to ICAM-1, LFA/MAC-1 or simply by using dapsone.

4 NEUTROPHIL MIGRATION

4.1 Introduction

Neutrophils play a major role in host defense because they rapidly migrate to sites of infection and destroy invading micro-organisms. Specific signal molecules (chemoattractants), released by bacteria or endogenously generated by the host, can elicit directed neutrophil migration (chemotaxis) towards the pathogen (Baker *et al* 1982, Barker *et al* 1986 and Levy and Kasper 1986). Since neutrophil accumulation is also observed in reperfused myocardium, it is thought to play a critical role in the reperfusion injury associated with acute myocardial infarction (Go *et al* 1988, Lucchesi *et al* 1989 and Lefer *et al* 1991) and other forms of ischaemic reperfusion injury.

The directed movement of neutrophils from the peripheral circulation to their destination is initiated by the specific interaction of chemoattractants with neutrophil plasma membrane receptors. The signal is then transmitted to the interior of the cell by a cascade of reactions that are collectively referred to as signal transduction. Biochemical events, including coupling of guanine nucleotide regulatory proteins (G proteins) to chemoattractant receptors, activation of phospholipases, generation of second messengers, and activation of protein kinases, occur during the signal transduction cascade (Omann *et al* 1987, Sha'afi 1988, Cockroft 1992, Thelen *et al* 1993, Gerard and Gerard 1994 and Murphy 1994).

Neutrophil chemotaxis requires chemoattractant binding to specific plasma membrane receptors, cell adherence to the endothelium, and reversible assembly of critical cytoskeletal elements. Chemoattractant-stimulated neutrophils undergo rapid morphological changes from rounded, relatively smooth cells to elongated, ruffled cells with pseudopodia. When stimulated neutrophils are attached to the endothelium, their pseudopodia form broad, thin lamellipodia that are extended anteriorly in the direction of an increasing chemoattractant gradient (Devreotes and Zigmond 1988 and Cassimeris and Zigmond 1990). A contractile uropod is formed posteriorly, which results in a polarized cell morphology. Neutrophil polarity is required for efficient directed migration. Neutrophils migrate by repetitive, complex events in which they extend lamellipodia in the

direction of the gradient and retract their uropodia toward the cell body (Cassimeris and Zigmond 1990).

Actin, a 43KDa globular protein of the microfilamentous cytoskeleton, is involved in cell migration (Singer and Kupfer 1986). The polymerization of globular actin (G-actin) monomers into actin filaments (F-actin) occurs within seconds after neutrophil stimulation with chemoattractant (Omann *et al* 1987). Dynamic alterations in F-actin polymerization correlate closely with chemoattractant-elicited neutrophil shape change and migration rates (Howard and Oresajo 1985 and Watts *et al* 1991). Actin assembly is regulated by a variety of actin-binding proteins that control the reversible gelation and solation of a three-dimensional actin network (Southwick and Stossel 1983, Stossel *et al* 1985 and Omann *et al* 1987). Agents that block actin polymerization, such as cytochalasins and botulism C2 toxin, inhibit neutrophil migration *in vitro* (Zigmond and Hirsch 1972 and Norgauer *et al* 1988). These findings demonstrate that actin is an essential protein for cell motility. Current models of cell migration propose that actin is involved in the force generation for cell movement (Singer and Kupfer 1986 and Cassimeris and Zigmond 1990). The precise mechanisms of neutrophil force generation remain unknown.

Neutrophil motility may be random, chemokinetic, or chemotactic, and is classified by *in vitro* assays which quantify the directional migration response (Bittleman *et al* 1996). Random migration (unstimulated motility) and chemokinetic migration (i.e., stimulated speed of cells) are motile responses which do not have consistent directionality. In contrast chemotactic responses are directional. Although the relative importance of random, chemokinetic, and chemotactic migration *in vivo* has not been determined, it is unlikely that all three types of motility are involved in neutrophil recruitment.

Neutrophils respond to a variety of chemoattractants, including N-formyl peptides, complement-derived C5a, leukotriene B4 (LTB_4), interleukin-8 (IL-8) and platelet-activating factor (PAF) (Baker *et al* 1982, Barker *et al* 1986 and Levy and Kasper 1986). Although C5a is believed to be one of the most important mediators after ischaemic reperfusion injury (section 1.6) more recent studies suggest that IL-8 is involved in the neutrophil infiltration of subendothelial tissue following ischaemic/reperfusion injury (Huber *et al* 1991 and Kuijpers *et al* 1992). IL-8, also known as neutrophil attractant/activation protein-1 (NAP-1), belongs to the C-X-C chemokine or chemokine-a family, all of which show chemoattractant activity for neutrophils. IL-8 is produced by

several cell types, including monocytes (Yoshimura *et al* 1987), neutrophils (Bazzoni *et al* 1991), endothelial cells (Strieter *et al* 1989), lung epithelial cells (Strieter *et al* 1989), fibroblasts (Standiford *et al* 1990), and cardiac myocytes (Seino *et al* 1993). Abe *et al* (1993) reported that serum IL-8 levels rise during the early phase of acute myocardial infarction. *In vitro* studies have also shown that IL-8 stimulates neutrophil chemotaxis, and that it increases binding activity and expression of adhesion molecules on neutrophils (Detmers *et al* 1990, Paccaud *et al* 1990 and Detmers *et al* 1991). Although Huber *et al* (1991) and Kuijpers *et al* (1992) reported that IL-8 enhances neutrophil adhesion to and transmigration across endothelial cell monolayers, the effects of IL-8 on neutrophil-endothelium interactions are still controversial. Gimbrone *et al* (1989) and Luscinskas *et al* (1992) for example showed that IL-8 had an inhibitory effect on neutrophil adhesion and transmigration.

There is also a growing body of evidence which implicates the chemoattractant PAF as a mediator of the microvascular dysfunction induced by ischaemic reperfusion injury (Kurose and Granger 1994). For example PAF levels rise in tissue exposed to ischaemia and reperfusion (Filep *et al* 1989), PAF receptor antagonists attenuate the microvascular alterations elicited by ischaemia and reperfusion (Kubes *et al* 1990) and the local administration of PAF elicits most of the microvascular alterations associated with ischaemia and reperfusion.

This chapter sets out to illustrate unequivocally that factor (s) produced by hypoxic or hypoxic/reoxygenated ECV cells which are capable of eliciting neutrophil migration. Furthermore because of the controversy surrounding their nature we have attempted to detail what these factors are and dissect out their involvement in the ischaemic/reperfusion story.

4.2 Results

4.2.1 HIZAS is a neutrophil chemoattractant

Neutrophils were loaded into the top deck of a migration chamber and allowed to migrate into PBS, zymosan activated complement (ZAC), zymosan activated serum (ZAS) and heat inactivated zymosan activated serum (HIZAS). Control migration was 6.6 +/- 1.6% (Figure 4.1). Migration into 0.5% HIZAS was significantly ($p<0.05$) increased to 12.9 +/-3.3%. In contrast ZAC and ZAS did not significantly heighten neutrophil migration though values for 2% ZAC were approaching significance at $p=0.059$. Figure 4.2 reinforces these finding and demonstrates that maximal neutrophil migration occurs using 1% HIZAS though 0.5% and 2% still give significant differences ($p<0.05$) from migration into PBS alone. Therefore, 1% HIZAS was used in all subsequent migration assays as a positive control

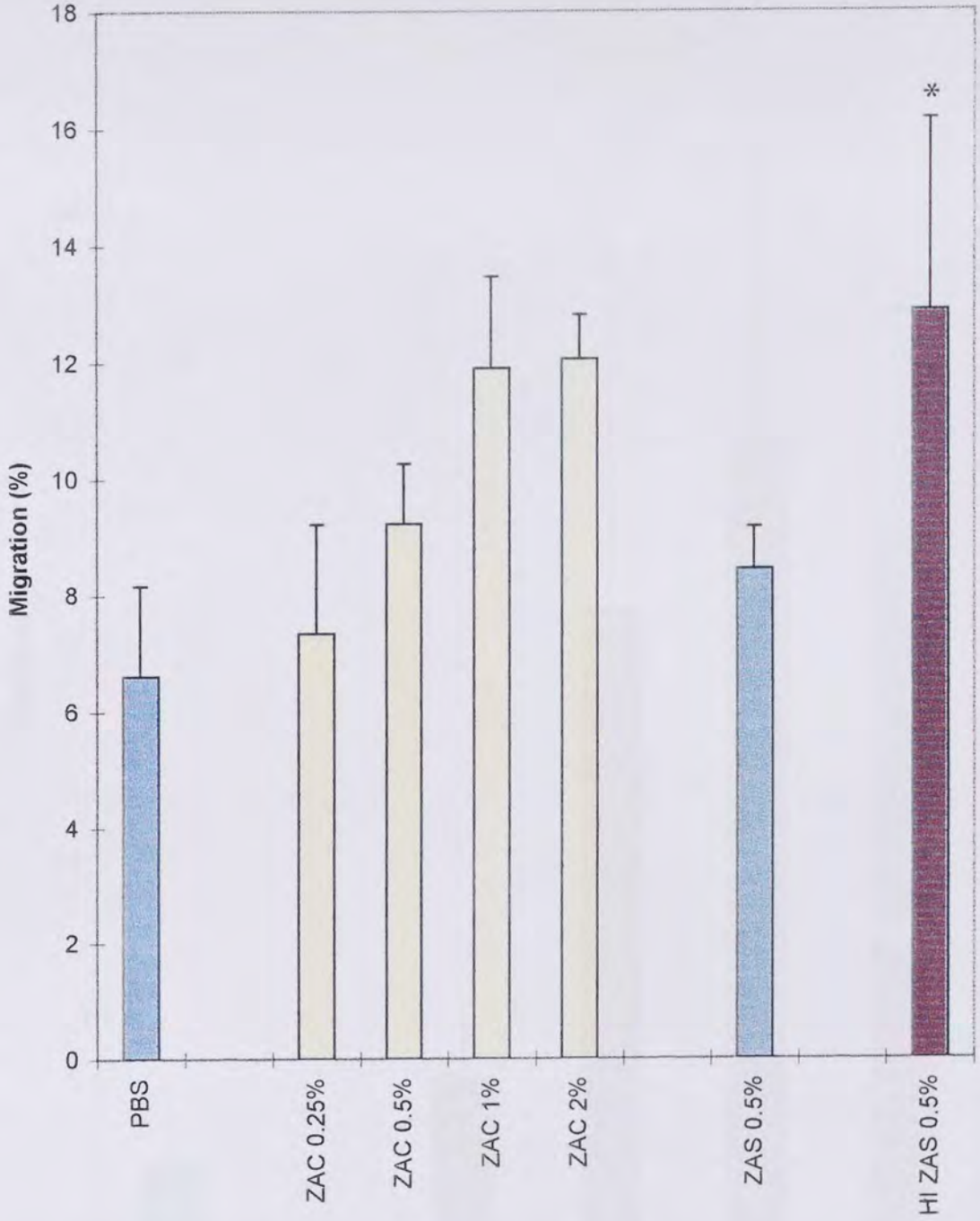
4.2.2 Neutrophil chemotaxis is time dependent

HIZAS (1%),possibly due to the production of C5a, was a strong neutrophil chemoattractant. Neutrophil chemotaxis is time dependent (Figure 4.3). Maximal neutrophil migration 15.9 +/- 3.2% occurs after 2 hours though incubation of the neutrophil with chemoattractant for as little as 30 mins produces a significant increase ($p<0.05$). A one hour incubation was therefore used in all subsequent migration assays.

4.2.3 ECV304 cells secrete a chemoattractant following hypoxia and reoxygenation that attracts neutrophils

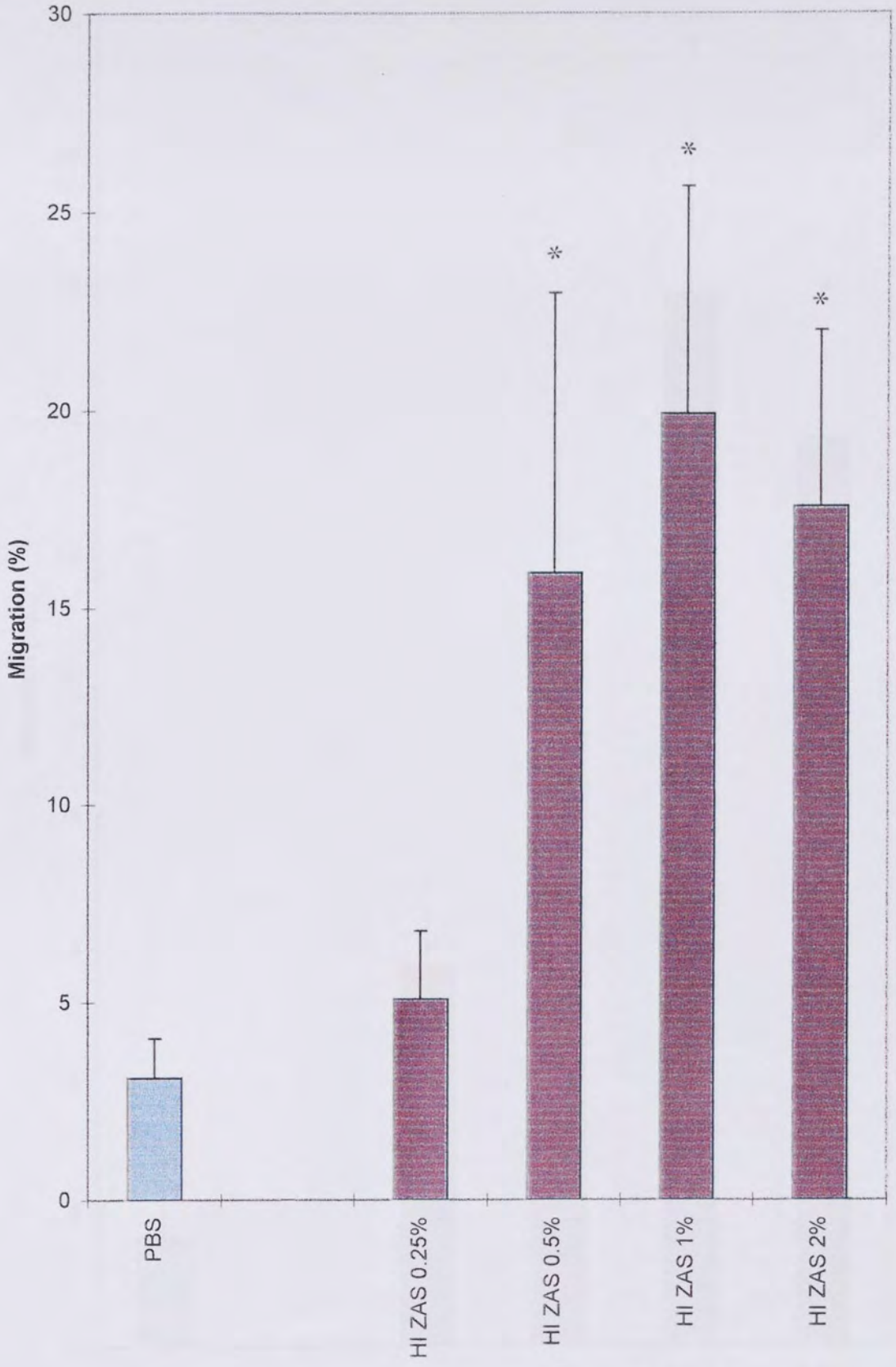
When endothelial cells were incubated for 4 hours in PBS a factor (s) was released into the supernatant which caused a moderate increase in migration (Figure 4.4). Although significant ($p<0.05$) the enhanced migration was not as large as that caused by the positive control (HIZAS). Even more chemoattractant was released during a 4 hour hypoxic period. If hypoxic endothelial cells were then reoxygenated in fresh PBS for only 30mins clearly large amounts of chemoattractant were now released as 17.8 +/- 0.4% of the

Figure 4.1 Effect of zymosan activated complement (ZAC), zymosan activated serum (ZAS) and heat inactivated zymosan activated serum (HIZAS) on neutrophil migration.



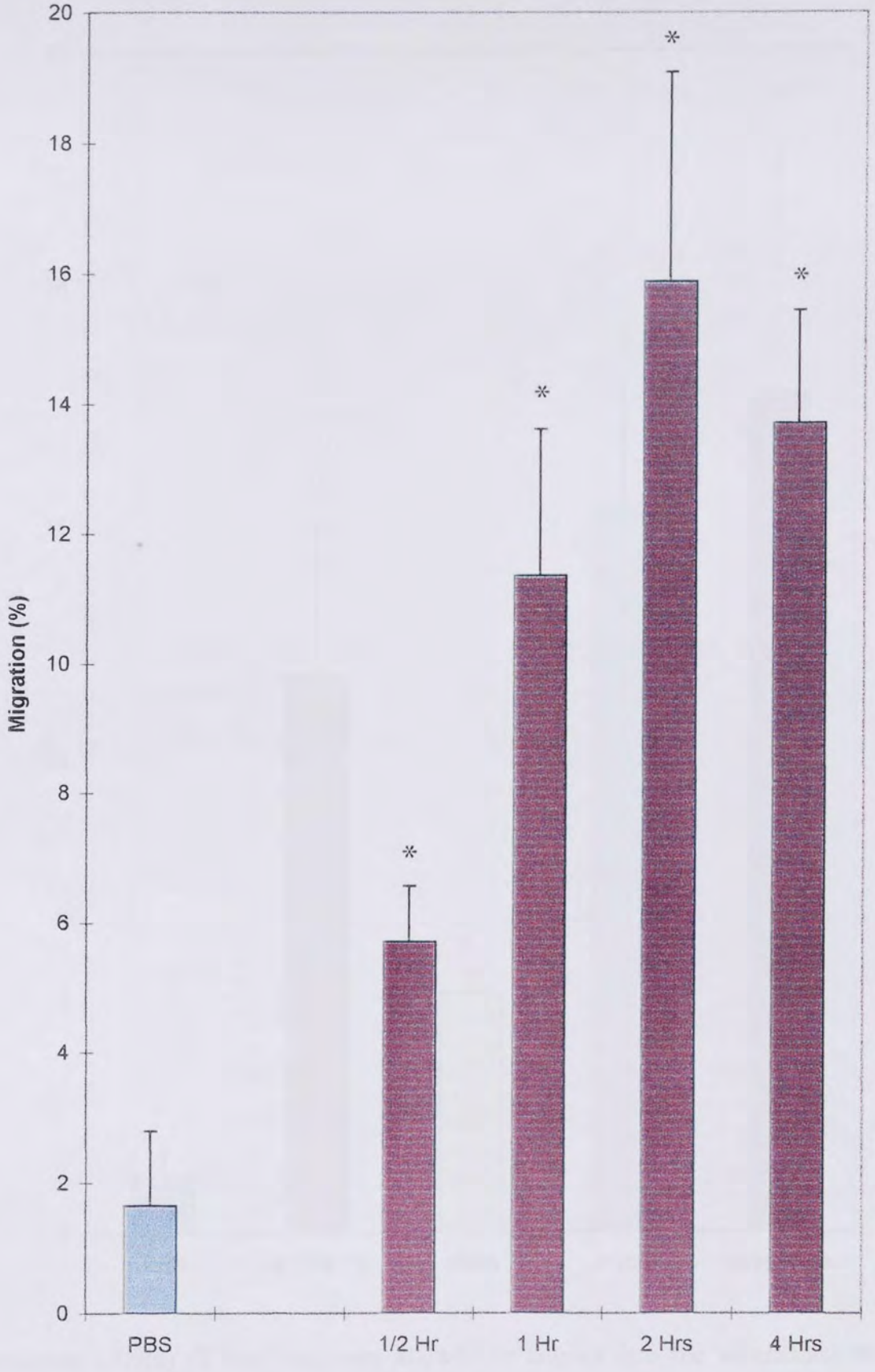
Neutrophils (200µl) ($2.5x10^6$/ml) were allowed to migrate into ZAC, ZAS or HIZAS for one hour and the number of migrant neutrophils in the lower compartment assessed via myeloperoxidase method 2.5.2. ZAC was prepared by incubating guinea pig complement with zymosan (5mg/ml) at 37°C for 20 mins before removing the zymosan. ZAS was obtained by allowing 10ml blood from healthy donors to clot for 30 mins. The red blood cells were then removed by spinning at 3000rpm for 30mins and the resulting serum incubated with zymosan (5mg/ml) for 20mins at 37°C. Once the zymosan was removed the ZAS was then incubated at 57°C for 30mins to produce HIZAS. Solutions of ZAC, ZAS, and HIZAS were made in calcium and magnesium free PBS. Values given are means +/- SEM where n=4 at each point. * indicates a significant difference ($p<0.05$) from PBS.

Figure 4.2 Effect of HIZAS on neutrophil migration



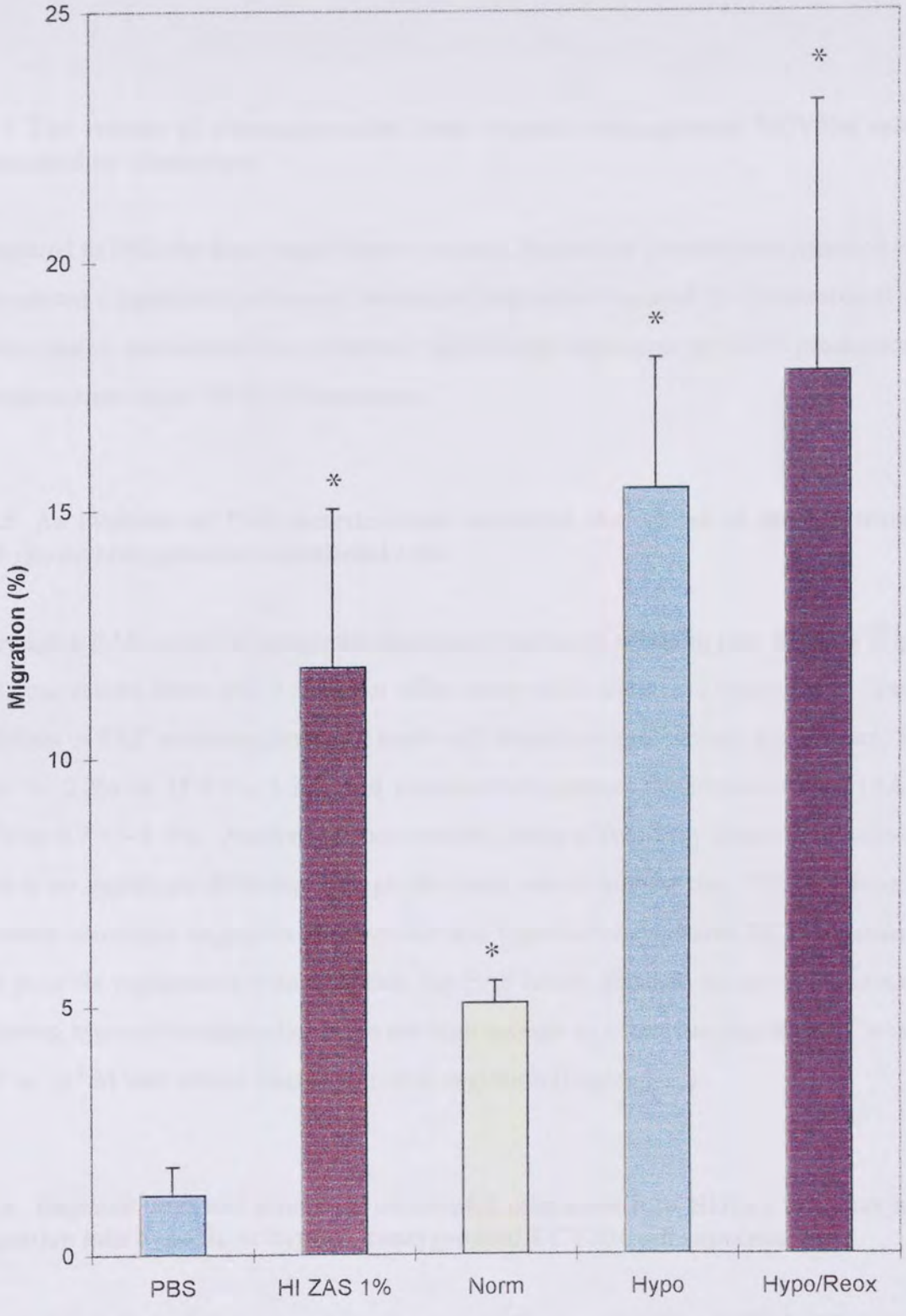
Neutrophils (200µl) ($2.5x10^6$/ml) were allowed to migrate into HIZAS (prepared as in figure 4.1) for 1 hour. Values givens are means +/- SEM where n=4 at each point. * indicates a significant difference ($p<0.05$) from PBS.

Figure 4.3 Effect of time on neutrophil migration into HIZAS



Neutrophils (200µl) ($2.5x10^6$/ml) were allowed to migrate into HIZAS (1%) (prepared as in figure 4.1) for 4 hours. Values given are means +/- SEM where n=4 at each point. * indicates a significant difference ($p<0.05$) from PBS.

Figure 4.4 Effect of the supernatant from normoxic, hypoxic and hypoxic/reoxygenated ECV304 cells on neutrophil migration



Neutrophils (200µl) ($2.5x10^6$/ml) were allowed to migrate into the supernatant from ECV304 cells that had either been made normoxic for 4 hours, hypoxic for 4 hours or hypoxic for 4 hours and reoxygenated for 30mins. Migration into HIZAS (1%) was used as a positive control. Values givens are means +/- SEM where n=4 at each point. * indicates a significant difference ($p<0.05$) from PBS

neutrophils migrated, which was significantly more ($p<0.05$) than into the positive control (HIZAS).

4.2.4 The release of chemoattractant from hypoxic/reoxygenated ECV304 cells is attenuated by allopurinol

Compared to PBS the supernatant from normoxic, hypoxic or hypoxic/reoxygenated ECV cells caused a significant increase in neutrophil migration (Figure 4.5). Pretreatment with Allopurinol, a xanthine oxidase inhibitor, significantly attenuates ($p<0.05$) production of cheoattractants under these circumstances.

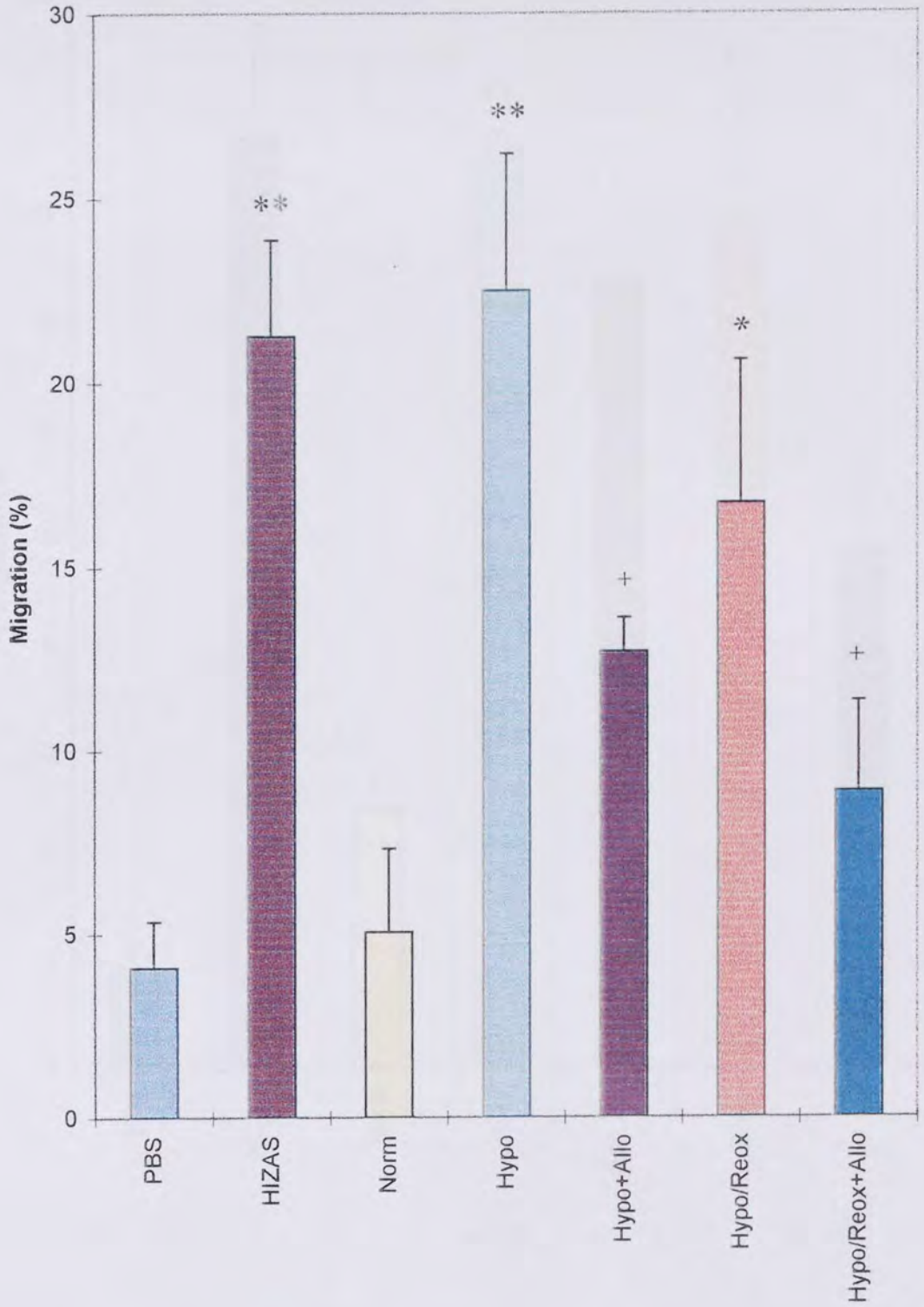
4.2.5 An inhibitor of PAF secretion does not affect the release of chemoattractant by hypoxic/reoxygenated endothelial cells.

Although a PAF secretion antagonist decreased neutrophil adhesion post hypoxia (Figure 3.11) our results show that it does not affect neutrophil migration (Figure 4.6). Specific inhibition of PAF secretion decreased neutrophil migration into hypoxic supernatant, from 17.3 +/- 0.2% to 15.8 +/- 1.7%, and hypoxic/reoxygenated supernatant, from 15.8 +/- 2.2% to 9.7 +/- 1.5%. Analysis of these results, using a Two-Way Anova, indicates that there is no significant difference though the trend would suggest that PAF inhibition can attenuate neutrophil migration into hypoxic and hypoxic/reoxygenated ECV supernatant. One possible explanation would be that the PAF levels obtained in our *in vitro* model following hypoxia/reoxygenation were not high enough to potentiate migration. Certainly PAF at 10^{-8} M was able to cause neutrophil migration (Figure 4.12).

4.2.6 Dapsone does not attenuate neutrophil migration into HIZAS but does alter migration into hypoxic or hypoxic/reoxygenated ECV304 cell supernatant

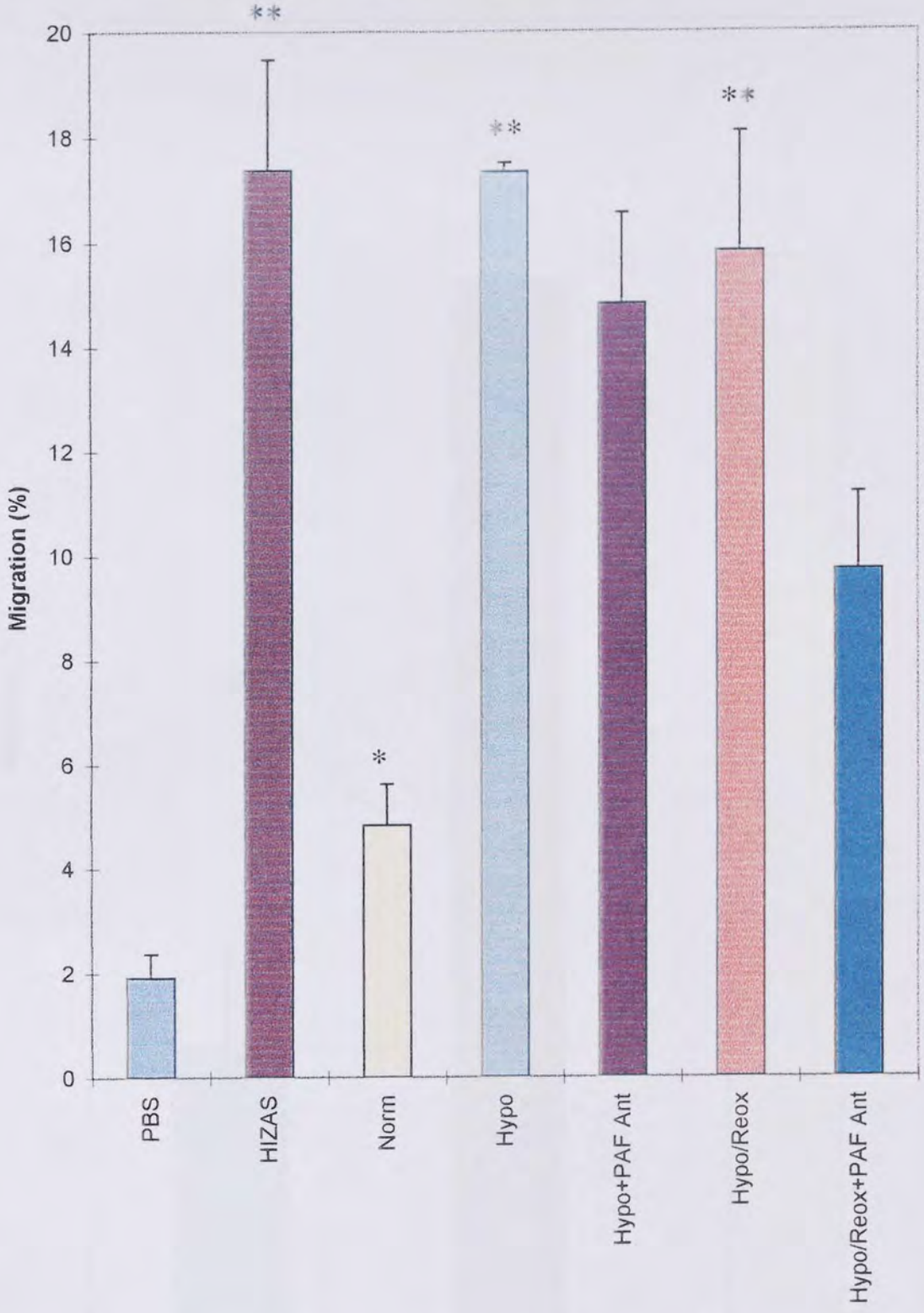
Figure 4.7 indicates that the enhanced neutrophil migration into HIZAS (1%) is not reduced by pre-treatment of the neutrophil with dapsone (0.1mM), an anti-inflammatory sulphone. However migration into hypoxic or hypoxic/reoxygenated ECV304 supernatant is significantly impaired ($p<0.05$ in both cases) (Figure 4.8). This suggest that there are different pathways through which different chemoattractants potentiate a response and that dapsone is selective as to which of these it is able to block.

Figure 4.5 Effect of allopurinol on the ability of hypoxic or hypoxic/reoxygenated ECV304 cells to secrete chemoattractants



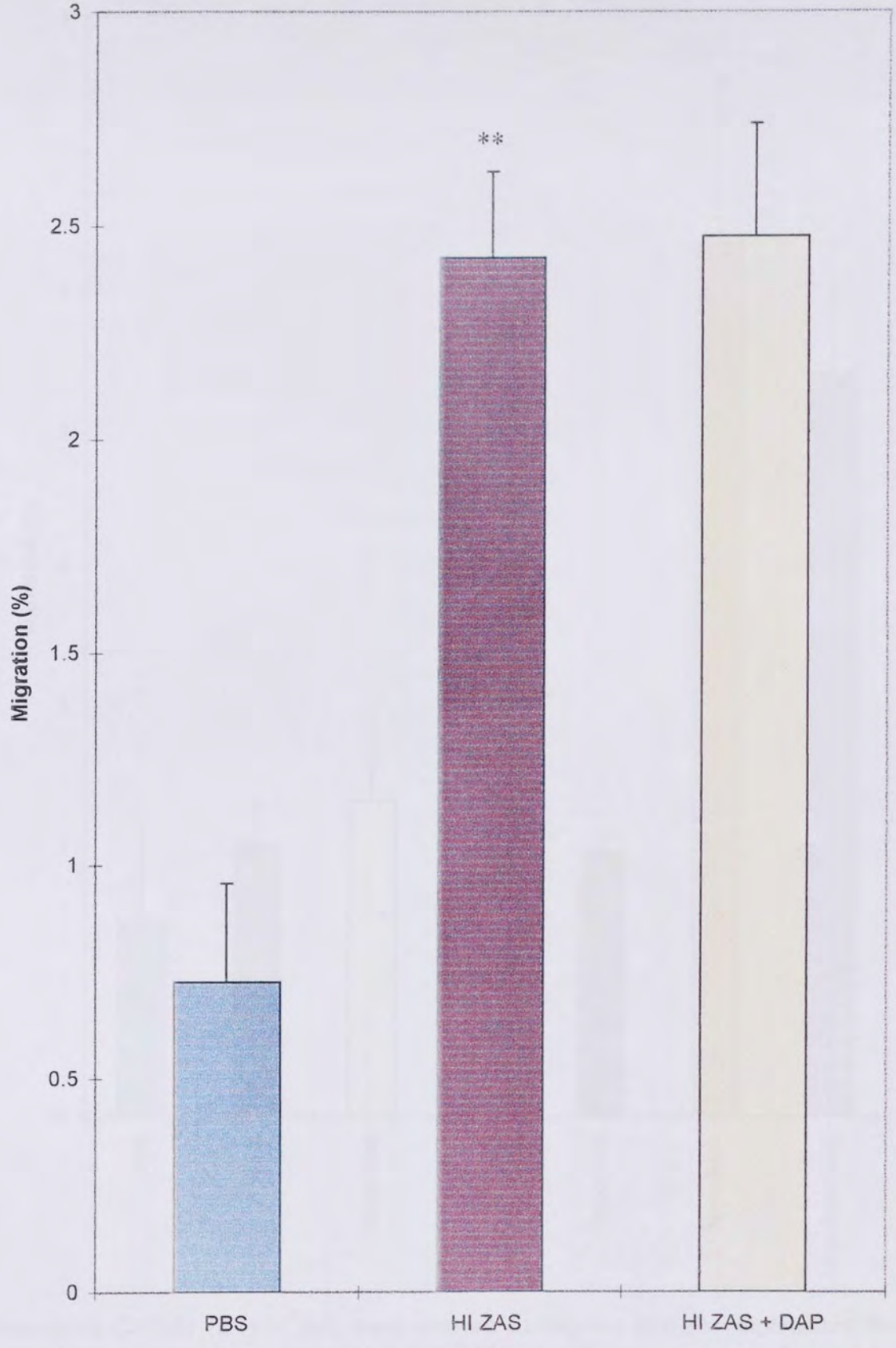
Neutrophils (200µl) ($2.5x10^6$/ml) were allowed to migrate into the supernatants derived from normoxic, hypoxic or hypoxic/reoxygenated ECV304 cells (prepared as in figure 4.4) in the presence or absence of allopurinol. Allopurinol (20µM) treatment of the ECV304 cells consisted of 1 hour pre-treatment before hypoxia and presence throughout the hypoxic period. HIZAS (1%) was used as a positive control. Values given are means +/- SEM where n=4 at each point. * indicates a significant difference ($p<0.05$) ** ($P<0.01$) from PBS while + indicates a significant difference ($p<0.05$) from hypoxia or hypoxic/reperfusion using a Two Way Anova.

Figure 4.6 Effect of an inhibitor of PAF secretion on the ability of hypoxic or hypoxic/reoxygenated ECV304 cells to secrete chemoattractants



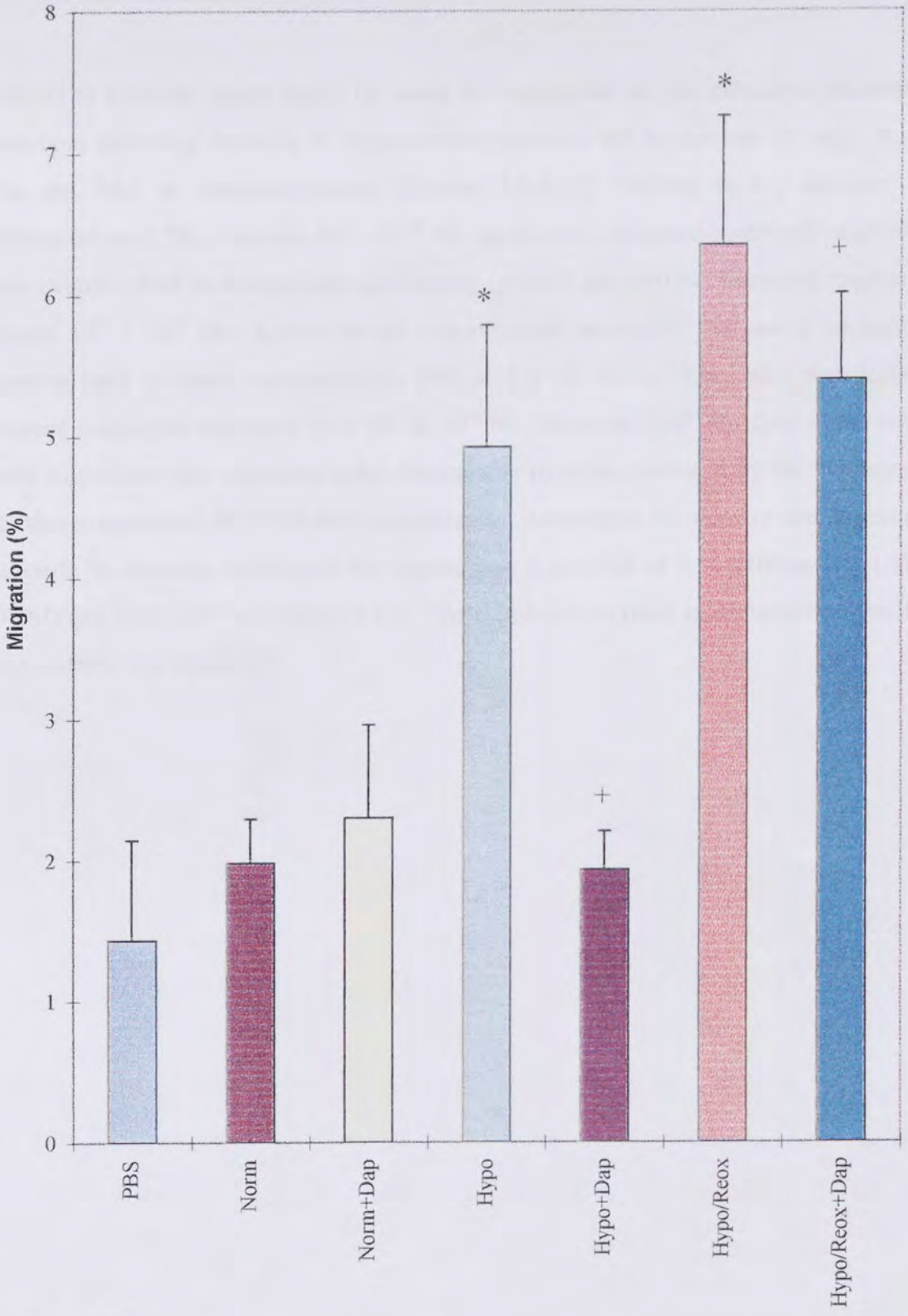
Neutrophils (200µl) ($2.5x10^6$/ml) were allowed to migrate into the supernatants derived from normoxic, hypoxic or hypoxic/reoxygenated ECV304 cells (prepared as in figure 4.4) in the presence or absence of a PAF secretion antagonist (10^{-8} M)(1-0-HEXADECYL-2-ACETYL-*sn*-GLYCERO-3-PHOSPHO-(N,N,N-TRIMETHYL)-HEXANOLAMINE). The PAF antagonist was present throughout the hypoxic and reoxygenation period. HIZAS (1%) was used as a positive control. Values given are means +/- SEM where n=4 at each point. * indicates a significant difference ($P<0.05$) ** ($p<0.01$) from PBS using a Two Way Anova.

Figure 4.7 Effect of Dapsone on the ability of neutrophils to migrate towards HIZAS



Neutrophils (200µl) ($2.5x10^6$/ml) were allowed to migrate into HIZAS (1%) with or without 30mins pre-treatment with 0.1mM dapsone. Values given are means +/- SEM where n=4. ** indicates a significant difference ($p<0.01$) from PBS using a Two-Way Anova.

Figure 4.8 Effect of dapsone on the ability of neutrophils to migrate towards the supernatant from hypoxic or hypoxic/reoxygenated ECV304 cells

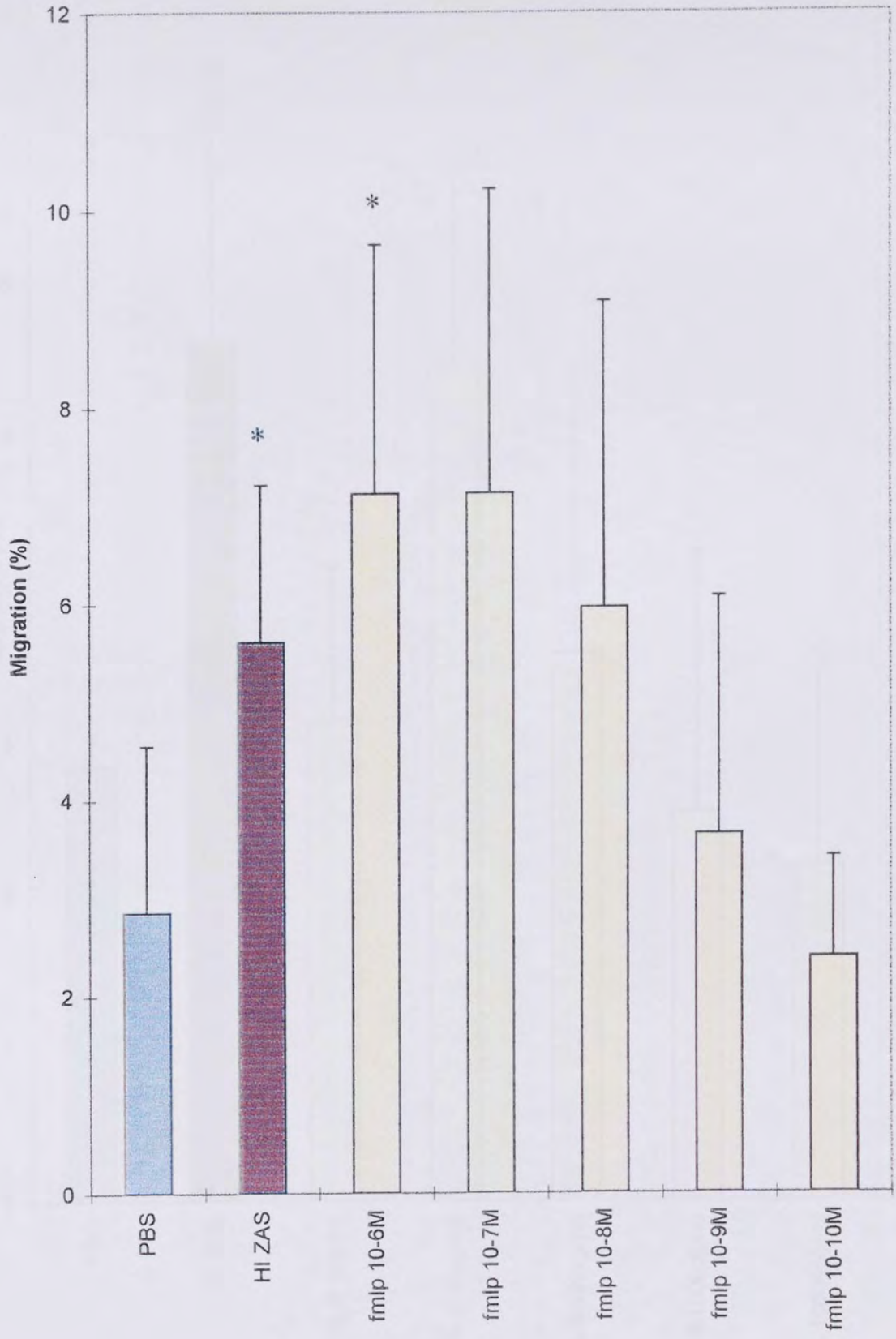


Neutrophils (200µl) ($2.5x10^6$/ml) were allowed to migrate into the supernatant from normoxic, hypoxic or hypoxic/reoxygenated ECV304 cells (prepared as in figure 4.4) in the presence or absence of 0.1mM dapsone. The neutrophils were pre-treated with dapsone for 30 mins before migration occurred. HIZAS (1%) was used as a positive control, not shown. Values given are means +/- SEM where n=4 at each point. * indicates a significant difference ($p<0.05$) from PBS while + indicates a significant difference ($p<0.05$) from hypoxia or hypoxia/reperfusion using a Two Way Anova.

4.2.7 PAF is a neutrophil chemoattractant in our *in vitro* model

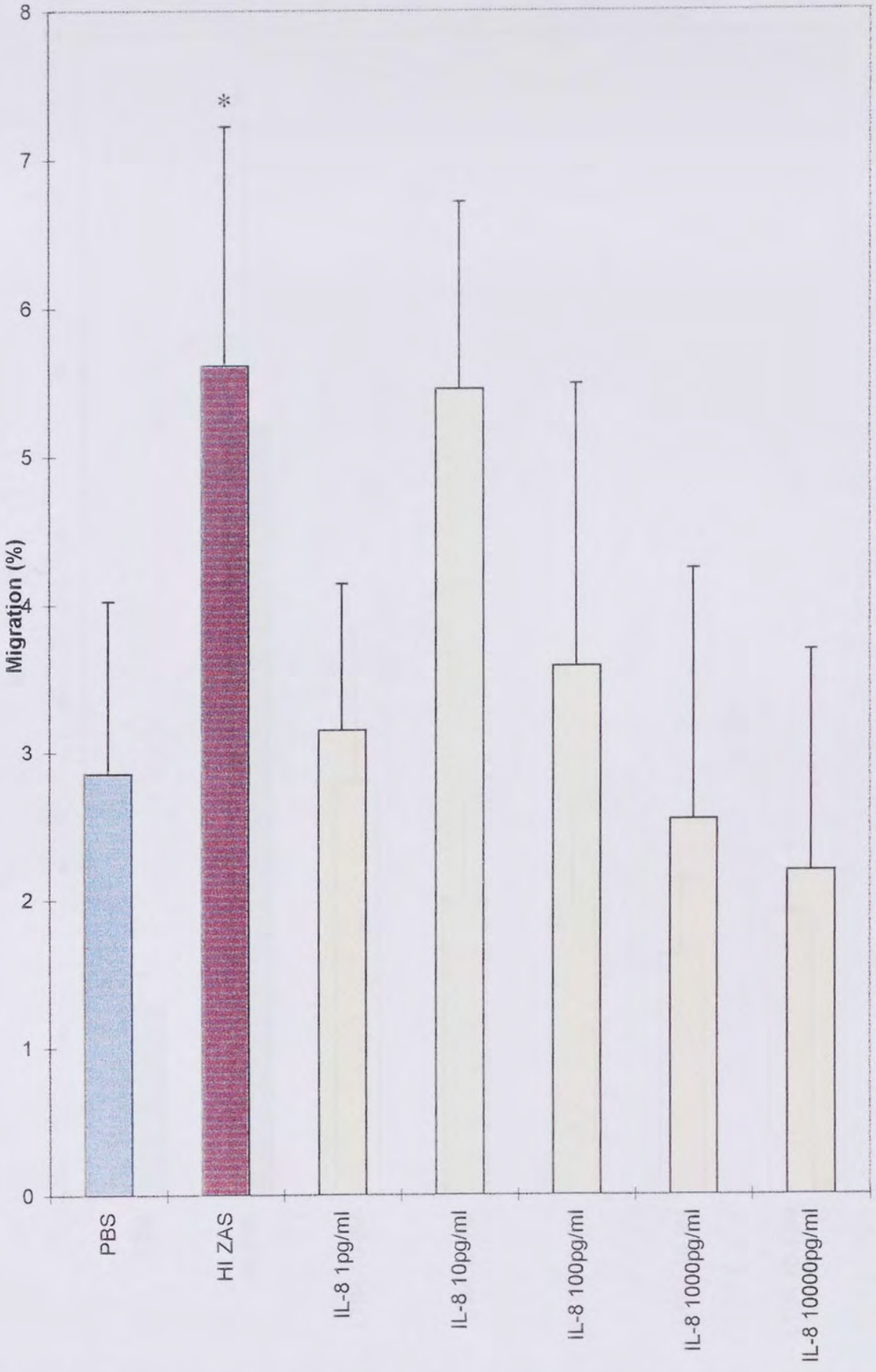
Attempts to establish which factor (s) could be responsible for the enhanced neutrophil chemotaxis following hypoxia or hypoxia/reoxygenation led to the use of fmlp, IL-8, LTB_4 and PAF as chemoattractants (Figures 4.9-4.12). Neither IL-8 , between 1-10000pg.ml nor LTB_4, between 10^{-6} - 10^{-10} M, significantly increased neutrophil migration above control. PAF in comparison significantly ($p<0.05$ and $p<0.01$) increased migration between 10^{-6} - 10^{-8} M. Lowering the concentration below 10^{-8} M saw a decline in migration back to those comparable to PBS at 1.5 +/- 0.1%. Fmlp also significantly increased neutrophil migration ($p<0.05$) at 10^{-6} M. However PAF nor fmlp alone were unable to produce any migration index comparable to those produced by the hypoxic or hypoxic/reoxygenated ECV304 cell supernatants. An attempt to recreate this migration was made by allowing neutrophils to migrate into a cocktail of IL-8 (100pg/ml), LTB_4 (10^{-6} M) and PAF (10^{-8} M) (Figure 4.13). No significant increase in neutrophil migration above control was observed.

Figure 4.9 Effect of fmlp on neutrophil migration



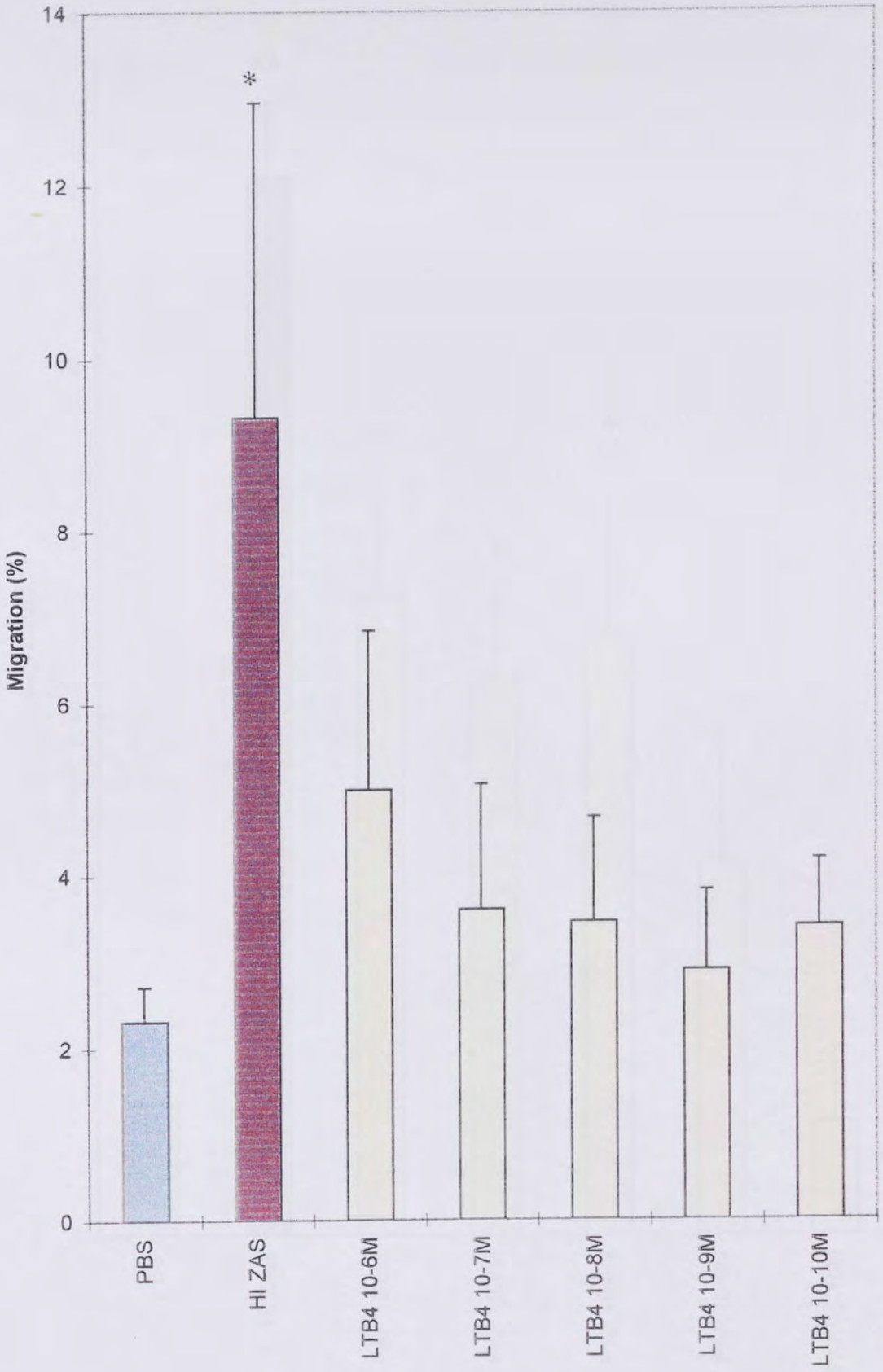
Neutrophils (200µl) ($2.5x10^6$/ml) were allowed to migrate into varying concentrations of fmlp. HIZAS (1%) was used as a positive control. Values given are means +/- SEM were n=4. * indicates a significant difference ($p<0.05$) from control using a Two-Way Anova.

Figure 4.10 Effect of IL-8 on neutrophil migration



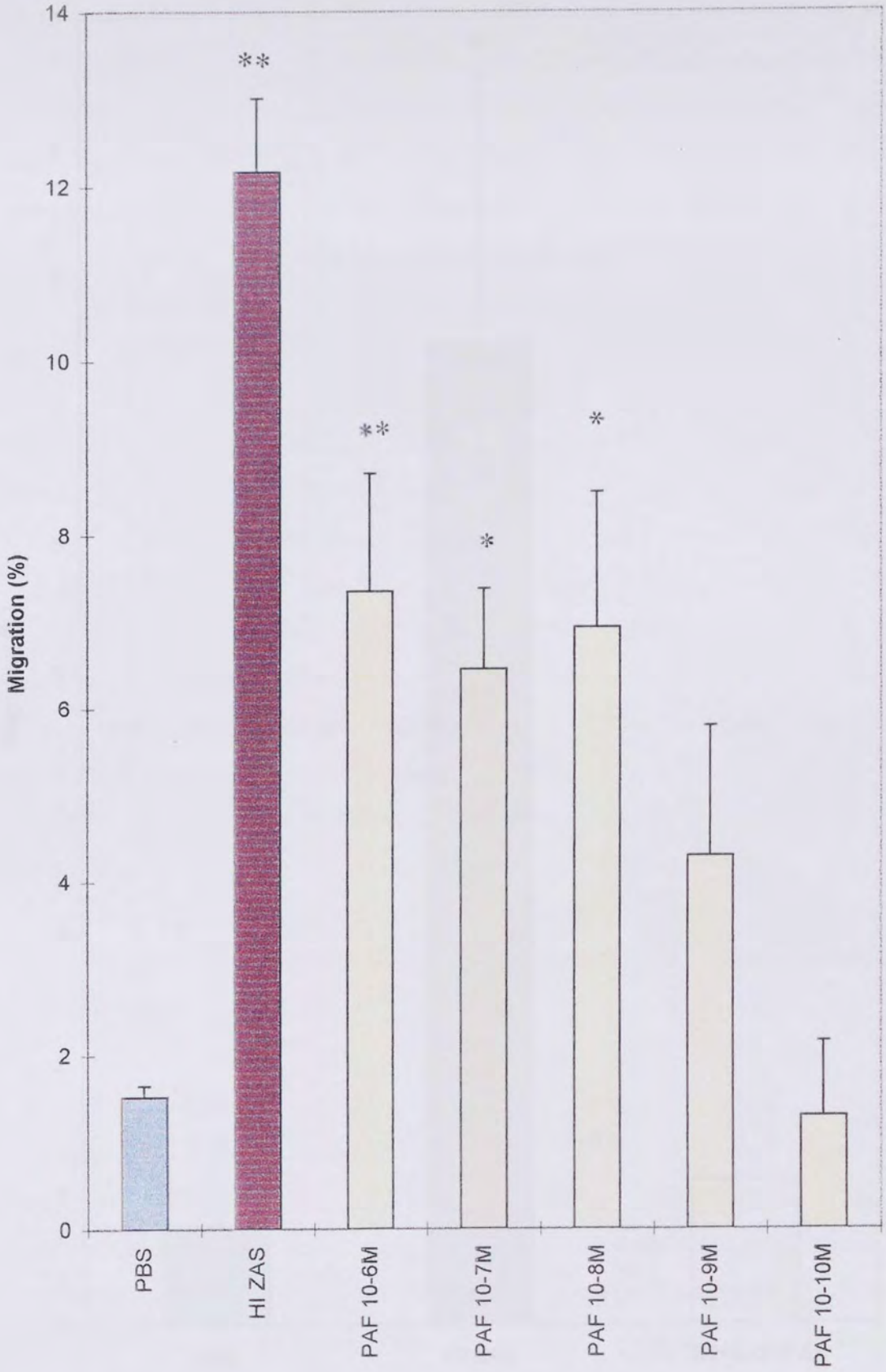
Neutrophils (200µl) ($2.5x10^6$/ml) were allowed to migrate into varying concentrations of IL-8. HIZAS was used as a positive control. Values given are means +/- SEM were n=4. * indicates a significant difference ($p<0.05$) from control using a Two-Way Anova.

Figure 4.11 Effect of LTB_4 on neutrophil migration



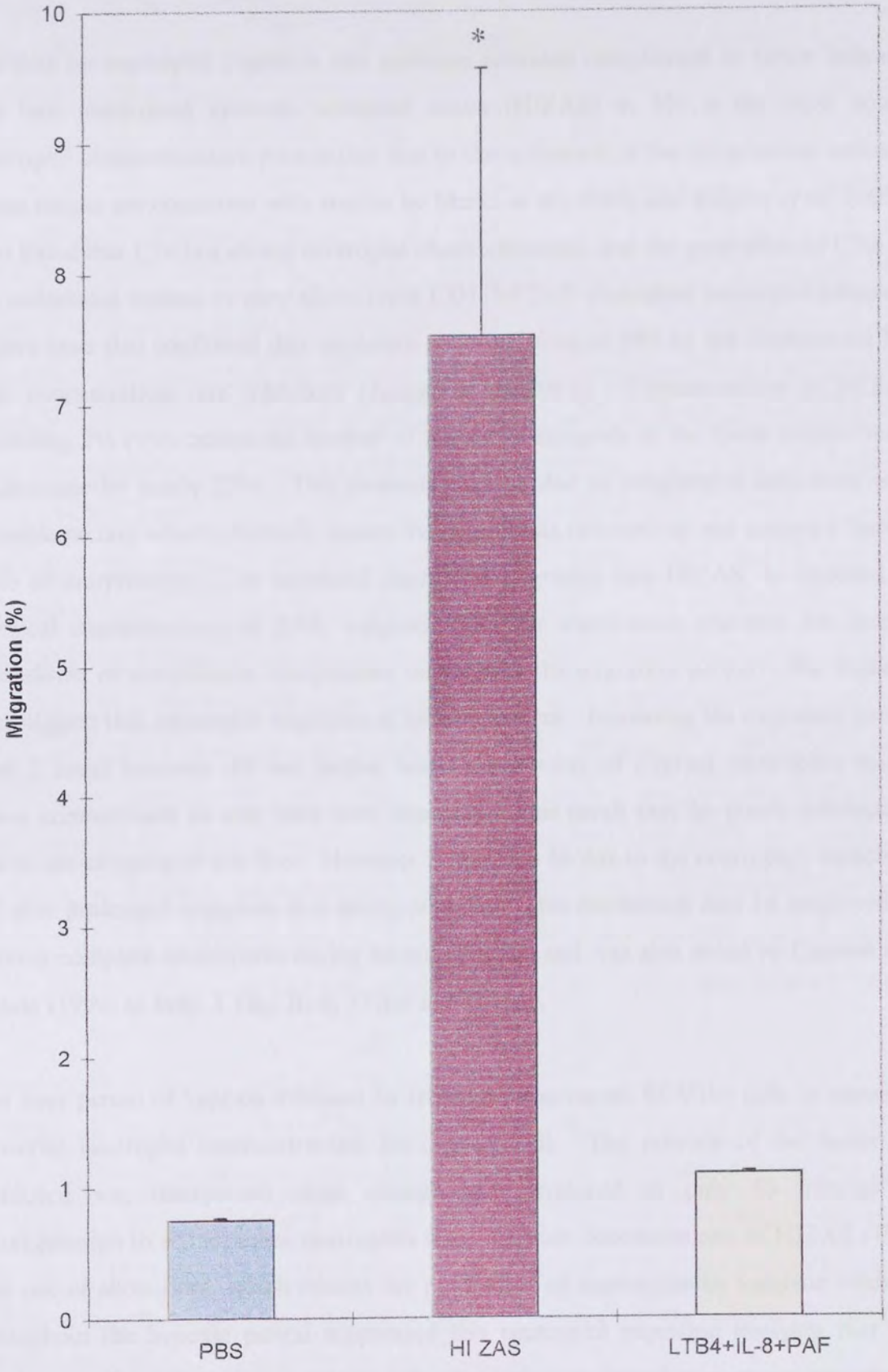
Neutrophils (200µl) ($2.5x10^6$/ml) were allowed to migrate into varying concentrations of LTB_4. HIZAS was used as a positive control. Values given are means +/- SEM were n=4. * indicates a significant difference ($p<0.05$) from control using a Two-Way Anova.

Figure 4.12 Effect of PAF on neutrophil migration



Neutrophils (200µl) ($2.5x10^6$/ml) were allowed to migrate into varying concentrations of PAF. HIZAS was used as a positive control. Values given are means +/- SEM were n=4. * indicates a significant difference ($p<0.05$) ** ($p<0.01$) from control using a Two-Way Anova.

Figure 4.13 Effect of IL-8, LTB4 and PAF on neutrophil migration



Neutrophils (200µl) ($2.5x10^6$/ml) were allowed to migrate into a combination of IL-8 (100pg/ml), LTB_4 (10^{-6} M) and PAF (10^{-8} M). HIZAS was used as a positive control. Values given are means +/- SEM were n=4. * indicates a significant difference ($p<0.05$) from control using a Two-Way Anova.

4.3 Discussion

The data for neutrophil migration into zymosan activated complement or serum indicates that heat inactivated zymosan activated serum (HIZAS) at 1% is the most potent neutrophil chemoattractant presumably due to the activation of the complement cascade. These results are consistent with studies by Marks *et al* (1989) and Kilgore *et al* (1994) who found that C5a is a strong neutrophil chemoattractant, and the generation of C3bi on the endothelial surface *in vitro* elicits rapid CD11b/CD18 dependent neutrophil adhesion. Others have also confirmed that moderate concentrations of HIZAS are chemotactic but high concentrations are inhibitory (Junger *et al* 1993). Concentrations of HIZAS exceeding 2% (v/v) caused the number of migrant neutrophils in the lower compartment to decrease by nearly 25%. This decrease may be due to progressive saturation with chemoattractant which ultimately causes the neutrophils to round up and assume a resting state of morphology. The increased neutrophil migration into HIZAS, as opposed to identical concentrations of ZAS, suggests that heat inactivation prevents the further breakdown of complement components throughout the migration period. Our findings also suggest that neutrophil migration is time dependent. Increasing the migration period over 2 hours however did not further boost the number of migrant neutrophils in the lower compartment as may have been expected. This result may be purely artefactual, due to the clogging of the filter. However it may also be due to the neutrophils switching off after prolonged exposure to a strong stimulus. This mechanism may be employed to prevent complete neutropenia during severe infection and was also noted by Carolan and Casale (1996) to fmlp, LTB_4, IL-8, TNFα and IL-1β.

A 4 hour period of hypoxia followed by reoxygenation causes ECV304 cells to release a powerful neutrophil chemoattractant (s) (Figure 4.4). The potency of the factor (s) produced was unexpected since enough was produced in only 30 minutes of reoxygenation to attract more neutrophils than optimum concentrations of HIZAS (1%). The use of allopurinol, which inhibits the production of superoxide by xanthine oxidase, throughout the hypoxic period suppressed this neutrophil migration implying that the production of reactive oxygen species following ischaemia/reperfusion causes the release of inflammatory chemokines which attract neutrophils. Allopurinol has been shown to reduce infarct size in animal models and some clinical trials (Granger *et al* 1981, Kurose and Granger 1994 and Varni and Ward 1994). However Reimer *et al* (1985), Werns *et al*

(1986), Rashid and Olison (1991) *in vivo* have found that allopurinol does not provide protection against ischaemic/reperfusion injury. There are two probable explanations for these conflicting findings. Firstly pre-treatment for 12-18 hours before ischaemia is necessary for allopurinol to be of benefit. This is the time required for accumulation of alloxanthine, the more active metabolite of allopurinol, to occur *in vivo*. Secondly allopurinol is less effective after prolonged ischaemia (>3hours) because irreversible ischaemia leads to cell death (Simpson *et al* 1987).

Another potential therapy for ischaemic/reperfusion injury is dapsone, an anti-inflammatory sulphone, that we have been able to show prevents neutrophil adhesion (Chapter 3, Figure 3.14). Initial attempts to prevent neutrophil migration into HIZAS were unsuccessful. However, further studies using normoxic, hypoxic or hypoxic/reoxygenated supernatant show that dapsone is able to suppress neutrophil chemotaxis into hypoxic and hypoxic/reoxygenated supernatant. Though dapsone treatment did not decrease neutrophil migration as dramatically following hypoxia/reoxygenation as it did following hypoxia the results were still significant. These results are consistent with published data that neutrophil migration towards complement components is not attenuated by pre-treatment with dapsone (Harveth *et al* 1986). Migration towards other factors such as fmlp and IL-8, in contrast, can be reduced by dapsone treatment of the neutrophils (Thuong-Nguyen *et al* 1993). Our results suggest that there are different pathways through which different chemoattractants potentiate a response and that dapsone is selective as to which of these it is able to block. Its inability to prevent migration into HIZAS does not preclude its use in ischaemic/reperfusion injury

Having illustrated conclusively that factors released from ECV cells following hypoxia and reoxygenation are capable of eliciting neutrophil migration we attempted to ascertain what factor (s) are responsible for the neutrophil sequestration. Early studies highlighted the role of complement as a neutrophil chemotactic factor (Chenowerth *et al* 1979) while later studies have focused more specifically on factors such as LTB_4 (Zimmerman 1990 and Carolan and Casale 1996), PAF (Kurose and Granger 1994), TNFα (Smith *et al* 1996), IL-1 (Furie and McHugh 1989), fmlp as a known positive chemokine (Bittleman *et al* 1996) and IL-8 (Ivey *et al* 1995, Takahashi *et al* 1995 and Utsunomiya *et al* 1996). As no complement is present in our system we focused solely on fmlp, IL-8, LTB_4 and PAF. In our hands fmlp and PAF stimulated migration. Fmlp is the major chemotactic factor produced by *Escherichia coli* (Marasco *et al* 1984). The fmlp findings are consistent with

other *in vitro* research that indicates neutrophils will migrate through a membrane towards fmlp (Bittleman *et al* 1996 and Carolan and Casale 1996). Suggestions that PAF alone may be responsible for the increased neutrophil migration following hypoxia and reoxygenation are not entirely substantiated by our findings. A PAF secretion antagonist showed an indication, by no means complete, of decreasing migration into hypoxic and hypoxic/reoxygenated supernatant hinting at the involvement of other factor (s). A role for PAF in ischaemic/reperfusion injury is certainly supported by several other lines of evidence; PAF levels rise in tissue exposed to ischaemia and reperfusion (Filep *et al* 1989), PAF receptor antagonists attenuate the microvascular alterations elicited by I/R (Kubes *et al* 1990) and the local administration of PAF elicits most of the microvascular alterations elicited by I/R injury (Kurose and Granger 1994). Therefore it is likely that in our *in vitro* system that either PAF was not produced at a high enough concentration to promote migration or the concentration of antagonist was too low to prevent secretion. Further investigation would be able to resolve these points.

IL-8 did not act as a neutrophil chemoattractant through a naked filter in our hands. The results surrounding IL-8 in models of neutrophil migration following ischaemia/reperfusion however are to say the least controversial. Abe *et al* (1993) reported that serum IL-8 levels rise during the early phase of myocardial infarction suggesting it could be involved in the neutrophil accumulation following ischaemia and reperfusion. Huber *et al* (1991) and Kuijpers *et al* (1992) also reported that IL-8 enhances neutrophil transmigration across cell monolayers whereas Gimbrone *et al* (1989), Luscinskas *et al*(1992) and Takaharshi *et al* (1995) all showed that IL-8 had an inhibitory effect on neutrophil adhesion and transmigration. There could be several explanations for these discrepancies. Firstly differences may be due to the different neutrophil separation techniques or migration chambers employed. Another likely explanation is that the chemotactic capacity of mediators is heavily dependent upon the barrier through which the neutrophil has to migrate. For example IL-1α will stimulate neutrophils to migrate through cellular barriers but not through naked filters (Bittleman *et al* 1996). An *in vivo* model by Utsunomiya (1996) accounts for these findings by showing that IL-1 can induce neutrophil chemotaxis indirectly via the synthesis of chemoattractant proteins including cytokine-induced neutrophil chemoattractant (CINC), from the endothelium. IL-1 is unable to promote neutrophil migration through monolayers that do not produce soluble chemoattractants in response to it. CINC, a 8 kDa polypeptide, is a member of the IL-8 family (Watanabe *et al* 1992). Originally identified in the conditioned

media of IL-1β stimulated rat glomerular epithelial cells and subsequently purified from NRK52E rat epithelial cells (Watanabe *et al* 1989), CINC resembles monocyte-derived neutrophil chemotactic factor (MDNCF) rather than human complement fragment C5a. CINC levels are known to rise following reperfusion reaching a peak at 6 hours (Hisama *et al* 1996). Finally our IL-8 may have been biologically inactive. However, this is unlikely as the same IL-8 used in parallel adhesion assays was able to potentiate neutrophil adhesion (Chapter 3, Figure 3.16).

Taking all these factors into account it is unlikely that IL-8 is the chemoattractant present in the hypoxic or hypoxic/reoxygenated ECV304 supernatant. What ever is liberated it is able to attract neutrophils directly through a naked filter, whereas IL-8 in our hands is not.

LTB_4, in contrast to several other studies (Zimmerman *et al* 1990 and Carolan and Castle 1996), was also unable to cause neutrophil migration. The most likely explanation for this is that the neutrophils used by those authors in the *in vitro* study cited were isolated using a modification of the density gradient technique (Hypaque-Ficoll) described by Ferrante and Thong (1978). Hypaque-Ficoll is known to activate neutrophils inappropriately which could then make them more responsive to LTB_4. All cells used in our studies were isolated using hespan and percoll which we demonstrated (Chapter 2, Figure 2.4) left the cells intact with quiescent morphology.

Clearly our studies unequivocally demonstrate that hypoxic endothelial cells will generate a powerful neutrophil chemoattractant. Manufacture of this material is further enhanced by a reperfusion period of as little as 30mins (Figure 4.3). Complement cannot be implicated in our experiment since there was no obvious source for generation of C3a or C5a. Furthermore we cannot stipulate that IL-8 has any role since it cannot promote migration through naked filters as used in our studies. Although others have suggested a role for LTB_4 we have been unable to demonstrate enhanced migration in response to this agent which is possibly only active if neutrophils are pre-primed as an artefact of isolation or by other agents. There may well be some contribution by PAF since we did find a moderate reduction in the release of chemoattractants by the hypoxic/reoxygenated endothelial cells in the presence of a PAF secretion antagonist (Figure 4.6). Perhaps higher concentrations of antagonist might have been more effective. Certainly PAF at high concentrations has the capacity to stimulate migration in our experimental system.

Whatever is produced by the hypoxic/reoxygenated endothelial cells a necessary prelude to this is xanthine oxidase activation and presumably the generation of ROS (Figure 4.5).

5 NEUTROPHIL ACTIVATION

5.1 Introduction

Neutrophils are antimicrobial because of their capacity to release up to 50 toxins within phagosomes and the extracellular fluid of their immediate surroundings (Babior *et al* 1973 and Anderson 1995). Unfortunately when triggered inappropriately during episodes of hypoxia/reoxygenation these same materials may cause extensive tissue damage (Wiess *et al* 1981, Dhôte-Burger *et al* 1995, Serizawa *et al* 1996 and Williams et al 1996). Such potential noxious materials may be ROS, products of arachiodonic acid metabolism and the contents of the neutrophil granules.

One of the earliest indications of neutrophil activation is the heightened O_2 consumption or respiratory burst which generates ROS. The enzyme responsible (NADPH oxidase) is inactive until the neutrophil is stimulated either by engagement of receptors for chemoattractants and cytokines or those receptors directly involved in phagocytosis (Anderson and Harken 1990). Indeed it may be that those factors responsible for heightened neutrophil adhesion and migration outlined in chapters 3 and 4 are also responsible for priming neutrophils to respond to other activating species or perhaps can even directly activate the neutrophil (Bender *et al* 1983, Baggiolini *et al* 1993 and Bokoch 1995). For example IL-8 is thought to cause neutrophil chemotaxis, shape change, degranulation, respiratory burst, increased cytosolic Ca^{2+}, and increased adhesion to endothelial cells, fibrinogen, and extracellular matrix proteins. As well as increased *Candida albicans* growth inhibition, increased expression of CD11a, CD11b, CD11c, and CD18 and lysosmal enzyme release (Djeu *et al* 1990, Oppenheim *et al* 1991, Schall 1991, Matsushima *et al* 1992 and Taub and Oppenheim 1993). Activation of PLA_2 and PAF production may also be an important mediator in neutrophil priming (Franciose *et al* 1996).

Furthermore the adhesion process itself whereby CD18 of the neutrophil integrin binds to the endothelium seems to be the signal required to release H_2O_2 and possibly proteases into the extracellular environment (Nathan *et al* 1989). Neutrophil-endothelial adhesion is thought to create a microenvironment which permits high concentrations

of injurious agents (Welbourne *et al* 1991, Crockett-Torabi *et al* 1995 and Liles *et al* 1995).

Whatever the triggers the signalling processes induced result in the rapid appearance of NADPH oxidase activity (Dewald and Baggiolini 1985). The active enzyme catalyses the following reaction and the production of superoxide anion.

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

Increased levels of $NADP^+$ activate the pentose phosphate pathway (hexose monophosphate shunt) and NADPH is regenerated. The O_2^- formed by NADPH oxidase activity can be rapidly converted to H_2O_2 and other toxic species (Chapter 1, Section 1.4). These ROS and reactive chlorine species cause damage to the cell membrane and surrounding tissue via lipid peroxidation (Curnutte and Babior 1987 and Weiss 1989). In addition to the individual toxicity's of ROS, granular substances, arachidonic acid derivatives, synergistic interactions between these toxins are likely and capable of greatly potentiating the damaging effects of the neutrophil (Ferretti *et al* 1994). This damage is further compounded by release of the contents of the neutrophil's specific and azurophilic granules; toxic peptides, proteins, and enzymes including MPO, elastase, cathespins, collagenase, cationic proteins, lysozyme, lactoferrin among others (Williams 1996). There are three neutrophil enzymes that have attracted particular interest as possible mediators of tissue damage: the serine proteinase elastase and the two metalloproteases collagenase and gelatinase. These enzymes are able to degrade key components of the extracellualr matrix. Thus elastase has been shown to alter barrier properties of endothelial monolayers *in vitro* and to cause detachment, or even lysis of cells (Harlan *et al* 1985, Harlan 1985 and Smedley *et al* 1986). When inhibitors of elastase and free radical scavengers are employed the tissue damage attendant upon hypoxia/reoxygenation is ameliorated (Zimmerman and Granger 1990, Dhôte-Burger *et al* 1995 and Franciose *et al* 1996). Zimmerman and Granger (1990) reported that the reperfusion induced increase in intestinal mucosal MPO activity is attenuated in animals pre-treated with elastase inhibitors. Thus proteases released from both stimulated endothelial cells and activated neutrophils may be an important component of ischaemic reperfusion injury.

Although several studies have now implicated neutrophil activation as being central to the genesis of tissue reperfusion injury and post-traumatic multiple organ failure (MOF) in

human subjects data about activation is scarce. This chapter details the enhanced neutrophil activation that occurs following hypoxia and reperfusion in our system. It attempts to dissect out the role of ROS and other factors involved in ischaemic/reperfusion. Furthermore it endeavors to establish the preliminary role that such factor (s) may play in neutrophil priming.

5.2 Results

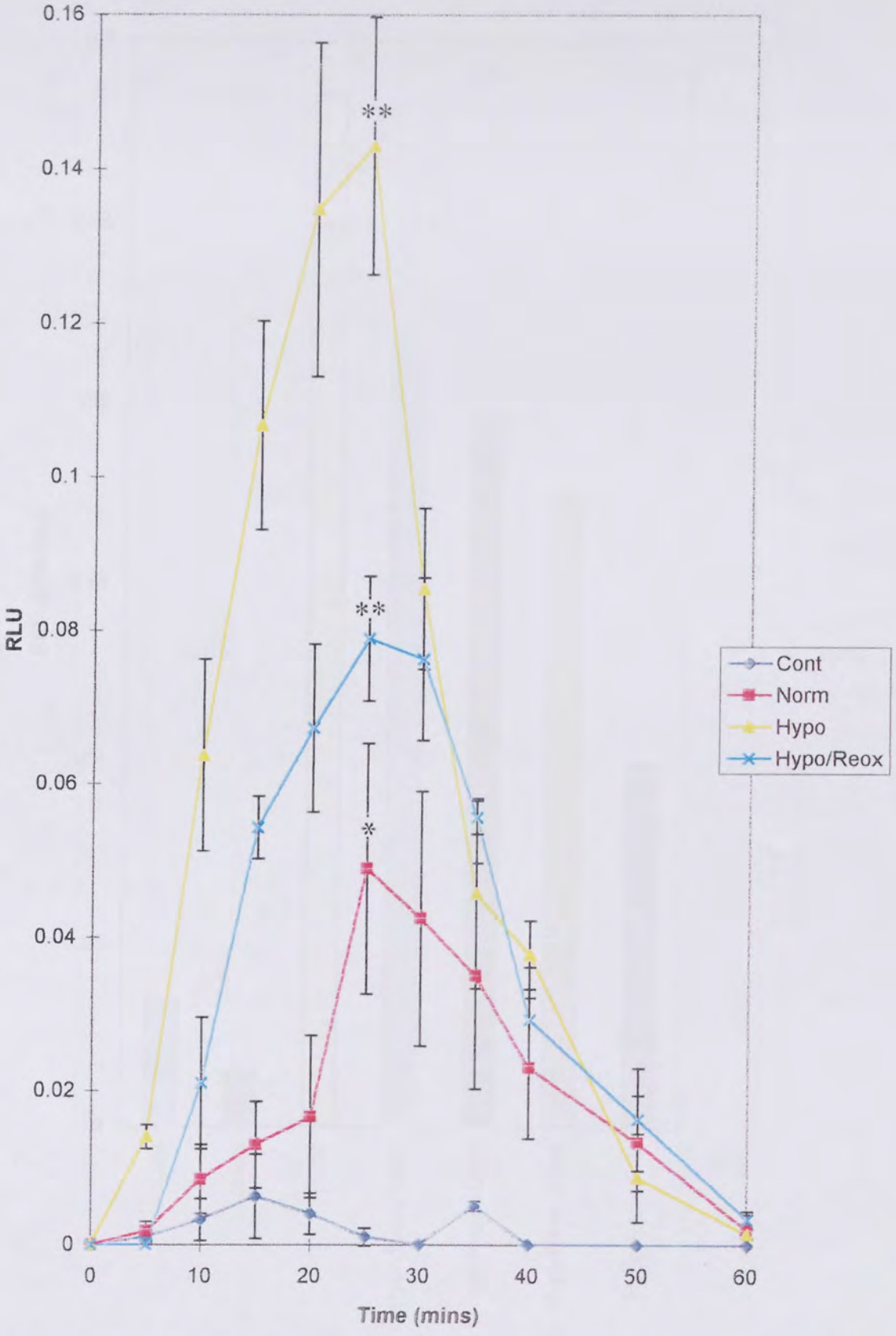
5.2.1 Hypoxic and hypoxic/reperfused ECV304 produce an oxidative burst from whole blood

When 100μl of whole blood was added to normoxic ECV304 cells grown to confluence on cytodex microcarriers a modest oxidative burst was generated, presumably a consequence of neutrophil activation (Figure 5.1). If the ECV304 cells were first subjected to hypoxia for 4 hours followed by 30mins reoxygenation in fresh medium a significant greater burst was generated ($p<0.01$). When whole blood was added to ECV cells immediately after the 4 hour hypoxic period a dramatic very powerful burst ensued which was significantly greater than that under all the other conditions ($p<0.01$). In all cases the peak of the response was seen at 25mins and had subsided by 1 hour. Thus hypoxic ECV must either alter their surface properties or release factor (s) into the surrounding medium (or both) which provokes neutrophil activation. The factor (s) involved must be labile or transient for as the period of reperfusion increased from zero to 1 hour the response progressively diminished (Figure 5.2)

5.2.2 Hypoxic and hypoxic/reperfused ECV304 produce an oxidative burst from isolated neutrophils

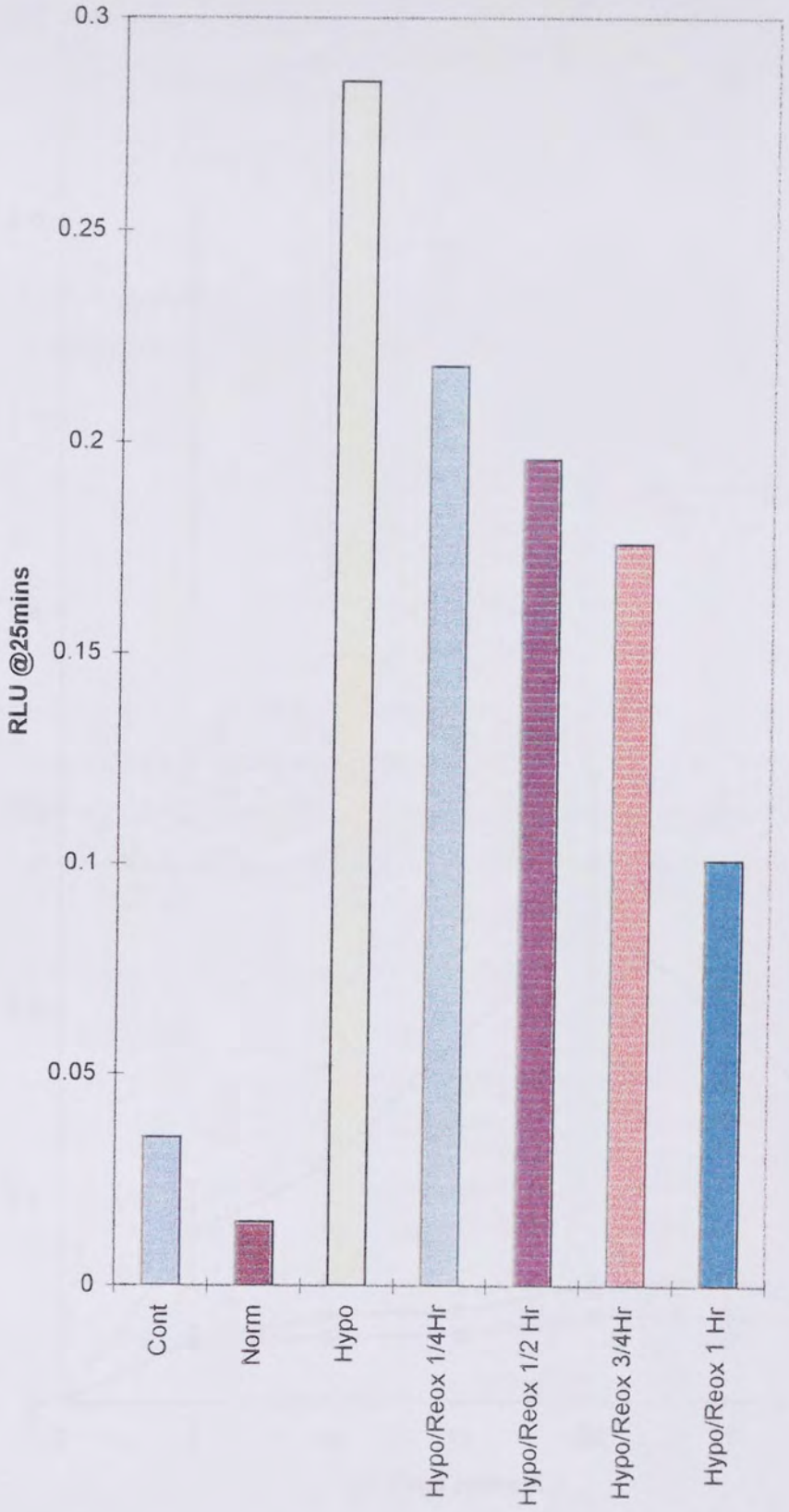
When purified neutrophils ($5x10^6$/ml) were employed under similar circumstances normoxic ECV produced a very modest but nevertheless significant response ($p<0.05$) whereas hypoxia and hypoxia/reoxygenation once again caused the neutrophils to generate a very powerful oxidative burst (0.540+/- 0.128 and 0.120 +/- 0.032 respectively). Although the experiments illustrated in Figure 5.1 and 5.3 are not strictly comparable it is apparent that the purified neutrophils react faster and more powerfully to the stimulus of hypoxic ECV304 cells.

Figure 5.1 Effect of ECV304 on the oxidative burst of whole blood



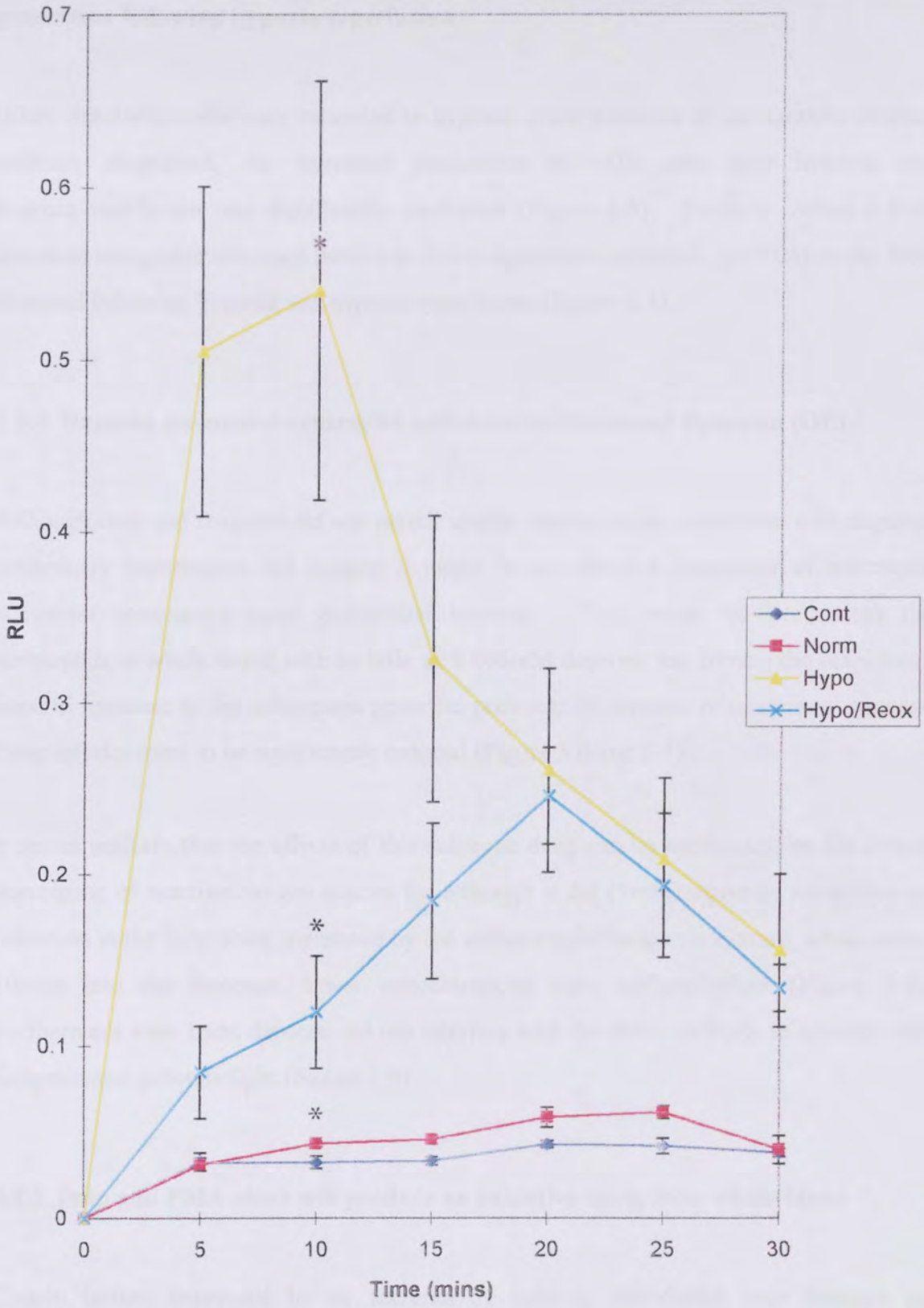
Whole blood (100µl) was added to ECV304 cells ($5x10^6$), grown on cytodex microcarriers, that had either been made normoxic for 4 hours, hypoxic for 4 hours or hypoxic for 4 hours and reoxygenated for 30mins. Values given are means +/- SEM where n=4 at each point. * indicates a significant difference ($p<0.05$) ** ($p<0.01$) from control using a Two Way Anova.

Figure 5.2 Effect of ECV304 on the oxidative burst of whole blood



Whole blood (100µl) was added to ECV304 cells ($5x10^6$), grown on cytodex microcarriers, that had either been made normoxic for 4 hours, hypoxic for 4 hours or hypoxic for 4 hours and reoxygenated for 15, 30, 45 or 60mins. Values given are means where n=3 at each point.

Figure 5.3 Effect of ECV304 cells on the oxidative burst of isolated neutrophils.



Neutrophils ($5x10^6$/ml) were added to ECV304 cells ($5x10^6$), grown on cytodex microcarriers, that had either been made normoxic for 4 hours, hypoxic for 4 hours or hypoxic for 4 hours and reoxygenated for 30mins. Values given are means +/- SEM where n=4 at each point. * indicates a significant difference ($p<0.05$) from control using a Two Way Anova.

5.2.3 Allopurinol and a PAF secretion antagonist attenuate the increased ROS generation following hypoxia/reperfusion

When endothelial cells were subjected to hypoxia in the presence of the xanthine oxidase inhibitor allopurinol, the increased production of ROS seen after hypoxia and hypoxia/reperfusion was significantly decreased (Figure 5.4). Similarly when a PAF secretion antagonist was used there was also a significant reduction ($p<0.05$) in the ROS liberated following hypoxia and hypoxia/reperfusion (Figure 5.5).

5.2.4 Dapsone attenuates neutrophil activation to Opsonized Zymosan (OZ)

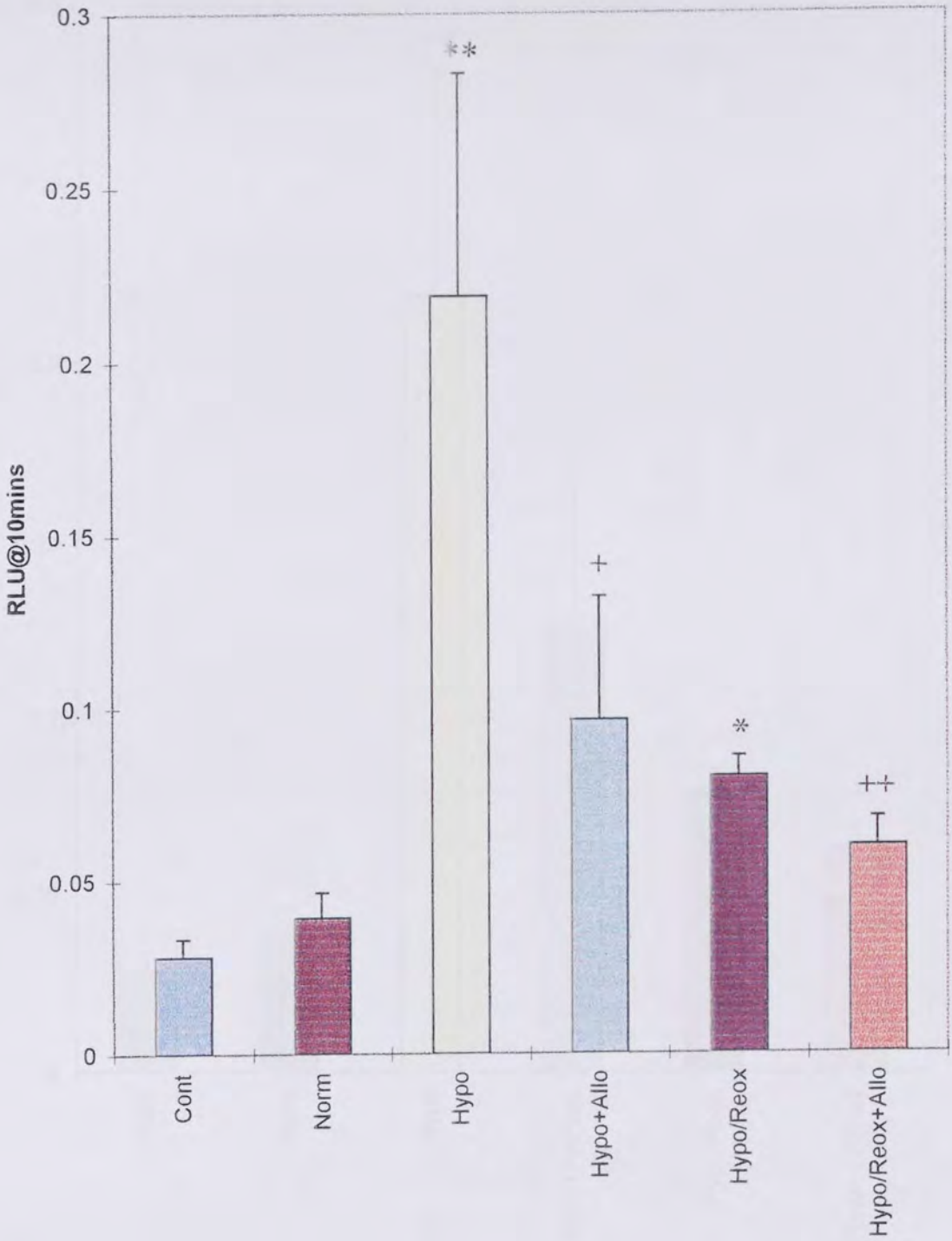
Although time and reagents did not permit similar studies to be undertaken with dapsone preliminary experiments did suggest it might be an effective attenuator of neutrophil activation consequent upon endothelial hypoxia. Thus when we pre-treated the neutrophils in whole blood with as little as 0.005mM dapsone for 20mins the respiratory burst in response to the subsequent powerful provocative stimulus of opsonised zymosan (5mg/ml) did seem to be significantly reduced (Figure 5.6 and 5.7)

It seems unlikely that the effects of this sulphone drug can be attributable to the simple scavenging of reactive oxygen species for although it did (1mM) cause an instantaneous reduction in the light being generated by the oz/neutrophil/lucigenin mixture, when added 10mins into the response, lower concentrations were without effect (Figure 5.8). Furthermore even 1mM dapsone did not interfere with the ability of H_2O_2 to interact with lucigenin and generate light (Figure 5.9).

5.2.5 fmlp and PMA alone will produce an oxidative burst from whole blood

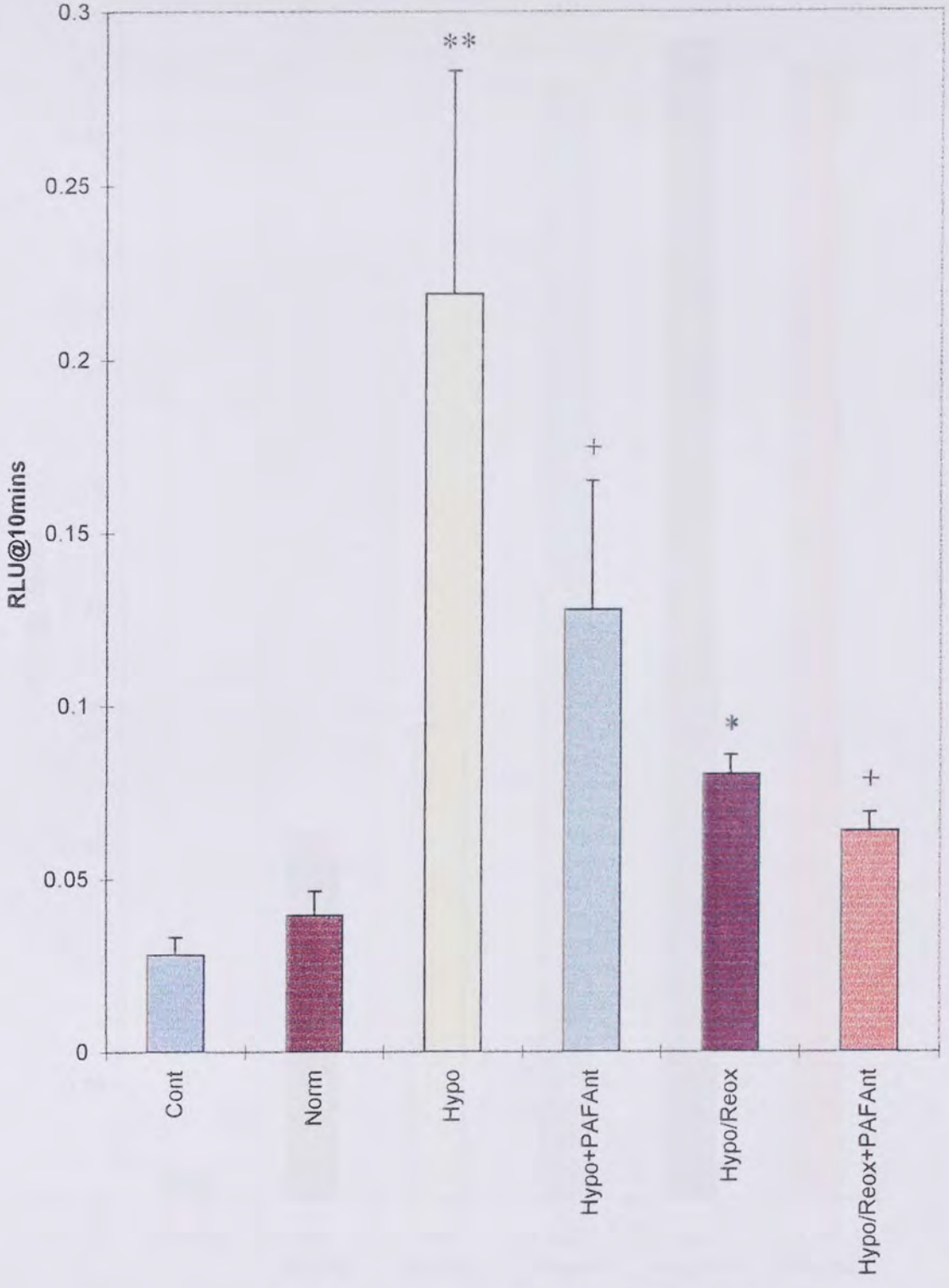
Clearly factors expressed by or secreted by hypoxic endothelial cells produce an inappropriate oxidative burst by neutrophils. In an attempt to establish the nature of the factor(s) involved we therefore screened a variety of compounds which the literature has suggested (see Chapters 3-5) could be implicated. We particularly wished to establish the chronology and magnitude of any oxidative burst in comparison to that elicited by hypoxic endothelial cells. As positive controls for these experiments to ensure the

Figure 5.4 Effect of Allopurinol on the ability of ECV304 cells to produce an oxidative burst from isolated neutrophils.



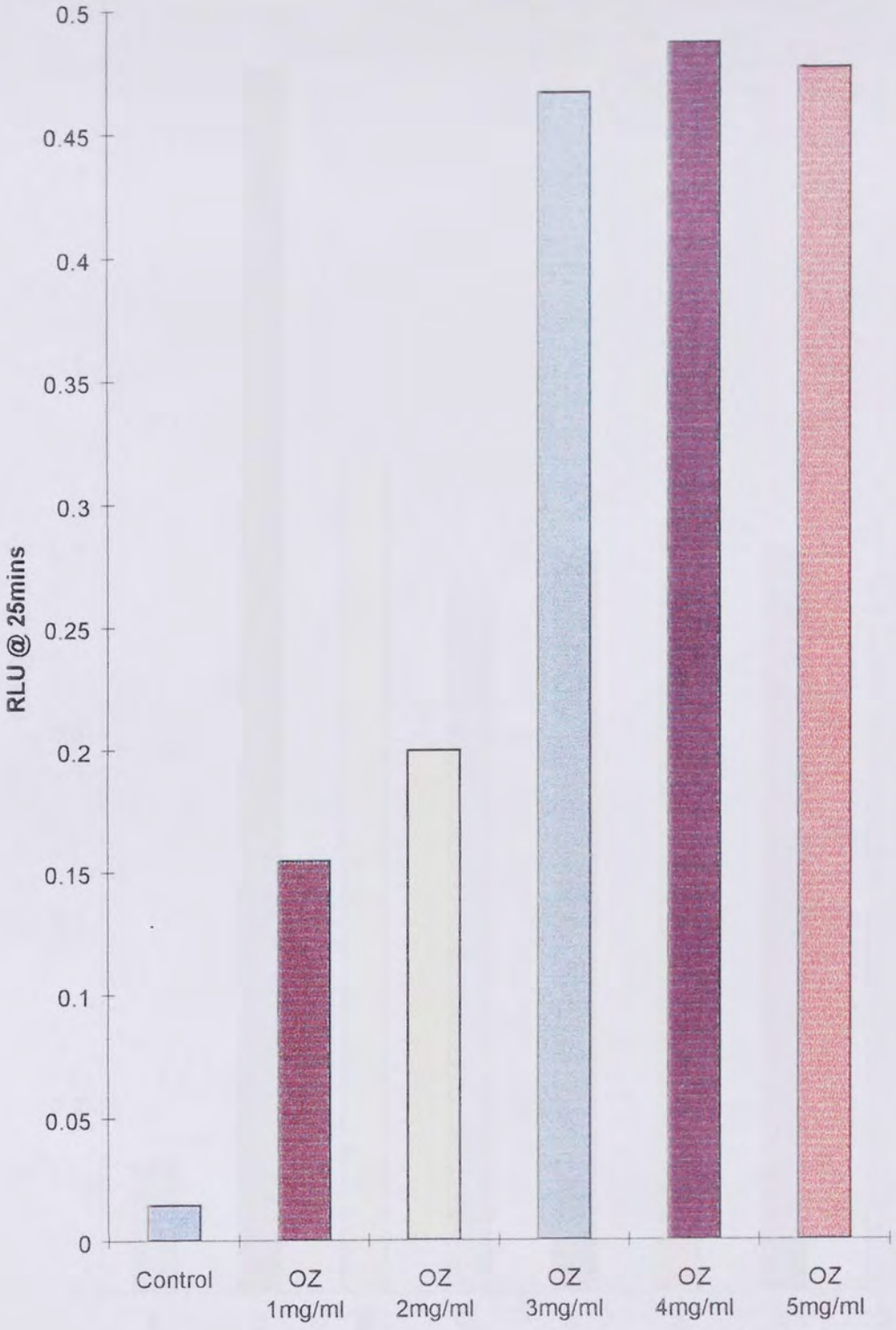
Neutrophils 100µl($5x10^6$/ml) were added to ECV304 cells ($5x10^6$), grown on cytodex microcarriers, that had either been made normoxic for 4 hours, hypoxic for 4 hours or hypoxic for 4 hours and reoxygenated for 30mins in the presence or absence of allopurinol. Allopurinol (20mM) treatment consisted of 1 hour pre-treatment before hypoxia and presence throughout the hypoxic period. Values given are means +/- SEM where n=4 at each point. * indicates a significant difference ($p<0.05$) ** ($P<0.01$) from normoxia while + indicates a significant difference ($p<0.05$) ++ ($p<0.01$) from hypoxia/reperfusion using a Two Way Anova.

Figure 5.5 Effect of a PAF secretion antagonist on the ability of ECV304 cells to produce an oxidative burst from isolated neutrophils.



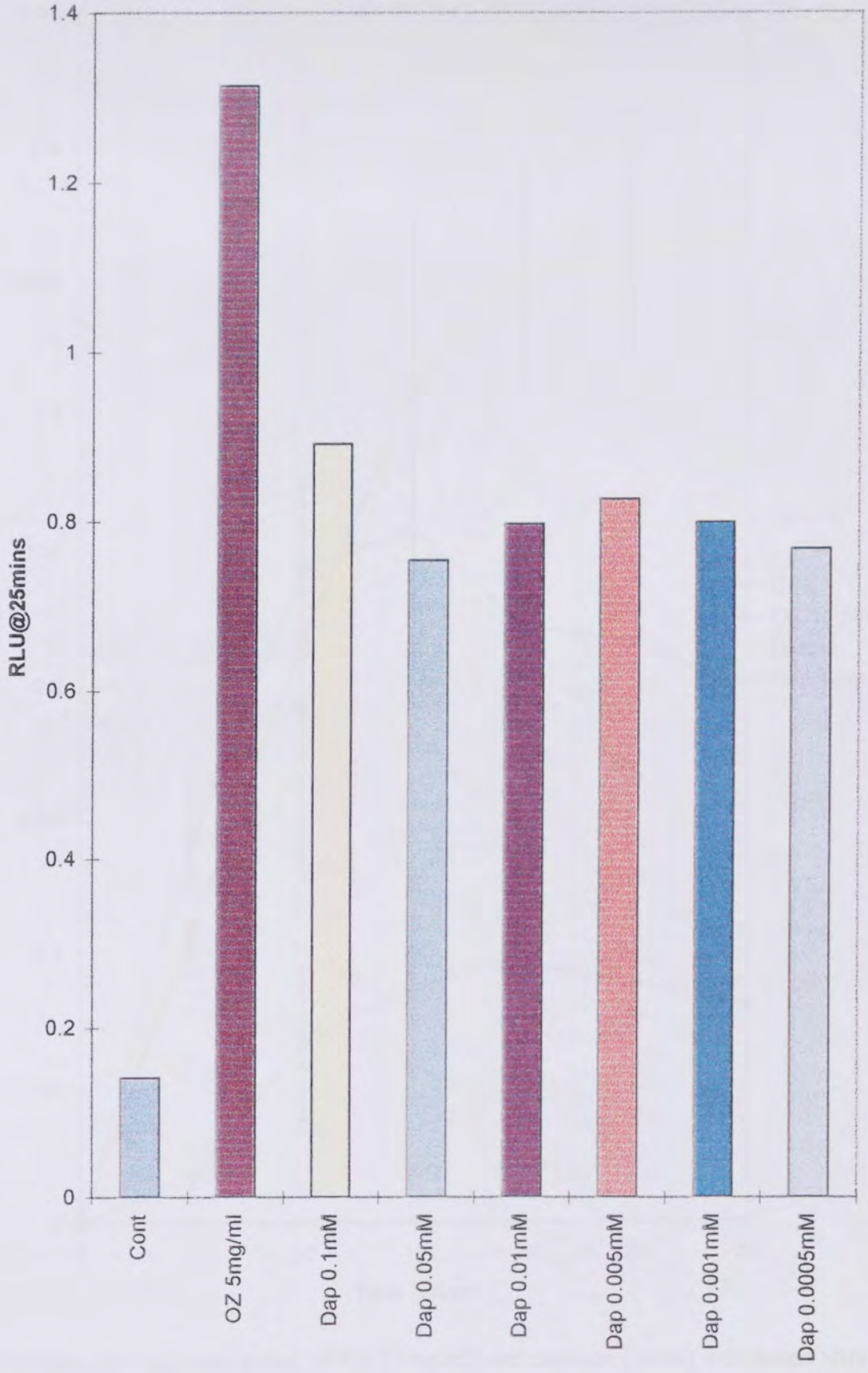
Neutrophils 100µl ($5x10^6$/ml) were added to ECV304 cells ($5x10^6$), grown on cytodex microcarriers, that had either been made normoxic for 4 hours, hypoxic for 4 hours or hypoxic for 4 hours and reoxygenated for 30mins in the presence or absence of a PAF secretion antagonist (10^{-8}M)(1-0-HEXADECYL-2-ACETYL-*sn*-GLYCERO-3-PHOSPHO-(N,N,N-TRIMETHYL)-HEXANOLAMINE). The PAF antagonist was present throughout the hypoxic and reoxygenation period. Values given are means +/- SEM where n=4 at each point. * indicates a significant difference ($p<0.05$) ** ($P<0.01$) from normoxia while + indicates a significant difference ($p<0.05$) from hypoxia/reperfusion using a Two Way Anova.

Figure 5.6 Effect of Opsonized Zymosan A on the oxidative burst of whole blood.



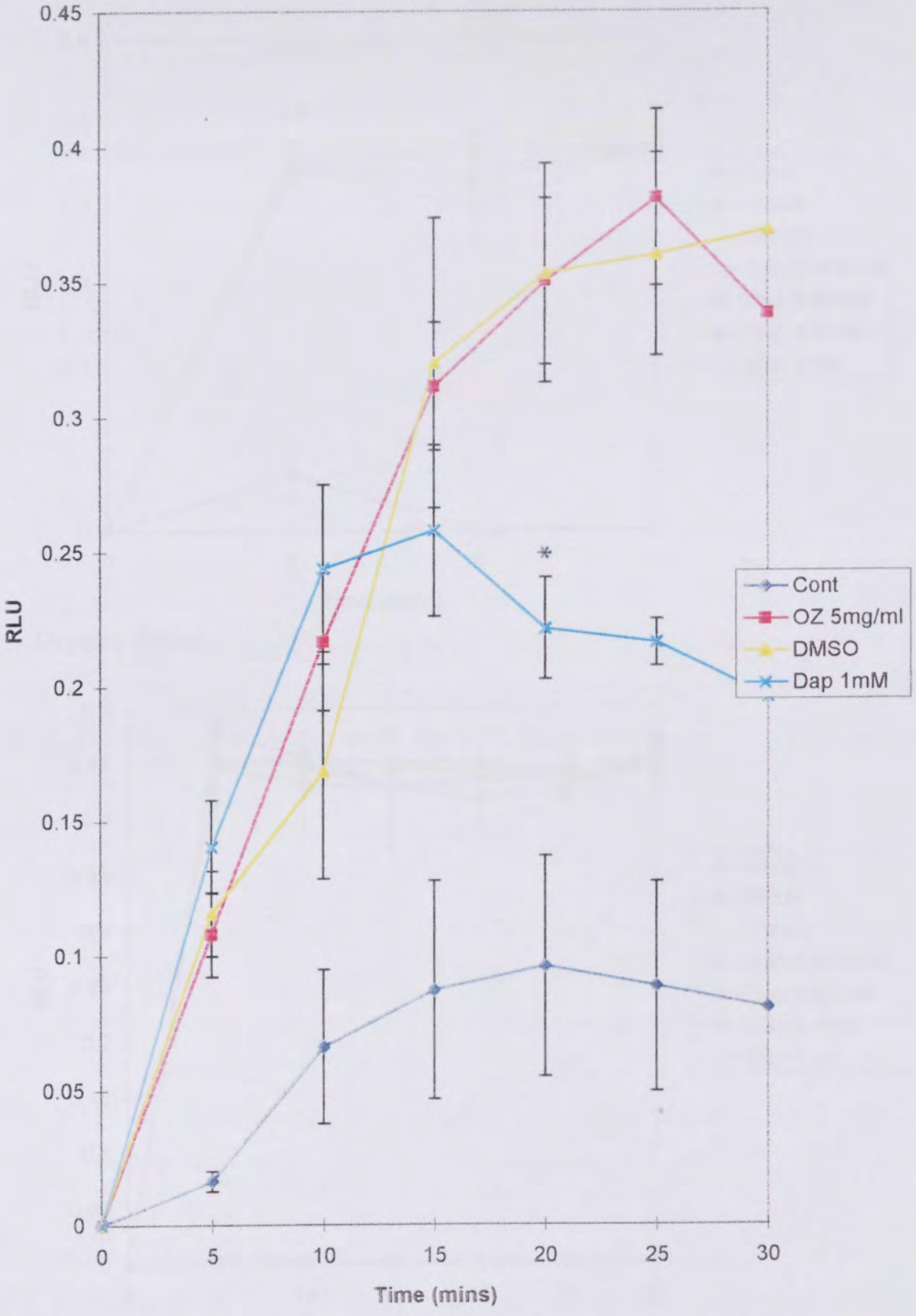
Whole blood (100µl) was added to varying concentrations of Opsonized Zymosan A (OZ). OZ was prepared by incubating 5mg/ml Zymosan with guinea pig serum for 20mins at 37°C. The OZ was then washed twice and resuspended at the appropriate concentration in unsupplemented medium. Values given are means where n=3 at each point.

Figure 5.7 Effect of Dapsone on the oxidative burst of whole blood to OZ.



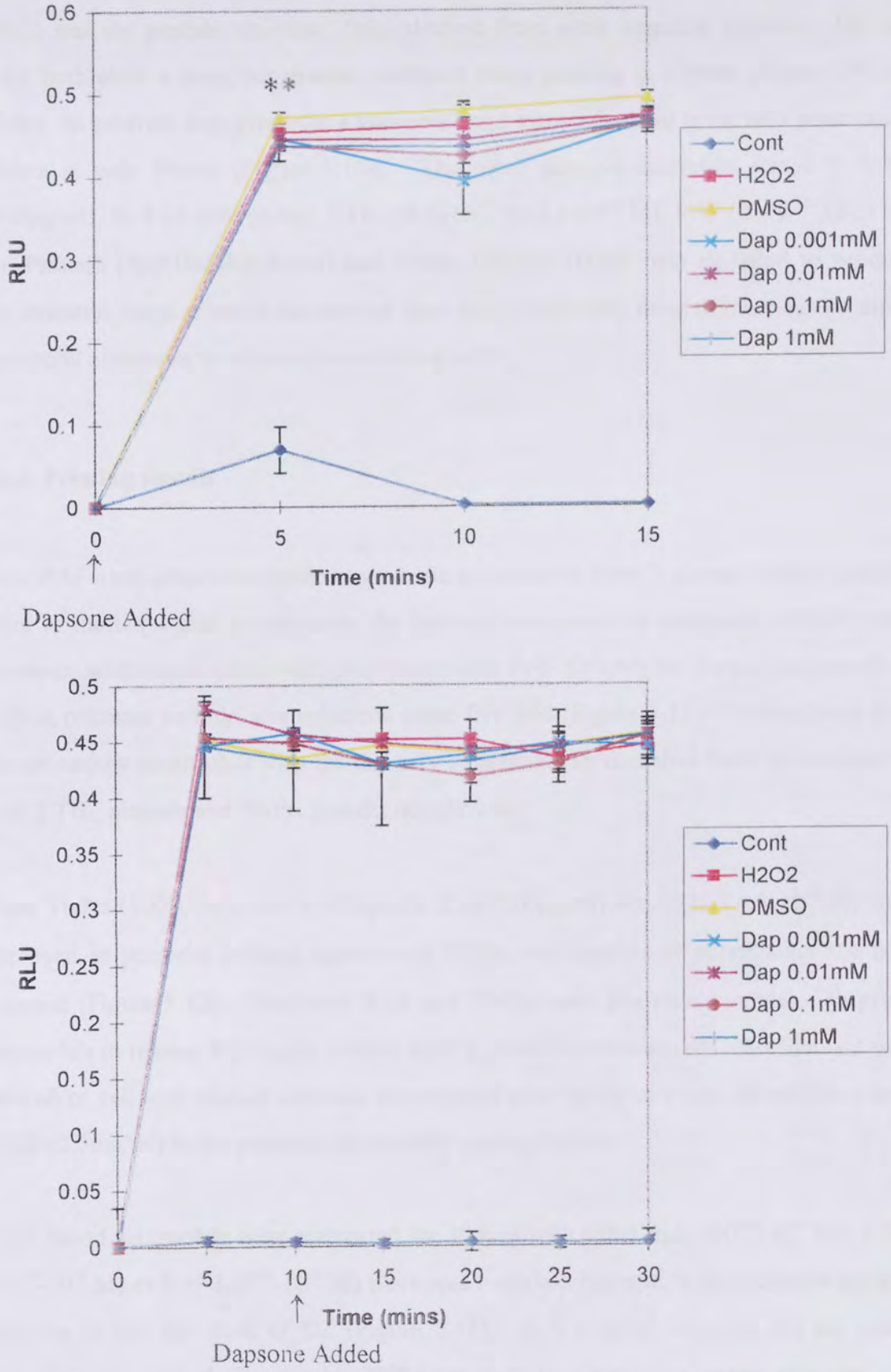
Whole blood (100µl) pre-treated with varying concentrations of dapsone for 20 mins was added to OZ (5mg/ml) and the oxidative burst recorded. OZ was prepared by incubating 5mg/ml Zymosan with guinea pig serum for 20mins at 37°C. Values given are means where n=3 at each point.

Figure 5.8 Effect of Dapsone on the oxidative burst of whole blood to OZ.



Whole blood (100µl) was added to OZ (5mg/ml) and dapsone (1mM) introduced after 10 mins. OZ was prepared by incubating 5mg/ml Zymosan with guinea pig serum for 20mins at 37°C. Values given are means where n=4 at each point. * indicates a significant ($p<0.05$) value from OZ using a Two-Way Anova.

Figure 5.9 Effect of varying concentrations of dapsone on the reaction between hydrogen peroxide and lucigenin.



Dapsone (varying concentrations) was added to hydrogen peroxide and lucigenin at 0 or 10 mins and the oxidative burst recorded. Values given are means +/- SEM where n=4 at each point. ** indicates a significant difference ($p<0.05$) from control using a Two-Way Anova.

neutrophils in the blood were active we included on occasion the particulate activator OZ, the soluble stimulant phorbol myristate acetate (PMA) which activates protein kinase C (PKC) and the peptide activator, fmlp, derived from gram negative bacteria. OZ and PMA both elicit a large but gradual oxidative burst peaking at 30mins (Figure 5.6 and 5.10b). In contrast fmlp produces a very small and extremely rapid burst with peak values evident at only 30secs (Figure 5.10a). The other putative activators tested IL-1 (1-1000pg/ml), IL-8 (1-1000pg/ml), LTB_4 ($4.5x10^{-12}$ M-$3.6x10^{-8}$ M), PAF ($2x10^{-10}$ M-$2x10^{-6}$ M), Plasmin (5μIU/ml-40μIU/ml) and TNFα (1IU/ml-1000IU/ml) all failed to produce any oxidative burst at either the early or later stages indicating none of them can be direct neutrophil activators in terms of an oxidative burst.

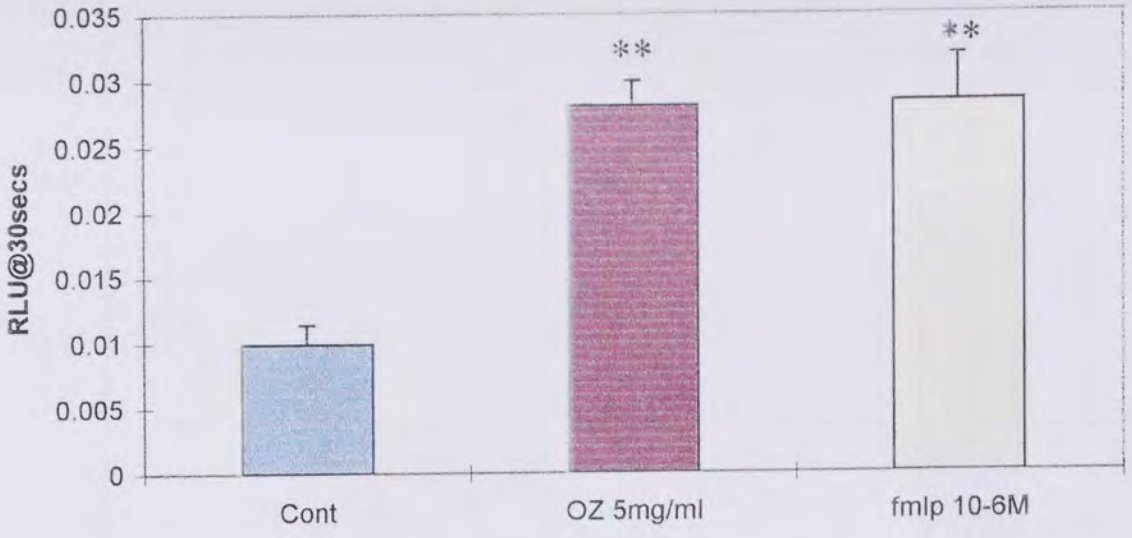
5.2.6 Priming signals

Since PAF used alone was unable to generate an oxidative burst it cannot be the causative agent in the burst that accompanies the hypoxic/reoxygenation treatment of ECV cells. However, when neutrophils were pre-treated with PAF (20nM) for 30mins the genesis of ROS in response to fmlp was enhanced some five fold (Figure 5.11). Priming with PAF did not endow neutrophils with the capacity to produce an oxidative burst in response to IL-1, LTB_4, plasmin and TNFα (results not shown).

When TNFα (100IU/ml), IL-1 (100pg/ml), IL-8 (100pg/ml) and LTB_4 ($4.5x10^{-9}$ M) were employed as potential priming agents only TNFα was capable of potentiating the fmlp response (Figure 5.12). Thus both PAF and TNFα seem plausible candidates to prime neutrophils to release ROS upon contact with hypoxic endothelial cells. To simulate such cell-cell or cell -cell surface contacts we exposed neutrophils to a low suboptimum dose of OZ (2.5mg/ml) in the presence of potential priming agents.

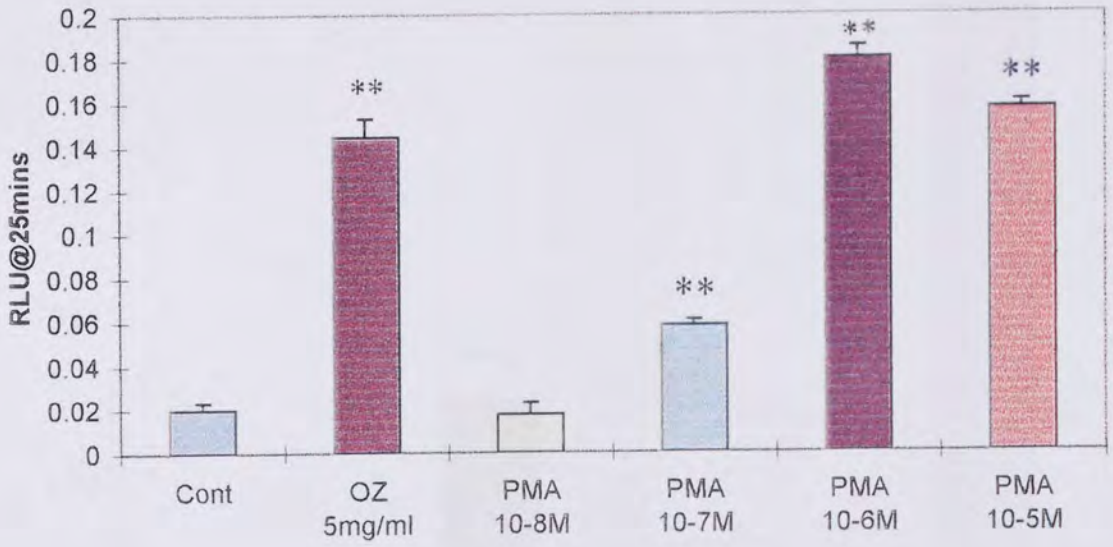
When blood neutrophils were pretreated for 30mins with either fmlp (10^{-10}-10^{-6} M), LTB_4 (10^{-10}-10^{-6} M) or PAF (10^{-10}-10^{-6} M) there was a marked increase in the oxidative burst in response to this low dose of OZ (Figure 5.13). IL-8 (1pg/ml-10ng/ml) did not prime neutrophils to respond. Regrettably TNFα and IL-1 were not tested on this occasion.

Figure 5.10a Effect of fmlp on the oxidative burst of whole blood.



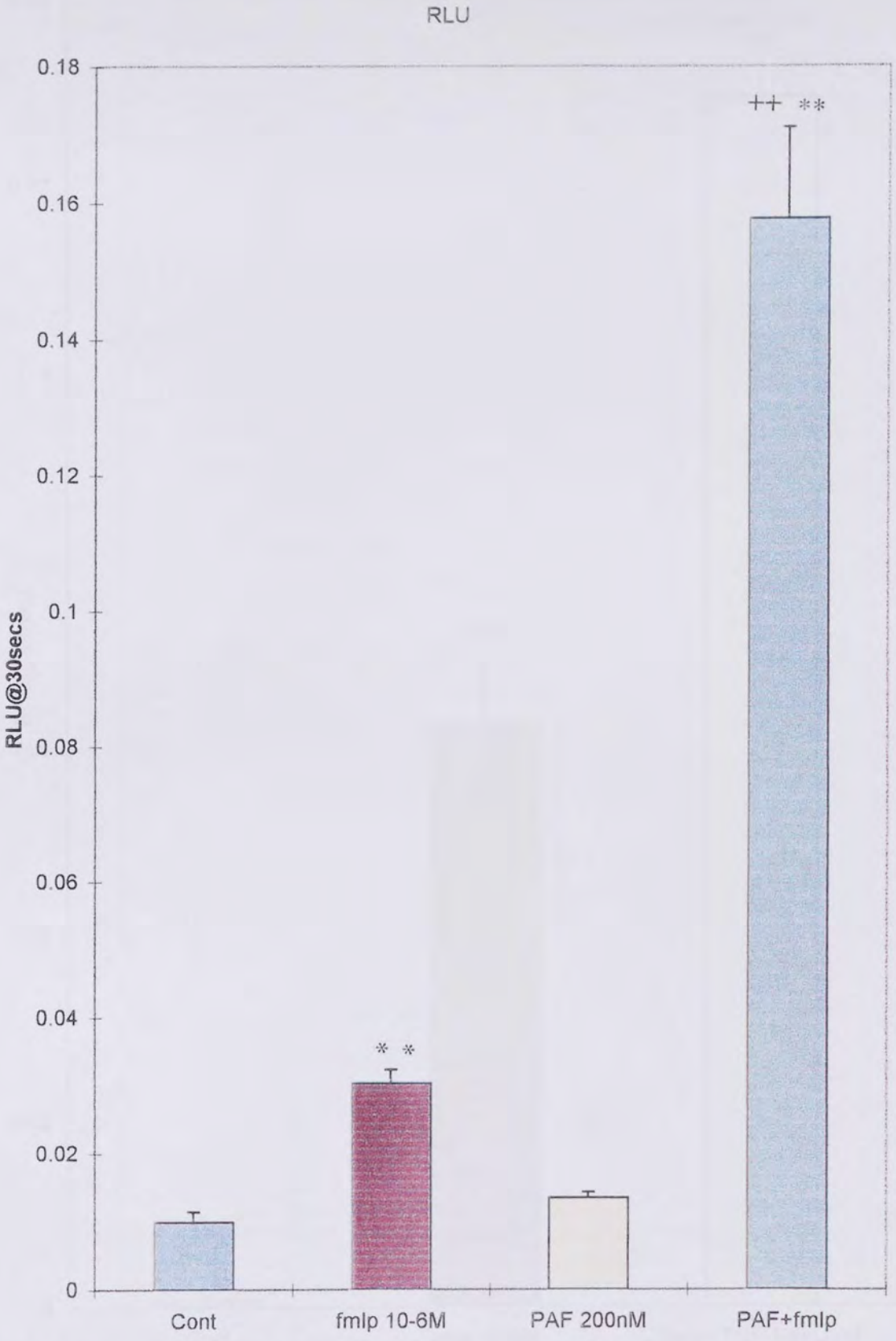
fmlp (10^{-6} M) was added to whole blood and the oxidative burst recorded after 30 secs. Values given are means +/- SEM where n=4 at each point. ** indicates a significant difference ($p<0.01$) from control using a Two-Way Anova.

Figure 5.10b Effect of PMA on the oxidative burst of whole blood.



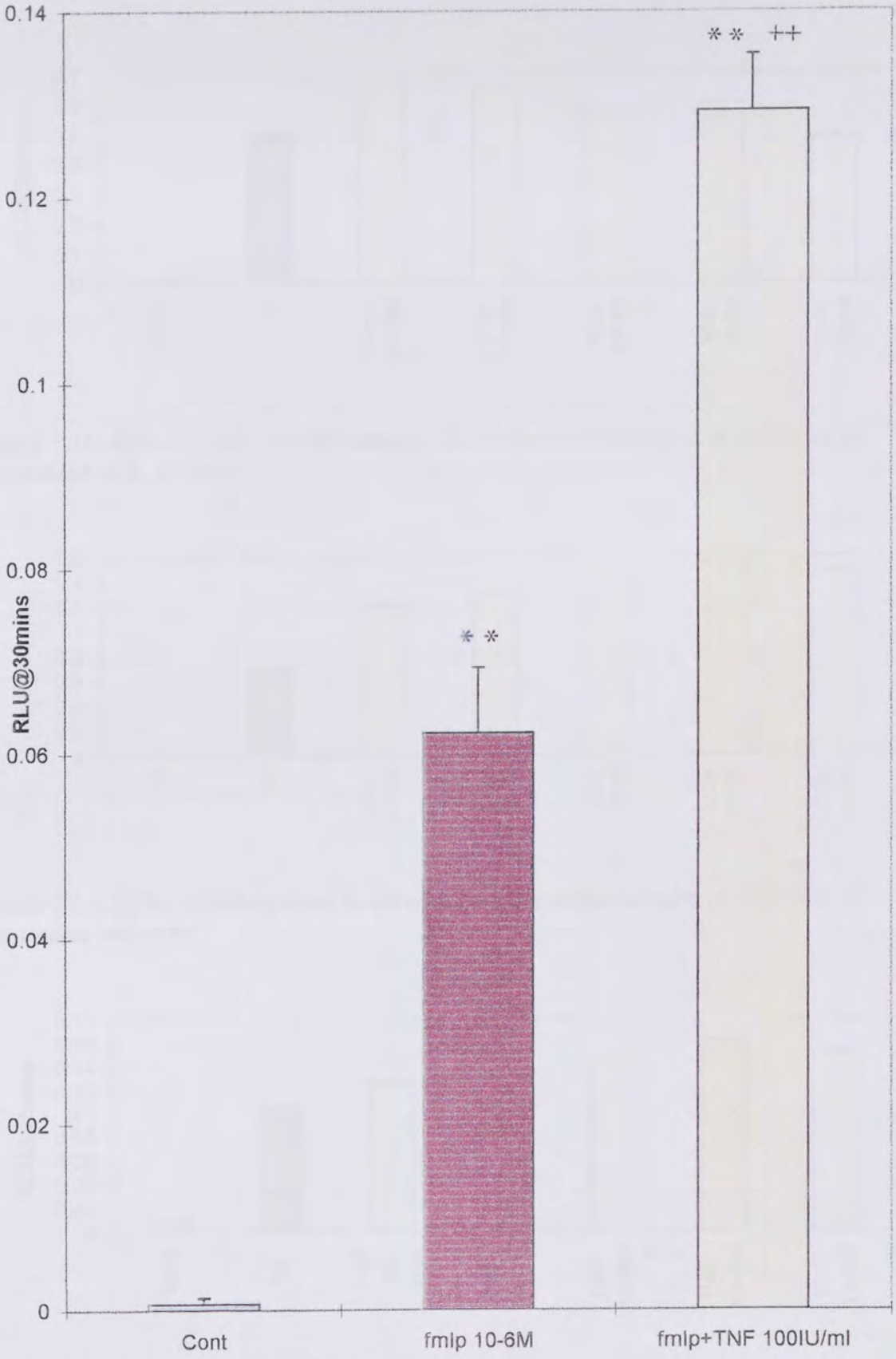
Varying concentrations of PMA were added to whole blood (100µl) and the oxidative burst recorded. Values given are means +/- SEM where n=4 at each point. ** indicates a significant difference ($p<0.01$) from control using a Two-Way Anova. In comparison but not shown here IL-1 (1-1000pg/ml), IL-8 (1-1000pg/ml), LTB_4 ($4.5x10^{-12}$ M-$3.6x10^{-8}$ M), PAF ($2x10^{-10}$ M- $2x\ 10^{-6}$ M), Plasmin (5µIU/ml-40µIU/ml) and TNFα (1IU/ml-1000IU/ml) were unable to produce an oxidative burst.

Figure 5.11 Effect of fmlp on whole blood pre-treated with PAF



Whole blood was pre-treated with PAF (20nM) for 30mins. Fmlp (10^{-6} M) was then added and the oxidative burst recorded. Values given are means +/- SEM where n=4 at each point. ** indicates a significant ($p<0.01$) difference from control while ++ indicates a significant ($p<0.01$) difference from PAF using a Two-Way Anova. In comparison IL-1 (100pg/ml), LTB_4 ($4.5x10^{-9}$M), Plasmin (20μIU/ml) and TNFα (100IU/ml) given after PAF pre-treatment did not increase the oxidative burst.

Figure 5.12 Effect of fmlp on whole blood pre-treated with TNFα



Whole blood was pre-treated with TNFα (100IU/ml) for 30mins. Fmlp (10^{-6} M) was then added and the oxidative burst recorded. Values given are means +/- SEM where n=4 at each point. ** indicates a significant ($p<0.01$) difference from control while ++ indicates a significant ($p<0.01$) difference from fmlp using a Two-Way Anova. In comparison pre-treatment with IL-1 (100pg/ml), IL-8 (100pg/ml)for 30mins or LTB_4 ($4.5x10^{-9}$M) for 5 or 30mins did not increase the oxidative burst of whole blood to fmlp.

Figure 5.13a Effect of sub-optimal doses of OZ on the oxidative burst of whole blood, pre-treated with fmlp.

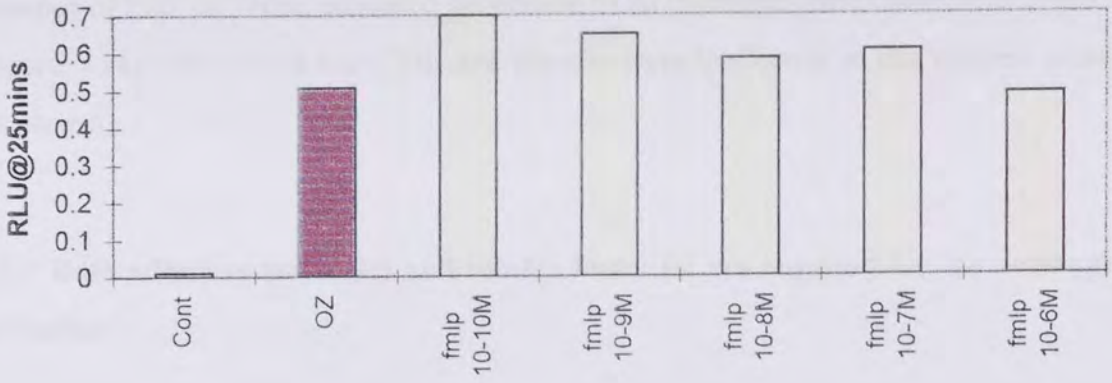


Figure 5.13b Effect of sub-optimal doses of OZ on the oxidative burst of whole blood, pre-treated with LTB4.

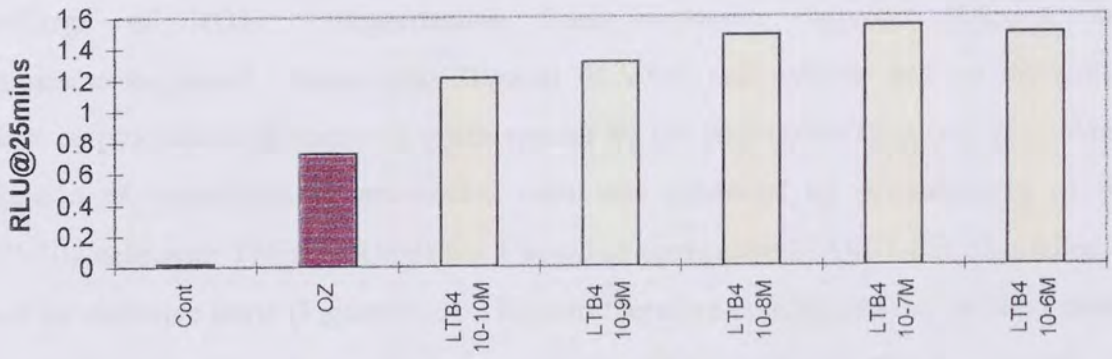
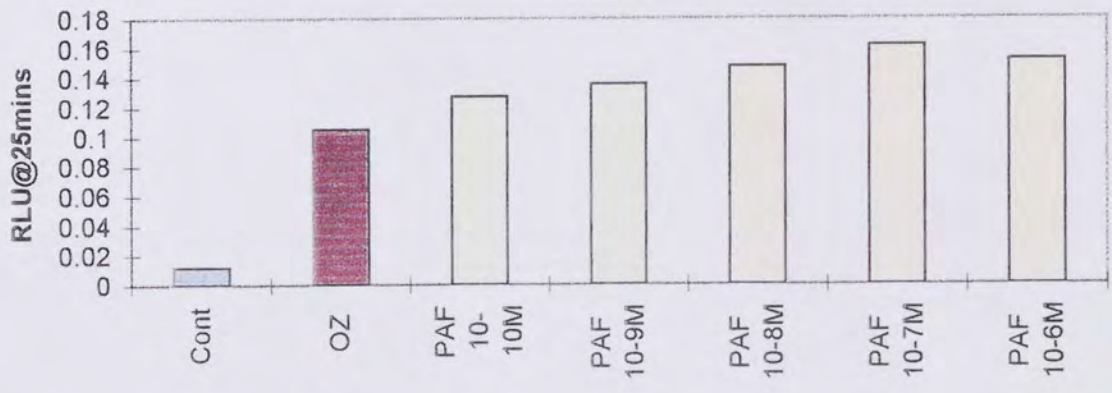


Figure 5.13c Effect of sub-optimal doses of OZ on the oxidative burst of whole blood, pre-treated with PAF.



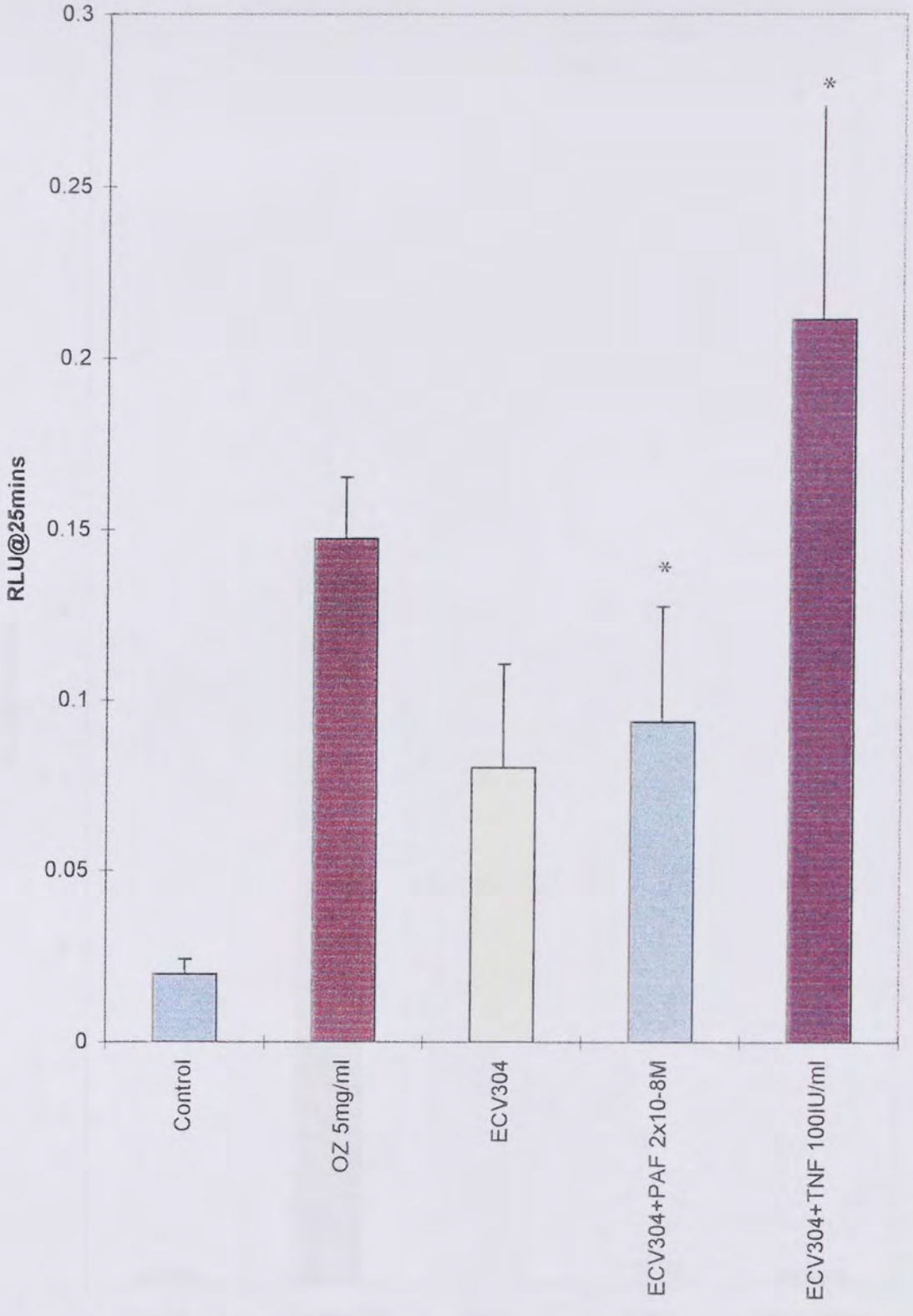
Whole blood was pre-treated with either fmlp, LTB_4 or PAF for 30mins and the oxidative burst to OZ (2.5mg/ml) recorded. Values givens are means where n=3 at each point. Not shown IL-8 (1-1000pg/ml) did not increase the oxidative burst further.

When cell-cell contacts between neutrophils and endothelial cells were possible by coincubating cytodex beads coated with ECV cells and whole blood the simultaneous presence of PAF or TNFα enhanced generation of ROS ensued particularly with TNFα (Figure 5.14). IL-1, IL-8 and LTB_4 and plasmin were ineffectual in this context (results not shown).

5.2.7 Both adhesion molecules and soluble factor (s) are required for the neutrophil activation

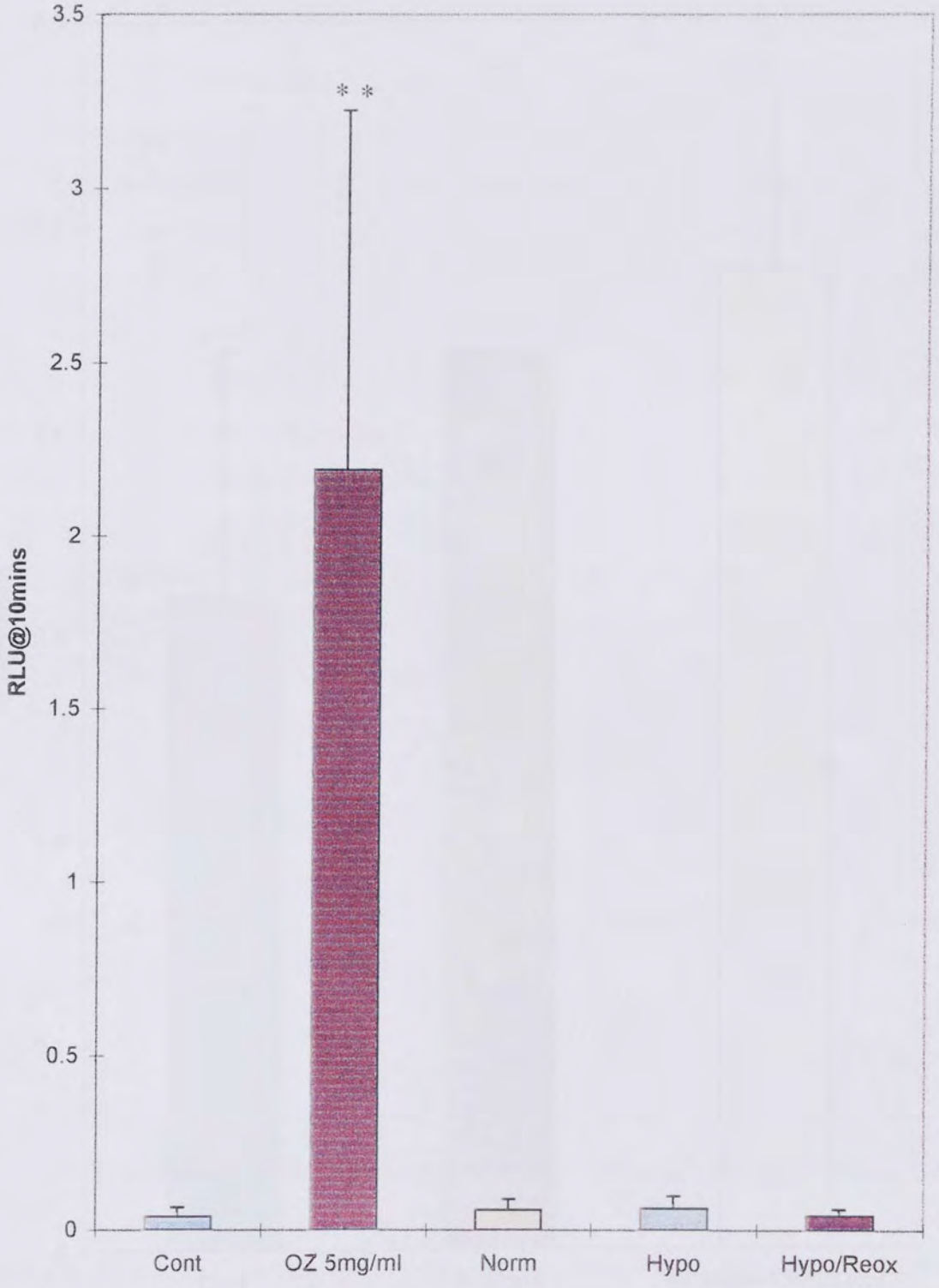
Thus it appears that some priming stimulus is required to prepare neutrophils to respond to a second signal which elicits an oxidative burst. Certain factors released into the supernatant from hypoxic endothelial cells are not in themselves sufficient to cause discharge of ROS. Supernatants from normoxic, hypoxic (4hours) and hypoxic/reoxygenated (4hours plus 30mins) ECV304 cell cultures had no discernible effect on production of reactive oxygen species by the neutrophils (Figure 5.15). When adhesion of neutrophils to endothelial cells was enhanced by pre-treatment of the ECV304 cells with TNFα (5IU/ml) for 4 hours to upregulate ICAM-1 this also failed to elicit an oxidative burst (Figure 5.16). We can therefore conclude in our *in vitro* model that both neutrophil adhesion and the presence of factor (s) in the supernatant are required for the enhanced neutrophil activation observed following hypoxia or hypoxia/reperfusion.

Figure 5.14 Effect of ECV304 cells and varying cytokines on the oxidative burst of whole blood.



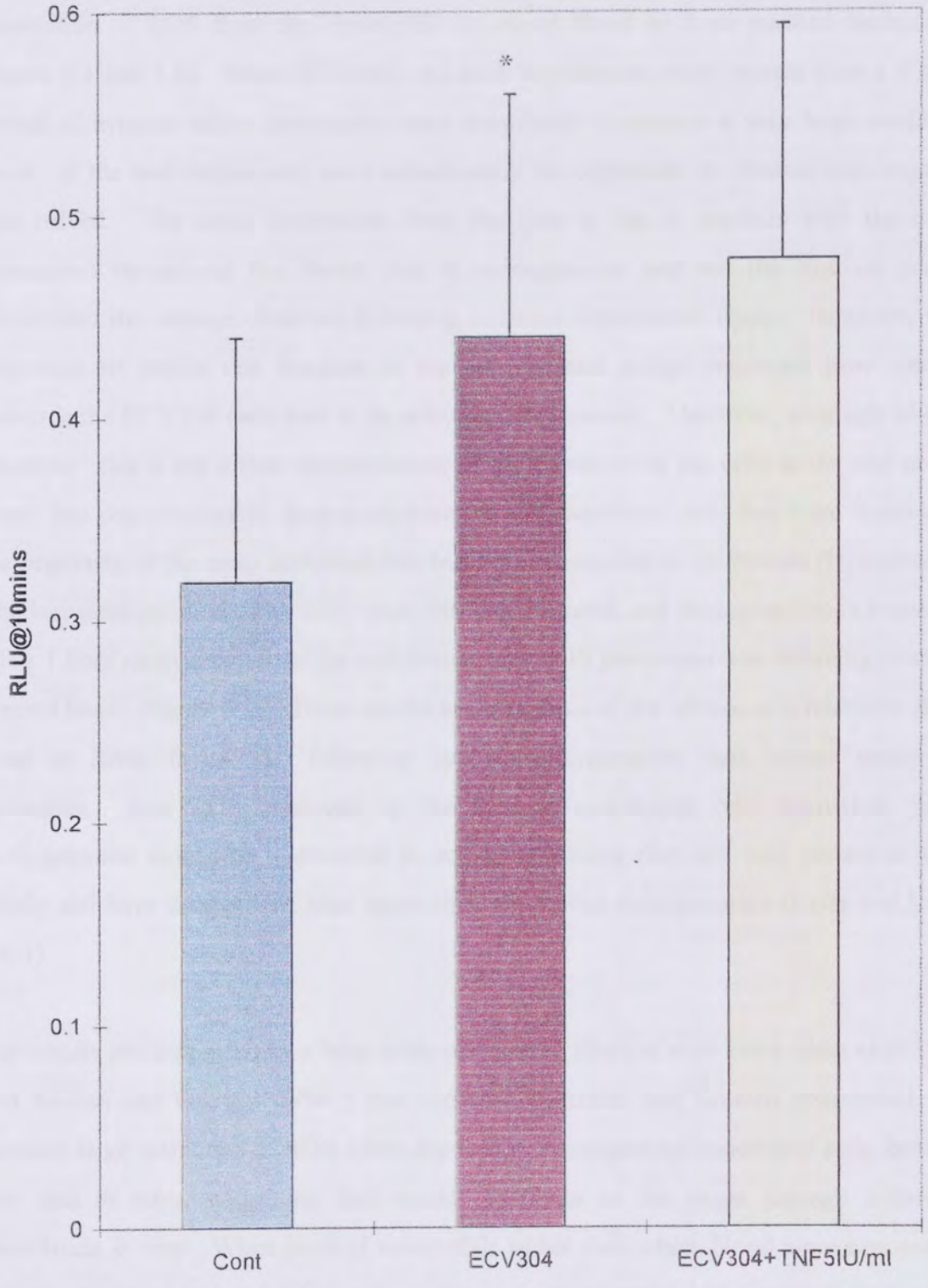
ECV304 cells ($5x10^5$/ml) grown of cytodex microcarriers were incubated with whole blood and either fmlp (10^{-8} M), IL-1 (100pg/ml), LTB_4 ($4.5x10^{-9}$ M), PAF ($2x10^{-8}$ M), TNFα (100IU/ml), IL-8 (100pg/ml) or plasmin (20μIU/ml). OZ (5mg/ml) is shown as a positive control. Values given are means +/- SEM where n=4 at each point. * indicates a significant ($p<0.05$) from ECV304 alone using a Two-Way Anova. The other factors were unable to elicit a response.

Figure 5.15 The effect of the supernatant from ECV304 cells on the oxidative burst of isolated neutrophils.



Isolated neutrophils ($5x10^6$) were incubated with the supernatant from ECV304 cells ($5x10^6$/ml) grown on cytodex microcarriers that had either been made normoxic for 4 hours, hypoxic for 4 hours or hypoxic for 4 hours and reoxygenated for 30mins. Values given are means +/- SEM where n=4 at each point. ** indicates a significant ($p<0.01$) from control using a Two-Way Anova.

Figure 5.16 Effect of upregulating ICAM-1 on ECV304 cells and their ability to produce an oxidative burst from isolated neutrophils



ECV304 ($5x10^6$)grown on cytodex microcarriers were pre-treated with TNFα (5IU/ml) for 4 hours to upregulate ICAM-1 before the addition of isolated neutrophils ($5x10^6$/ml). Values given are means +/- SEM where n=4 at each point. * indicates a significant ($p<0.05$) from control using a Two-Way Anova.

5.3 Discussion

This chapter illustrates conclusively that in our *in vitro* model endothelial cells and/or factor (s) produced by them following hypoxia/reoxygenation are capable of eliciting the production of ROS from the neutrophils in whole blood or from purified neutrophils (figure 5.1 and 5.3). When ECV cells and their supernatants were present after a 4 hour period of hypoxia added neutrophils were stimulated to produce a very large oxidative burst. If the endothelial cells were subsequently reoxygenated for 30mins this response was halved. The initial impression from this data is that it conflicts with the trend concurrent throughout this thesis; that is reoxygenation and not the hypoxic period potentiates the damage observed following ischaemic/reperfusion injury. However, it is important to realize that because of the experimental design employed from time 0 onwards the ECV304 cells start to be actively reoxygenated. Therefore, although labeled "hypoxic" this is not a true representation of the condition of the cells at the end of the assay, but was required to distinguish between the endothelial cells that were hypoxic at the beginning of the assay and those that had been reoxygenated for 30mins (Hypo/Reox). The increased production of ROS seen following hypoxia and reoxygenation is transient. After 1 hour reoxygenation of the endothelial cells ROS generation was returning towards control levels (Figure 5.2). These results are indicative of the release of a relatively short lived or labile factor (s), following hypoxia/reoxygenation, that causes neutrophil activation. Any ROS produced by the hypoxic endothelial cells themselves upon reoxygenation would be undetected in our system since they are only produced very briefly and have disappeared after approximately 3 mins reoxygenation (Lefer and Lefer 1993).

Our results are supported by a large body of research (Burton *et al* 1984, Hess *et al* 1984 and Kurose and Granger 1994) that certainly illustrates that isolated neutrophils will produce large quantities of ROS when exposed to reoxygenated endothelial cells, both *in vivo* and *in vitro*, suggesting they could contribute to the tissue damage following reperfusion *in vivo*. When purified neutrophils rather than whole blood were exposed to the hypoxic/reoxygenated ECV cells a much more powerful burst was elicited when responses were compared on a cell for cell basis. This may be because our isolation technique partially primed the neutrophils even though they appeared quiescent (see Chapter 2). Also the ECV304 cells may also have released a priming agent that binds

non-specifically to the excess red blood cells or the red blood cells may simply quench some of the light produced. Winterbourn and Stern 1987 demonstrated that erythrocytes are excellent scavengers of neutrophil derived reactive oxygen species.

When ECV cells were pre-treated with the xanthine oxidase inhibitor allopurinol (Varni and Ward 1994) before and during the hypoxic period the response of added neutrophils was much attenuated (Figure 5.4). Therefore, it is reasonable to conclude that the neutrophil's oxidative burst following 4 hours hypoxia is certainly the consequence of reoxygenation, rather than hypoxia. This confirms similar findings by Phan *et al* (1989) who showed that by blocking endothelial xanthine oxidase with allopurinol they were able to prevent stimulated neutrophil killing of endothelial cells by 61% suggesting a direct link between the release of ROS from the endothelium and tissue damage (as discussed in Chapter 4 Section 4.3)

It has been suggested that PAF release from endothelial cells post ischaemia is a consequence of ROS production and that it may promote adhesive interaction between circulating neutrophils and endothelium in ischaemia/reperfusion injury (Lewis *et al* 1988 and Kubes *et al* 1990). Our own results (Chapter 3, Figure 3.11 and Chapter 4, Figure 4.6) have shown that the use of a PAF secretion antagonist indeed attenuates neutrophil adhesion and migration post hypoxia. We therefore investigated what effect blocking PAF secretion would have on the neutrophil's ability to produce an oxidative burst (Figure 5.5). Pre treatment lowered the neutrophils ability to generate an oxidative burst following hypoxia and hypoxia/reoxygention. This result is supported by Yin *et al* (1995) who also found, *in vitro*, that the use of a PAF receptor antagonist decreased endothelial injury. From these results it is possible to conclude that PAF is involved in the neutrophil activation associated with hypoxia and reoxygention in our system. As such the use of PAF antagonists *in vivo* could be a potential treatment for ischaemic/reperfusion injury.

Another possible treatment for ischaemic/reperfusion injury could be dapsone, the drug of choice in the treatment of dermatitis herpetiformis and other similar inflammatory conditions which are characterized by neutrophil infiltration (Zone 1991 and Uetrecht 1992). Our results so far (Chapter 3, Figure 3.14 and Chapter 4, Figure 4.7) have shown that dapsone inhibits neutrophil adhesion and chemotaxis. Kettle and Winterbottom (1991), van Zyl *et al* (1991), Booth *et al* (1992), Thuong-Nguyen *et al* (1993) and Wozel and Lehmann (1995) have also been able to illustrate that dapsone attenuates neutrophil

adhesion, chemotaxis, lipoxygenase activity and the cells ability to generate ROS. We therefore investigated the ability of dapsone to inhibit the oxidative burst of neutrophils to OZ (Figure 5.6) and therefore its potential to prevent tissue damage following ischaemia/reoxygenation (Figure 5.7). Pre-treatment of whole blood with dapsone levels as low as 0.0005mM, for 20mins, reduced their ability to respond to OZ stimulation.

Currently dapsone's mode of action is not understood though it has been suggested that it could work by scavenging ROS. Kettle and Winterbottom (1991) proposed that dapsone inhibits HOCl production by trapping MPO as an inactive compound. MPO catalyses the oxidation of Cl- by H_2O_2 to yield HOCl. To determine whether dapsone is scavenging the products of neutrophil activation, so they are not detected in our system, or truly affecting neutrophil function we therefore modified the previous experiment by not pre-treating with dapsone but starting the burst off and adding it after 10mins(Figure 5.8). If dapsone is working by scavenging ROS a dramatic reduction in RLU should be seen at 15mins. A very slight reduction, however, was only seen using large amounts of dapsone (1mM). Furthermore dapsone was unable to prevent H_2O_2 directly oxidizing lucigenin (Figure 5.9). Consequently we concluded that dapsone's actions at high concentrations (1mM), may be due to its ability to scavenge some ROS, but not H_2O_2. It is unlikely that such a high concentration could be achieved *in vivo* without causing gross adverse events. Therefore *in vivo* dapsone could prevent neutrophil activation directly. As such subsequent studies need to be carried out to investigate what effect dapsone has on the oxidative burst elicited by neutrophils exposed to hypoxic/reoxygenated ECV cells. Even so our results suggest that dapsone may be a possible treatment for ischaemic/reperfusion injury through its ability to prevent neutrophil adhesion, migration and activation. However, in our activation assays we were unable to reduce the oxidative burst to anywhere near control levels so dapsone may best be used in conjunction with other therapies.

Having established that neutrophil activation occurs when exposed to ECV304 cells subjected to hypoxia/reoxygenation we then attempted to ascertain if factor (s) present in the supernatant could be responsible. Initial screening of a variety of implicated factors and known stimulators (Figure 5.10) showed that only fmlp and PMA will directly activate whole blood. These findings are concurrent with published literature (Dewald and Baggiolini 1985, Gay 1993, Wozniak *et al* 1993 and Franciose *et al* 1996) which has shown that fmlp and PMA will directly activate neutrophils.. Fmlp is the chemotactic

factor produced by *E coli* that causes superoxide anion production (O_2-) (Ferretti *et al* 1994). PMA is a receptor-independent activator which enters the cell independently as a DAG analogue and directly activates PKC. PMA activation of neutrophils results in the assembly and activation of NADPH oxidase. In contrast fmlp is a receptor dependent activator of neutrophil NADPH oxidase and functions via a G-protein linked chemotactic receptor (Patrick *et al* 1996). Although fmlp and PMA are not present in our ECV/neutrophil system they show that different activators have different mechanisms and time courses of activation. From these results it looks as if the neutrophil pathway activated by the ECV cell's resembles that triggered by PMA or OZ rather than the rapidly inducible response triggered by fmlp.

It is possible that because the ECV response is slow, cell to cell contact may occur with other factors priming the response. Of the many factor (s) suggested we have found that PAF and TNFα will potentiate priming to both the protracted and rapid activation signals, OZ and fmlp respectively, while LTB_4 will only amplify the response to OZ (Figures 5.11--5.14). Walker and Ward (1992) and Fletcher and Halpern (1990) have also shown that LTB_4 is able to prime neutrophils.

Certainly PAF has also been implicated by many other studies (Dewald and Baggiolini 1985, Gay 1993 and Franciose *et al* 1996). Reperfused gut certainly elaborates PAF that attracts and then primes neutrophils for O_2- generation (Kim *et al* 1995). Small amounts of PAF are known to prime (Vercellotti *et al* 1988) while this effect can be inhibited by a PAF receptor antagonists. Although a relatively weak activating agent PAF is a potent priming agent (Patrick *et al* 1996). PAF can prime neutrophils by a variety of potential mechanisms, either directly by PLA_2 activation or by promoting the production of TNFα and CINC (Serizawa *et al* 1996). In contrast the data surrounding TNFα is far from conclusive.

TNF acts via two cell-surface receptors; the p55-TNF receptor (TNF-R55) and the p75-TNF receptor (TNF-R75). Both TNF-R55 and TNF-R75 are involved in the TNF-induced activation of the respiratory burst (Richter *et al* 1995). Our results illustrate conclusively that TNFα will prime neutrophils to fmlp.(Figure 5.12). This result is supported by Dularay *et al* (1990), and Barnes *et al* (1995) who all found that TNFα enhances neutrophil stimulus-induced MPO release. However, in direct conflict to these

findings Sample and Czuprynski (1991) found that TNFα alone will induce bovine neutrophils to produce an oxidative burst. Earlier studies by Larrick *et al* (1987) also showed that TNFα will directly cause neutrophil activation while Shalaby *et al* (1985) found that TNFα will induce neutrophils to phagocytosis.

These inconsistencies may be due to differences in the methodology employed, particularly in the techniques used for neutrophil isolation which may themselves result in partial activation or priming. Both Sample and Czuprynski (1991) and Larrick *et al* (1985) isolated their neutrophils by lysing the red blood cells and using dextran density gradients. We have been able show that dextran itself will prime neutrophils to respond to stimuli (Chapter 2, Figure 2.2). Therefore, in the earlier studies the investigators were unknowingly priming their neutrophils to respond to TNFα. However, if great care is taken in the isolation procedures TNFα will prime to additional signals but not activate, as our results show.

Walker and Ward (1992) divide the priming agents PAF, LTB_4, fmlp, IL-8, TNFα and IL-1 into two main classes. Firstly those that are receptor-dependent and achieve priming within 1-2mins by increasing intracellular calcium (PAF, LTB_4, fmlp and IL-8). These priming agents will cause neutrophil activation directly at high enough concentrations. Secondly TNFα and IL-1 which are receptor-dependent and take 15-30mins preincubation to elicit a primed state that remains for 15mins. This second type do not increase intracellular calcium. Our findings challenge some of these classifications.

We have not been able to show priming by IL-1 or IL-8. Bailey *et al* (1994) in support of our findings also conclude that IL-1 is unable to prime neutrophils. However, Sample and Czuprynski (1991) found it could cause neutrophil activation directly. Again I feel the neutrophil isolation technique is at fault as Sample and Czuprynski (1991) also showed that TNFα will directly activate neutrophils in contrast to many other studies. Similar complexities surround neutrophil activation by IL-8. In our studies we have not been able to demonstrate any direct activation or priming effect by IL-8. These results are supported by Wozniak *et al* (1993) who found that IL-8 will not produce a burst directly and Djeu *et al* (1990) who illustrated that IL-8, from 1-1000ng/ml, enhanced neutrophil-mediated anti-*Candida* activity though it failed to stimulate them directly to produce superoxide or prime the respiratory burst of neutrophils exposed to fmlp. However,

Wozanick *et al* (1993) did find that IL-8 primed neutrophils to respond to fmlp, PMA and PAF while Elbim *et al* (1994) showed that IL-8 primes to fmlp.

Again the conflicts between these results may be a consequence of the neutrophil isolation techniques employed. Another explanation for these findings could also be the relative instability and difficulties associated with storing IL-8. We prepared, stored and used the IL-8 as recommended by NIBSC. However, it must be noted that IL-8 did promote neutrophil adhesion (Chapter 3, Figure 3.16) in our hands, and was therefore deemed to be biologically active. Consequently, we can only conclude that in our model IL-8 does not cause neutrophil activation or priming at the concentrations used, as measured by the release of ROS.

To conclude this set of experiments whole blood was added to IL-1, IL-8, TNFα, LTB_4 and PAF in the presence of endothelial cells, to see if it was possible to prime whole blood to respond to ECV304 cells (Figure 5.15). Under these circumstances we found that both PAF and TNFα will again increase the burst. Liles *et al* (1995) also found that CD18 cross-linking primes neutrophils to activate the respiratory burst after stimulation with TNFα. It is likely therefore that the presence of TNFα and/or PAF could provoke neutrophil activation following ischaemic/reperfusion injury. This result is also consistent with our findings that the use of a PAF secretion antagonist attenuates activation (Figure 5.5).

Finally we set out to determine if factor (s) released from hypoxic/reoxygenated ECV cells or adhesion molecules on their cell surface were provoking neutrophil activation, as up to this point we have been unable to directly activate the neutrophils with any of the implicated molecules. Our results show conclusively that both are required (Figure 5.16 and 5.17). Assays carried out in parallel showed that the supernatant from hypoxic/reoxygenated ECV304 cells will not cause activation, though they will still provoke neutrophil migration, and likewise upregulating adhesion molecules will not cause activation, but will still increase neutrophil adhesion. This points towards a dual signal hypothesis. Neutrophil priming, through the presence of factor (s) in the supernatant, and adhesion are required to provoke the respiratory burst seen in our system.

We have been able to show that *in vitro* activation does occur following hypoxia/reoxygenation. This can be attenuated by the use of allopurinol, a PAF secretion antagonist and possibly dapsone. Furthermore not only is the presence of factor (s) vital in the supernatant but also neutrophil adhesion, either as a priming or activating signal, is required to cause the oxidative burst. Our studies thus far have implicated PAF, TNFα and LTB_4 as causative agents in this activation though preliminary findings and a more detailed analysis of the supernatants may highlight other or unknown factors. What is evident at this point is that a complex signal transduction mechanism is in play during neutrophil activation and it is unlikely that any one factor is exclusively involved.

6 HL60 CELLS

6.1 Introduction

The previous result chapters have shown that neutrophil adherence to ECV304 cells is significantly increased following hypoxia and reoxygenation (Chapter 3, Figure 3.3). Furthermore isolated neutrophils will preferentially migrate into the supernatant from hypoxic/reoxygenated ECV304 cells (Chapter 4, Figure 4.4) and the presence of factor (s) in this supernatant, along with adhesion molecules on the ECV304 cell surface, will cause the neutrophil to produce an oxidative burst (Chapter 5, Figure 5.1). In this chapter we have explored whether by using a neutrophil like cell line (HL60), in our *in vitro* models, we could obtain similar results. A positive result would not only strengthen the case for our model being representative of the situation *in vivo* but it would also provide us with a reliable alternative model.

The HL60 cell line was established in 1977 from a patient with acute myeloid leukemia (Collins *et al* 1977). HL60 cultures comprise maturation-arrested cells many of whose properties are similar to those of myeloblasts and promyelocytes. In all cultures of HL60 cells, however, the block in differentiation is spontaneously overcome in a small proportion of cells (5-10%) which display morphological and other characteristics of more mature cells (Fontana *et al* 1981 and Collins 1987). Some reagents cause HL60 cells to differentiate to granulocyte-like cells, others to monocyte/macrophage-like cells. Dimethyl sulphoxide (DMSO), and other compounds as diverse as retinoic acid and actinomycin D, all induce differentiation to granulocyes, while 1,25 dihydroxy-vitamin D3, phorbol esters and sodium butyrate induce monocyte/macrophage differentiation (Brietman *et al* 1980, Fibach *et al* 1982).

The course of the differentiation induced by any of these compounds is accompanied by a large number of changes in the cells (Tsiftsoglou and Robinson 1985 and Collins 1987) and is easily monitored by morphological, histochemical and immunological criteria. Thus, incubation with DMSO or retinoic acid leads, over a period of five days, to a progressive decrease in the size of HL60 cells and condensation of nuclear material with the appearance of kidney-shaped nuclei characteristic of the myelocyte and, later, lobed

nuclei characteristic of banded and segmented nuclei. The nuclear/cytoplasmic ratio decreases and the cytoplasm becomes more diffuse. In parallel with these changes there occur marked changes in histochemistry and the ability to reduce nitroblue tetrazolium, a marker of granulocyte function (Collins 1987). A large number of changes in cell surface antigens can also be detected, (Graham *et al* 1985) all of them characteristic of increased maturity of the cells.

As expected, the marked morphological and other changes seen when HL60 cells are induced to differentiate are reflections of large qualitative and quantitative changes in the relative abundances of many mRNAs. Several lines of evidence suggest that the protein encoded by *c-myc* is intimately involved in controlling gene expression, in particular , some of the genes that regulate differentiation and proliferation (Land *et al* 1983, Kohl and Ruley 1987). The HL60 cell genome contains amplified *c-myc* proto-onocogene; *c-myc* mRNA levels are also correspondingly high in undifferentiated cells but decline rapidly following induction of differentiation (Collins 1987).

Until now the HL60 cell line has proved useful for studies looking at the nature and control of changes that occur when immature cells differentiate. Therefore, it will be fascinating to see how they behave in our models.

6.2 Results

6.2.1 HL60 cells contain myeloperoxidase

HL60 cells cultured with DMSO (1.25%) for 5 days to promote differentiation contained significantly measurable amounts of MPO (Figure 6.1). The enzyme content was directly proportional to cell numbers. Thus this method could be used to assess numbers of adherent or migrating cells as for our freshly isolated neutrophils (Chapter 2, Method 2.5.2).

6.2.2 TNFα treatment of ECV304 cells increases HL60 cell adhesion

When $1x10^6$ cells/ml were added to confluent ECV304 cells 26.2 +/- 14.3% stuck (Figure 6.2). After up regulation of adhesion molecules on the endothelial cell surface with TNFα (5IU/ml) this significantly increased ($p<0.05$) to 40.9 +/- 13.6%. This result was similar to the response seen using freshly isolated neutrophils (Chapter 3, Figure 3.2) so the TNFα treatment was therefore used as a positive control throughout.

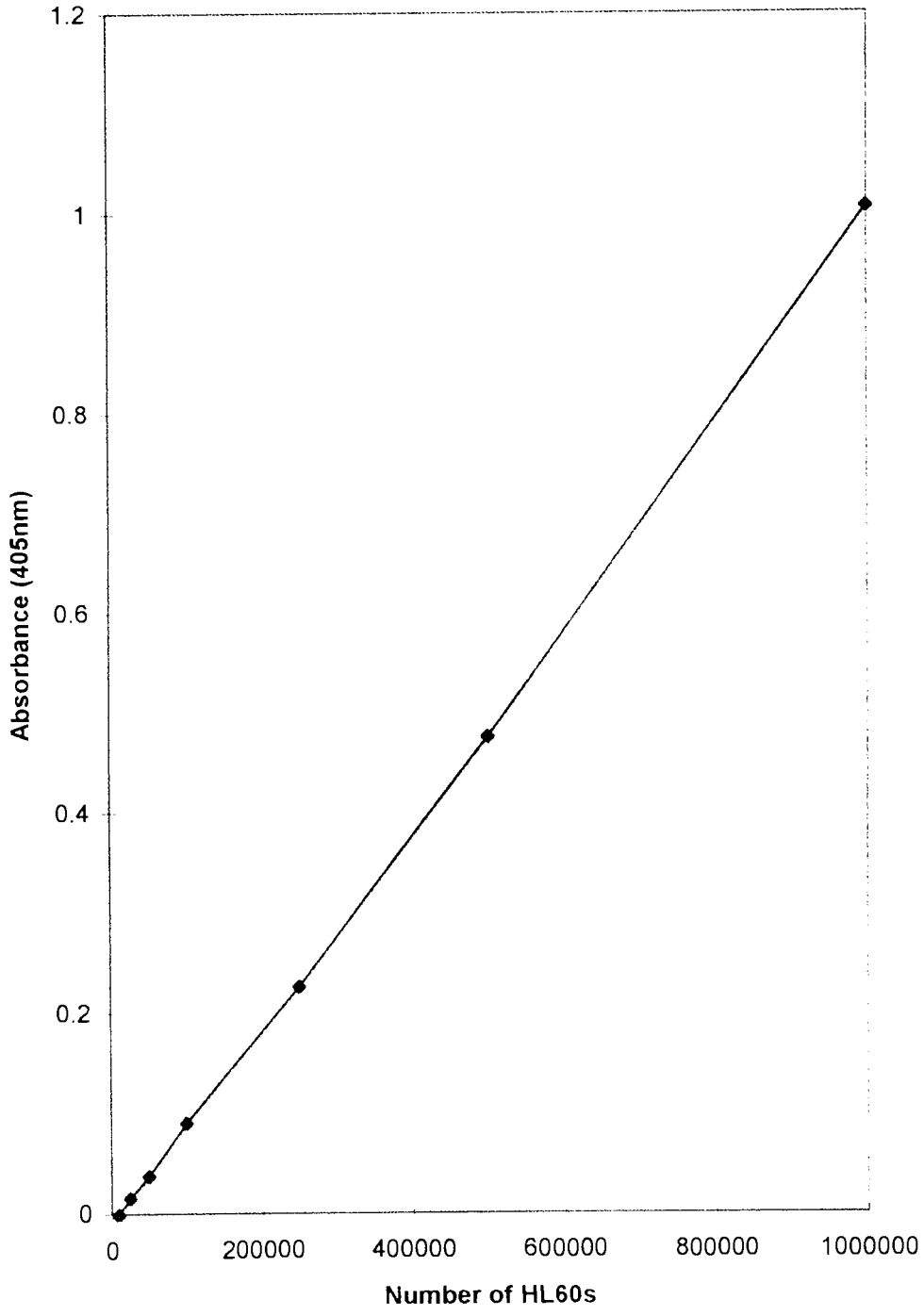
6.2.3 Hypoxia and reoxygenation causes increased HL60 cell adhesion to ECV304 cells

If ECV304 cells were subjected to 4 hours hypoxia and 30mins reoxygenation a significant increase in HL60 adhesion was noted ($p<0.05$) once again demonstrating that HL60 were behaving as neutrophil-like adhesive cells in this assay (Figure 3.3).

6.2.4 HIZAS is a HL60 cell chemoattractant

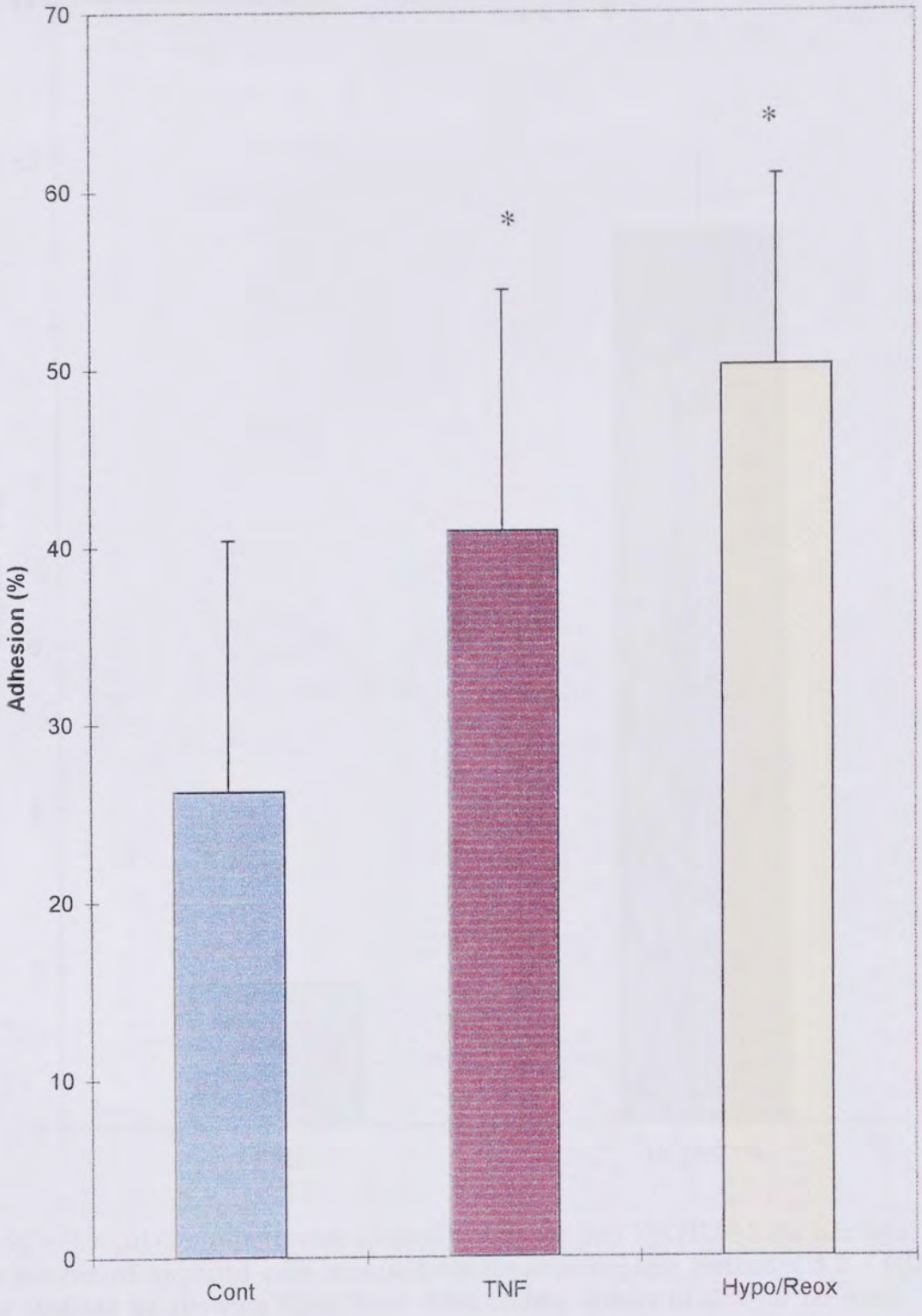
HL60 cells were placed in a migration chamber and allowed to migrate towards calcium- and magnesium-free PBS or 1% HIZAS for 1 hour. Figure 6.3 demonstrates that 1.8 +/- 0.3% HL60 cells migrated towards PBS while in comparison 11.1 +/- 1.0% migrated into HIZAS producing a significant difference ($p<0.05$).

Figure 6.1 Assessment of HL60 numbers by the myeloperoxidase technique



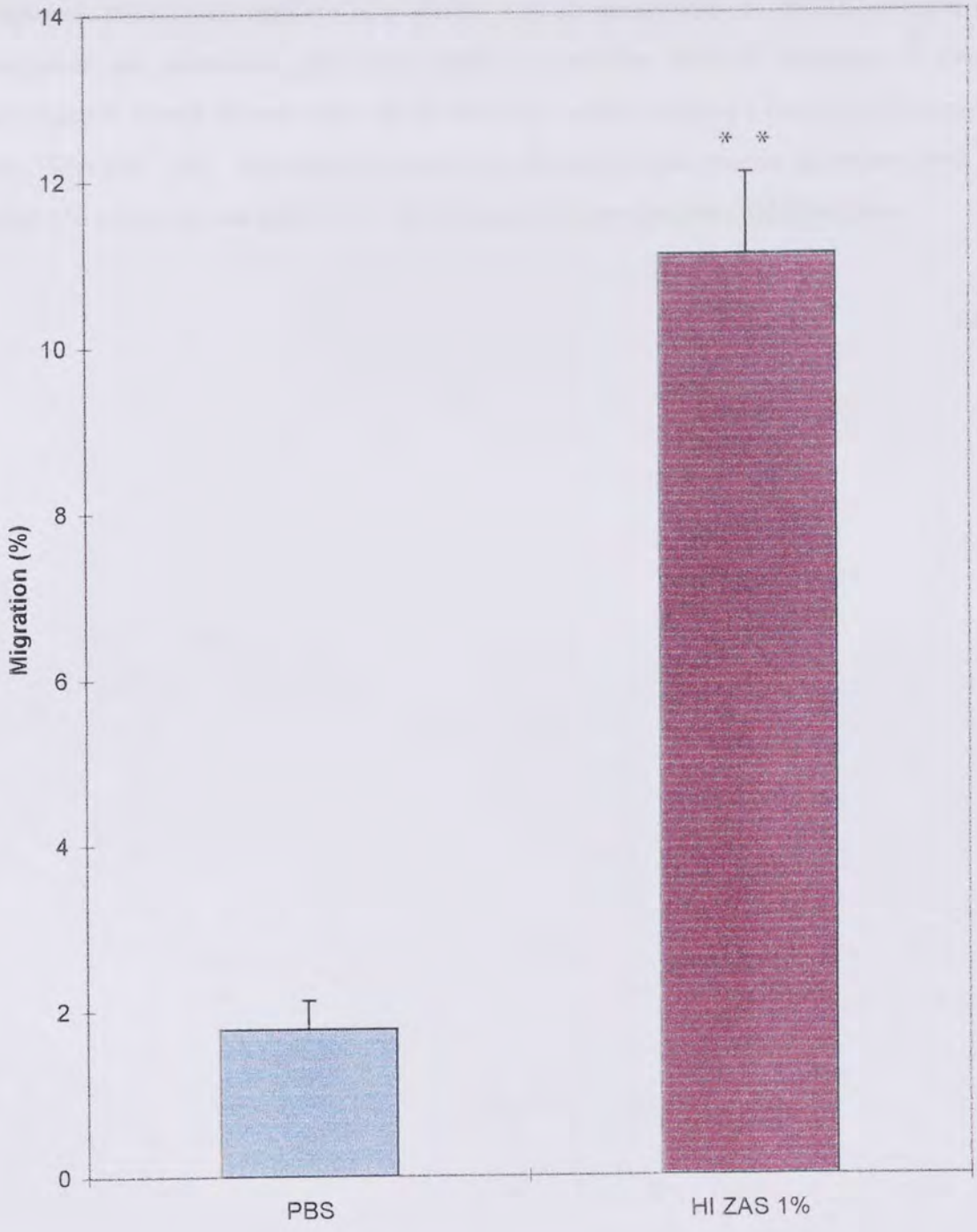
Known numbers of HL60's were added to wells to assess their myeloperoxidase content as in section 2.5.2. Values given are means where n=3 at each point.

Figure 6.2 Effect of hypoxia and reoxygenation of endothelial cells on HL60 adhesion.



HL60 adhesion (100µl) (1x106 cells per ml) to ECV304 cells made hypoxic for 4 hours and reoxygenated for 30 mins. Values are means +/- SEM where n=4. * indicates a significant difference ($p<0.05$) from normoxic using a Two Way Anova.

Figure 6.3 Effect of heat inactivated zymosan (HIZAS) on HL60 migration.

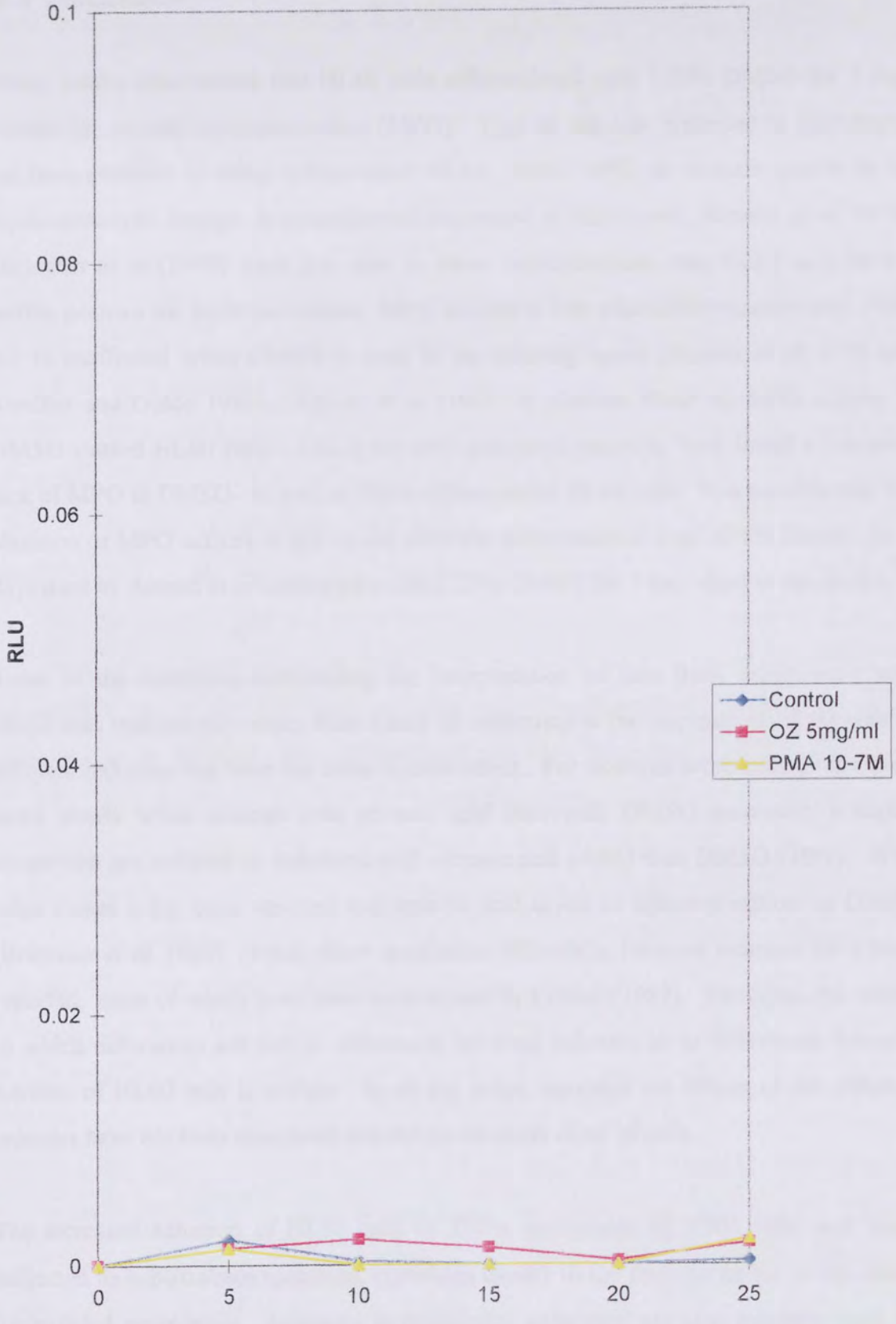


HL60's (200µl) ($2x10^6$/ml) were allowed to migrate into 1% HIZAS for one hour and the number of migrated cells assessed via myeloperoxidase method 2.5.2. HIZAS was obtained by allowing 10ml blood from healthy donors to clot for 30 mins. The red blood cells were then removed by spinning at 3000rpm for 30mins and the resulting serum incubated with zymosan (5mg/ml) for 20mins at 37°C. Once the zymosan was removed the zymosan activated serum was then incubated at 57°C for 30mins to heat inactivate. A 1% solution of the serum was then prepared in calcium and magnesium free PBS. Values givens are means +/- SEM where n=4 at each point. ** indicates a significant difference ($p<0.01$) from PBS.

6.2.5 HL60 cells differentiated with 1.25% DMSO will not produce an oxidative burst

Although HL60 cells perform in a similar way to neutrophils, as far as adhesion and migration are concerned, they are unable to generate ROS in response to known provocative stimuli (Figure 6.4) HL60 cells will neither produce a burst to OZ (5mg/ml) nor PMA (10^{-7} M). Although not shown in Figure 6.4 neutrophils stimulated with the same OZ (5mg/ml) and PMA (10^{-7} M) in tandem did produce an oxidative burst.

Figure 6.4 The effect of Opsonized Zymosan (OZ) and PMA on HL60 activation



HL60's were incubated with OZ (5 mg/ml) and PMA (10-6M) for 25 mins and activation, as assessed by the oxidative burst, measured. OZ was prepared by incubating guinea pig complement with zymosan (5mg/ml) for 20mins. The complement was then removed and a 5mg/ml solution of the opsonized zymosan in unsupplemented medium prepared. Values given are means where n=3.

6.3 Discussion

These results demonstrate that HL60 cells differentiated with 1.25% DMSO for 5 days contain the enzyme myeloperoxidase (MPO). Thus all the data presented in this chapter has been obtained by using differentiated HL60. Infact MPO, an enzyme specific to the myelomonocytic lineage, is constitutively expressed in HL60 cells (Rovera *et al* 1979). Gallagher *et al* (1979) were also able to show histochemically that HL60 cells have a profile positive for myeloperoxidase. MPO activity is lost after differentiation with PMA but is unaffected when DMSO is used as an inducing agent (Rovera *et al* 1979 and Koeffler and Golde 1980). Ahmed *et al* (1991) in contrast found no MPO activity in DMSO treated HL60 cells. This is the only published report to have found a complete lack of MPO in DMSO- as well as PMA-differentiated HL60 cells. It is possible that the abolition of MPO activity is due to the different differentiation dose of 1% DMSO for 6 days used by Ahmed *et al* compared to the 1.25% DMSO for 5 days used in our studies.

Some of the confusion surrounding the interpretation of data from experiments with HL60 cells undoubtedly arises from a lack of uniformity in the response of HL60 cells to different inducers that have the same overall effect. For example some cells differentiate more slowly when induced with retinoic acid than with DMSO moreover, a higher proportion are resistant to induction with retinoic acid (40%) than DMSO (10%). With other clones it has been reported that retinoic acid is just as effective inducer as DMSO (Brietman *et al* 1980). Many other qualitative differences between inducers have been reported, some of which have been summarized by Collins (1987). However, the extent to which differences are due to differences between inducers or to differences between sublines of HL60 cells is unclear. In all but a few instances the effects of the different inducers have not been compared directly on the same clone of cells.

The increased adhesion of HL60 cells to TNFα pre-treated ECV304 cells, and those subjected to hypoxia/reoxygenation, correlates closely to the findings earlier in this thesis for isolated neutrophils. Increases in endothelial adherence are also regularly seen on HL60 maturation (Collins *et al* 1979). The migration of differentiated HL60 cells into HIZAS (1%) was also comparable to that of isolated neutrophils. HL60 cells constitutively possess few receptors for C3b but an increase in expression of receptors for

C3b has been demonstrated following DMSO treatment (Novogrodsky *et al* 1983 and Harris and Ralph 1985). This could account for the increased migration we observed.

In contrast to these results differentiated HL60 cells, unlike neutrophils, did not produce an oxidative burst to PMA or OZ in this study. Phagocytosis of latex beads or opsonized yeast and the capacity to kill microorganisms is an inducible function in myelomonocytic cell lines like HL60. Collins *et al* (1979) and Harris and Ralph (1985) have both shown that the neutrophilic end products of HL60 cell differentiation acquire the ability to form ROS, as determined by the superoxide dismutase inhibitable reduction of cytochrome c or the reduction of nitroblue tetrazolium dye. Ahmed *et al* (1991) have also shown that HL60 cells differentiated maximally for 9 days with 1% DMSO release superoxide anion in response to PMA using the same assay systems. Suprisingly though when Ahmed *et al* (1991) measured the formation of reactive oxygen species by luminol-dependent chemiluminesence the response to PMA was much lower than that found in control human neutrophils. This chemiluminescence response can be amplified by taking advantage of the co-oxidation of luminol by H_2O_2 and HOCL generated during the respiratory burst, although the precise details of the chemical mechanism are not known. Hence Ahmed *et al* (1991) found that when horseradish peroxidase (HRP) was added to substitute for a possible lack of MPO in the HL60 cells the chemiluminesence response of the DMSO treated cells increased 6-7 times over that in its absence. The much higher chemiluminesence responses of the differentiated cells to oxidative-burst stimulants in the presence but not in the absence of HRP reflects an increase in H_2O_2 production that is not matched by an increased rate of synthesis of MPO. The induced cells are evidently unable to synthesize MPO at a higher rate, which is consistent with the observed absence of increased MPO mRNA production found by Novogrodsky *et al* 1983.

This is unlikely to be the case in our studies though as the differentiated HL60's contained high levels of MPO in comparison to human neutrophils (Chapter 2, Figure 2.8 and Figure 6.1). Even so further studies would have been able to confirm this hypothesis simply by the addition of HRP to the chemilluminesence assays. If the assay was affecting our ability to measure activation by measuring cytochrome c or nitroblue tetrazolium dye reduction we may have been able to detect activation. However, we feel that our inability to illustrate an oxidative burst does not lie with a lower level of MPO or the method of detection but with the inducer. As stated before different sublines of HL60 require different concentrations of inducers (Bunce *et al* 1983). We do not know what sublines

Collins *et al* (1979), Harris and Ralph (1985) or Ahmed *et al* (1991) used but Bunce *et al* (1983) found that HL60AST3, the subline we used, require 1.75% DMSO treatment for 7 days to induce maturity. The guidelines given by the ECACC, from whom we obtained the cells, recommend 1.25%. As they do not guarantee the viability of the cells unless cultured in this way we used the lower dose. This seems unusual as Dr. Bunce deposited the cells with the ECACC. Unfortunately the time was not available to carry out the oxidative burst assays using the higher concentration of DMSO though it is likely if we had for 6 days the activation assay would have worked.

Therefore, in conclusion this chapter indicates that HL60 cells look good as a possible substitute for neutrophils in our *in vitro* models though, for many reasons, leukemia cells show some distortion of phenotype as compared to their normal counterparts (Greaves *et al* 1986). The close correlation between the two *in vitro* studies, adhesion and migration, for both neutrophils and HL60 cells demonstrates the reproducibility of these studies and suggests their equivalence to *in vivo* ischaemia/reperfusion injury. They also highlight that HL60 cells can be used in the screening of possible adhesion or chemoattractant agents and may point to areas responsive to pharmacological manipulation to broaden our basic understanding of ischaemic/reperfusion injury. Furthermore with additional manipulation it is likely that we would have been able to use HL60 cells for the third and final facet of our *in vitro* models, activation. However, time was a limiting factor.

7 ADHESION MOLECULES, CYTOKINES AND CONTRACTILITY

7.1 Introduction

This final results chapter attempts to assess a number of apparently random disparate factors that from time to time have been implicated in the ischaemia/reperfusion story.

Firstly since neutrophil adhesion was dramatically heightened (Chapter 3, Figure 3.3) following hypoxia/reoxygenation we attempted to monitor potential ligands on the endothelial surface. Certainly some *in vivo* studies have indicated that the neutrophil adherence and emigration observed in postcapillary venules exposed to ischaemia and subsequent reperfusion is mediated by both LFA-1 and Mac-1 on neutrophils and that ICAM-1 on endothelial cells acts as the ligand for both heterodimers of CD11/CD18 (Oliver *et al* 1991). The results obtained from this *in vivo* model are both qualitatively and quantitatively consistent with the data obtained from *in vitro* models (Yoshida *et al* 1992). Therefore, it was of some importance to assess such factors in our own ECV304 /neutrophil model.

Secondly since earlier chapters (Chapter 3-5) have provided inferential evidence of roles for IL-8, TNFα, LTB_4 and PAF we wished to assess the chronology and magnitude of their release from the ECV304 cells.

Finally in addition to a possible role in causing tissue necrosis following reperfusion, neutrophils have also been implicated in other less severe changes in tissue function namely "myocardial stunning" and the "no-reflow phenomenon". Myocardial tissue that is reperfused following even a brief period of ischaemia shows evidence of impaired contractile function. This myocardial dysfunction is referred to as "stunning" (Heyndrickx *et al* 1975) and full recovery of contractile function can take a period of hours or days, depending on the duration of ischaemia (Braunwald and Kloner 1985). Calcium overload and free radical generation have both been implicated in causing stunning but the mechanisms involved in causing this dysfunction have not been fully resolved. Certainly neutrophils could contribute to myocardial dysfunction either through the release of ROS or by capillary plugging. Some studies clearly show that neutrophil depletion alleviates

the reduction in contractile function following reperfusion (Engler and Covell 1987 and Westlin and Mullane 1989).

Although normal flow may quickly be restored it subsequently disminshes once again to levels significantly lower than that prior to occlusion (Ames *et al* 1968). This phenomenon is termed "no-reflow". Amongst the several suggested causes of "no-reflow" are tissue oedema causing compression of arterioles or capillaries (Flores *et al* 1972 and Kloner *et al* 1974), swelling of endothelial cells causing narrowing of capillaries (Kloner *et al* 1974), occlusion of microvessels by hemorrhage (Higginson *et al* 1982) and activated neutrophils lodging in capillaries, (Schmid-Schonbein and Engler 1986 and Litt *et al* 1989).

Both impaired cardiac contractility and the "no-reflow" phenomenon could of course be the consequence of arteriolar constriction. We therefore examined the effects of supernatants derived from hypoxic/reoxygenated ECV304 cells on contractility of vascular smooth muscle using rings of rat aorta as our experimental model. Again it must be stressed that the experiments in this chapter are isolated observations. As such they are far from exhaustive but purely preliminary studies in these areas.

7.2 Results

7.2.1 ECV304 cells basally express ICAM-1

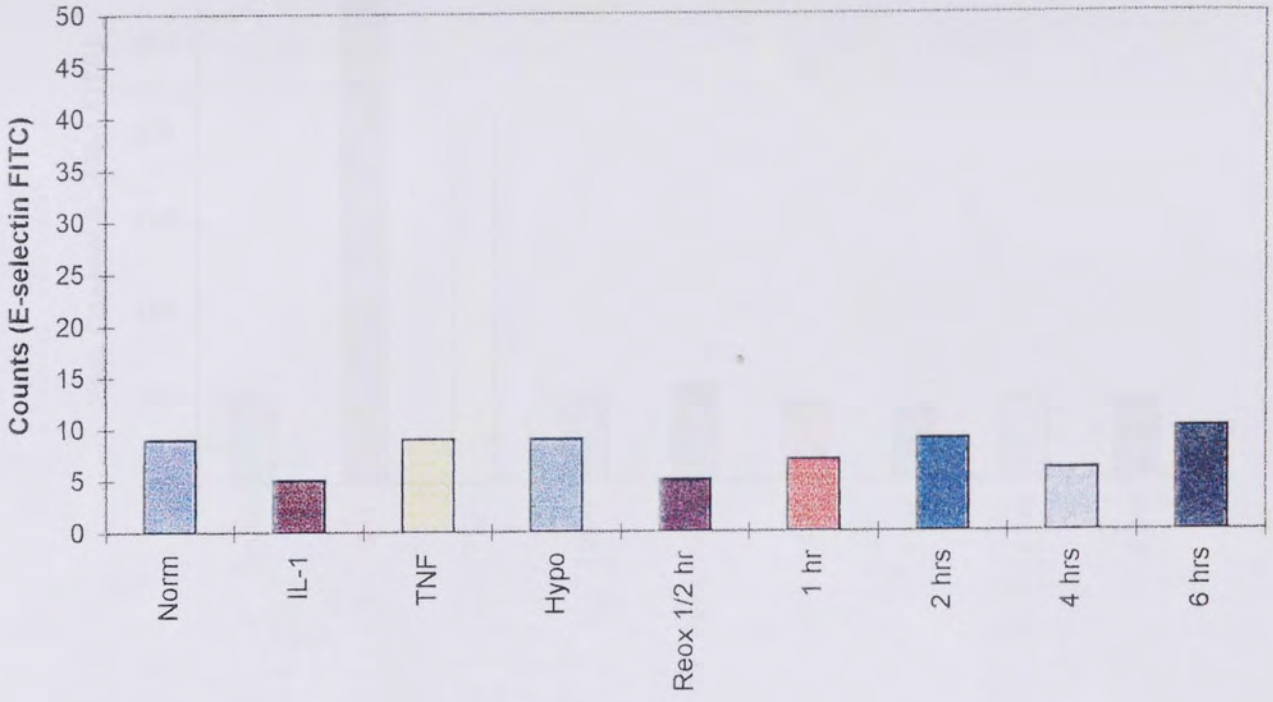
ICAM-1 is the only ligand present in significantly measurable amounts on the ECV304 cell surface (Figure 7.1). It can be upregulated by 4 hours treatment with IL-1 (100pg/ml) and TNFα (5IU/ml). Hypoxia itself or 4 hours hypoxia followed by up to 6 hours reoxygenation had no effect on the expression of ICAM-1. Similarly there was no change in the levels of E and P-selectin and VCAM-1, which in any case were only just on the threshold of detection in normal culture conditions. It is important to remember that at these times we have been able to demonstrate increased neutrophil adhesion, migration and activation (Chapters 3-5). Therefore, it is reasonable to conclude that factors released by the ECV allow or stimulate the neutrophils to adhere, migrate and activate, possibly by LFA-1/MAC-1 interactions with the abundant ICAM-1 counter-ligands.

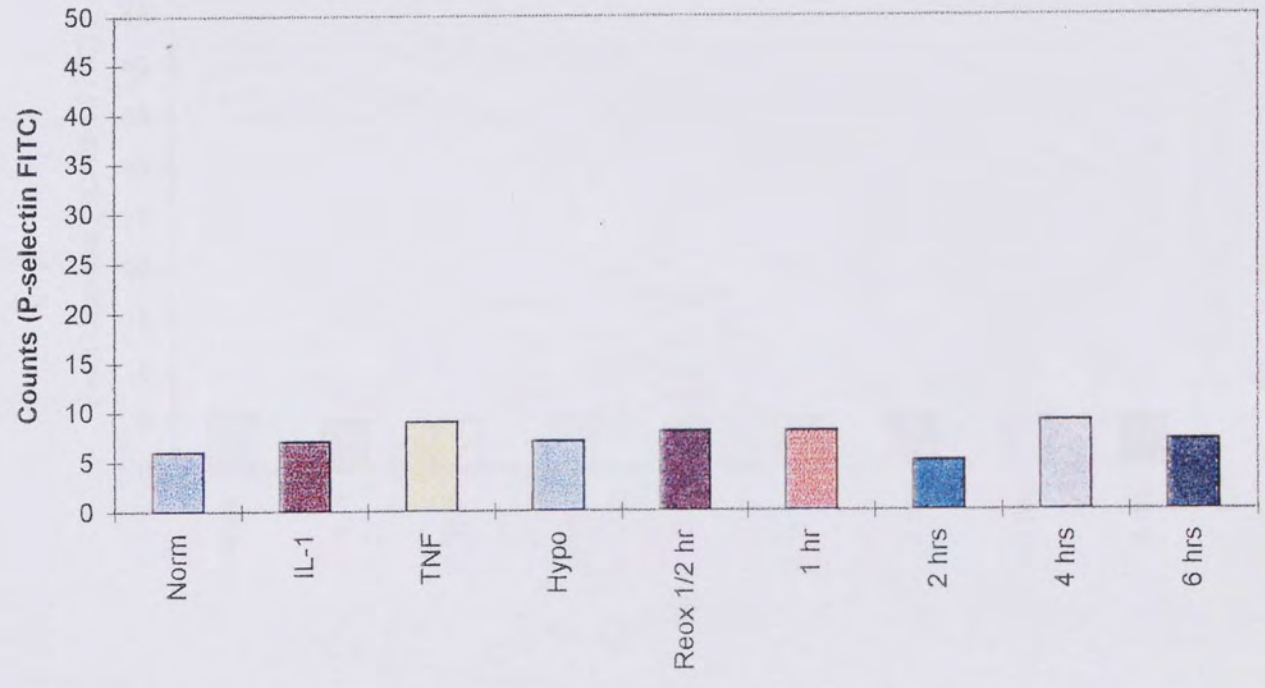
7.2.2 Factors present following hypoxia/reoxygenation

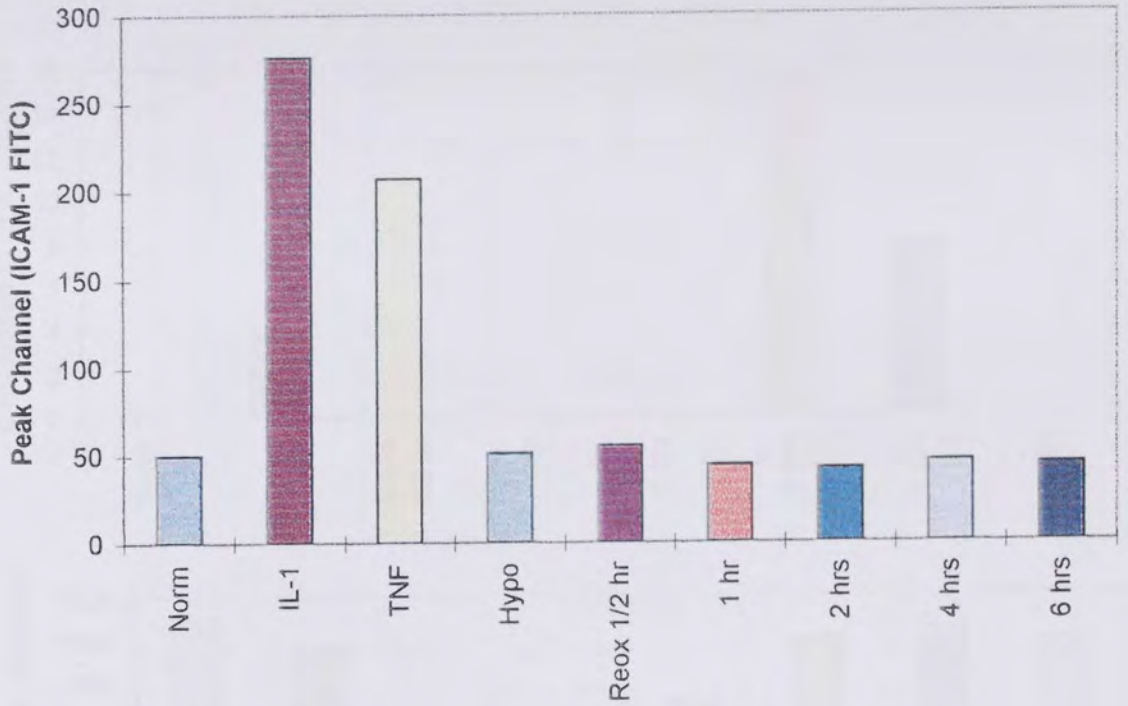
IL-1 secretion by ECV is not detectable at early times after hypoxia (Figure 7.2). However a 4 hours reoxygenation period does seem to show some episodic secretion (14.7pg/ml), though this is reduced following 6 hours reoxygenation. Presumably enzyme degradation causes this later reduction in the accumulated or secreted IL-1. During a 4 hour incubation under normoxic conditions IL-8 is secreted in large amounts. The concentration in the culture medium reached 1100pg/ml. This represents 275pg/hr. When the ECV cells were made hypoxic and then cultured in fresh medium for only 30mins the IL-8 concentration had already reached 250pg/ml. This is equivalent to 500pg/hr. TNFα release was also detectable after 2 hours reoxygenation though no LTB_4 was found. The increased IL-8 production detected after hypoxia/reoxygenation was reduced by allopurinol (20μM) and cycloheximide (5mg/ml). MMPI (1μM), dexamethasone (5μM) and a PAF secretion antagonist (10^{-6} M) had little effect.

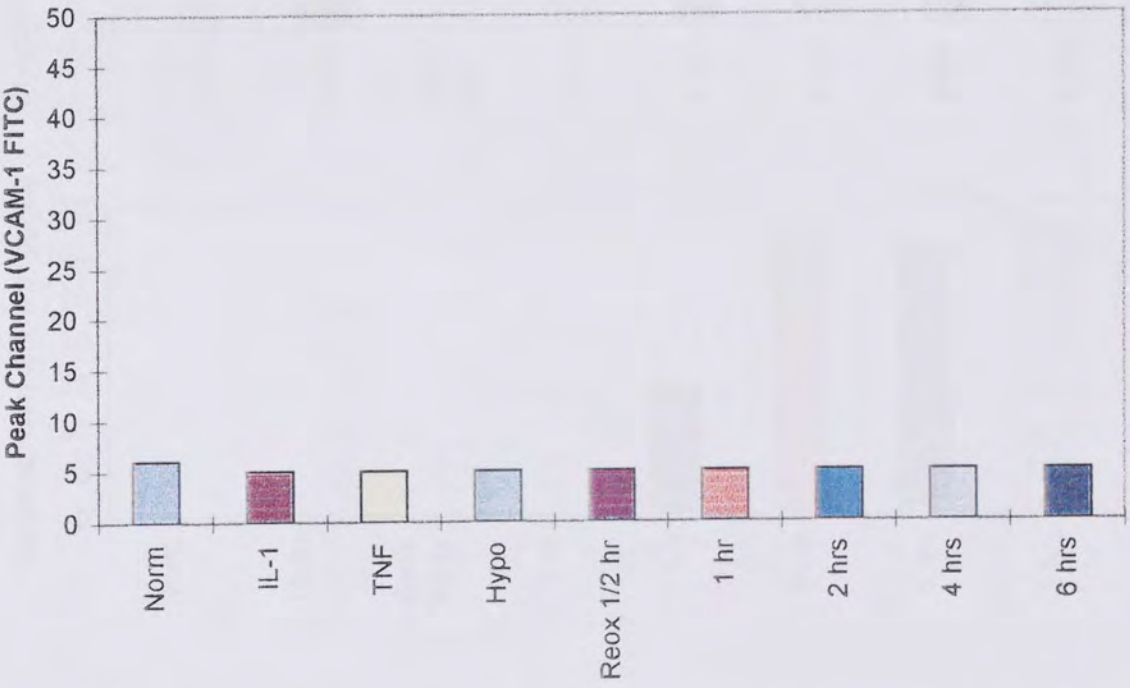
Given the intimate nature of the neutrophil/endothelial cell interaction the possibilities of autocrine and paracrine effects and extensive cross-talk between the two cell types must be countenanced. Therefore IL-1, IL-8, TNFα and LTB_4 production was measured with neutrophils present throughout the reoxygenation (Figure 7.4). Presence of both cell

Figure 7.1 Effect of Hypoxia/Reoxygenation on the expression of E and P-selectin, ICAM-1 and VCAM-1.







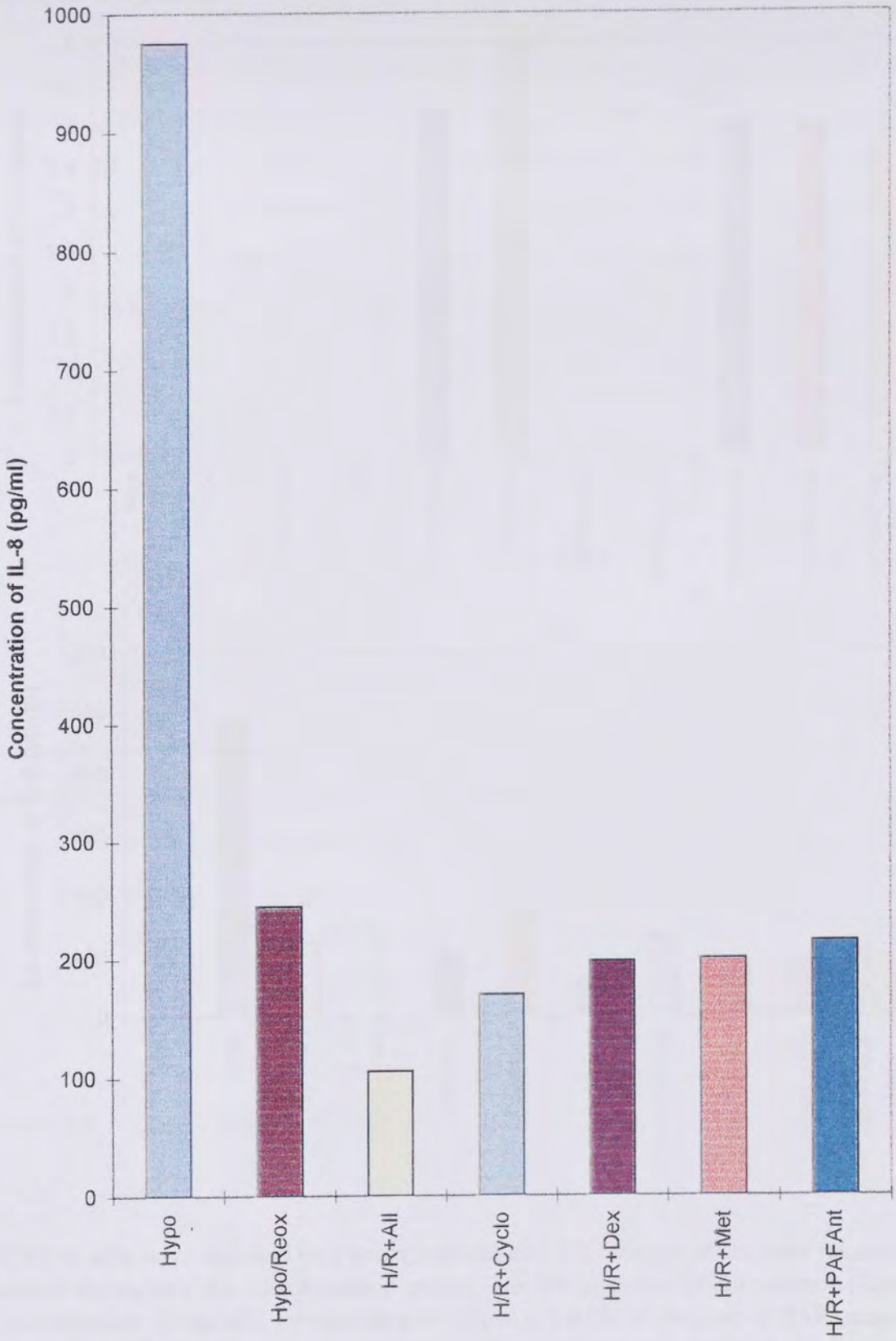


ECV304 cells ($1x10^6$) grown on cytodex microcarriers were exposed to hypoxia for 4 hours and reoxygenated for up to 6 hours. TNFα (5IU/ml) and IL-1(100pg/ml) were used as positive controls. The expression of E and P-selectin, ICAM-1 and VCAM-1 on the cell surface was then measured by FAC analysis. The peak channels, where >95% of the cells had that number of FITC counts, have been shown. The higher the peak channel therefore the more adhesion molecules expressed per cell. Values given are means where n=2.

Figure 7.2 Effect of Hypoxia/Reoxygenation on the IL-1, IL-8 and TNFα secreted by ECV304 cells.

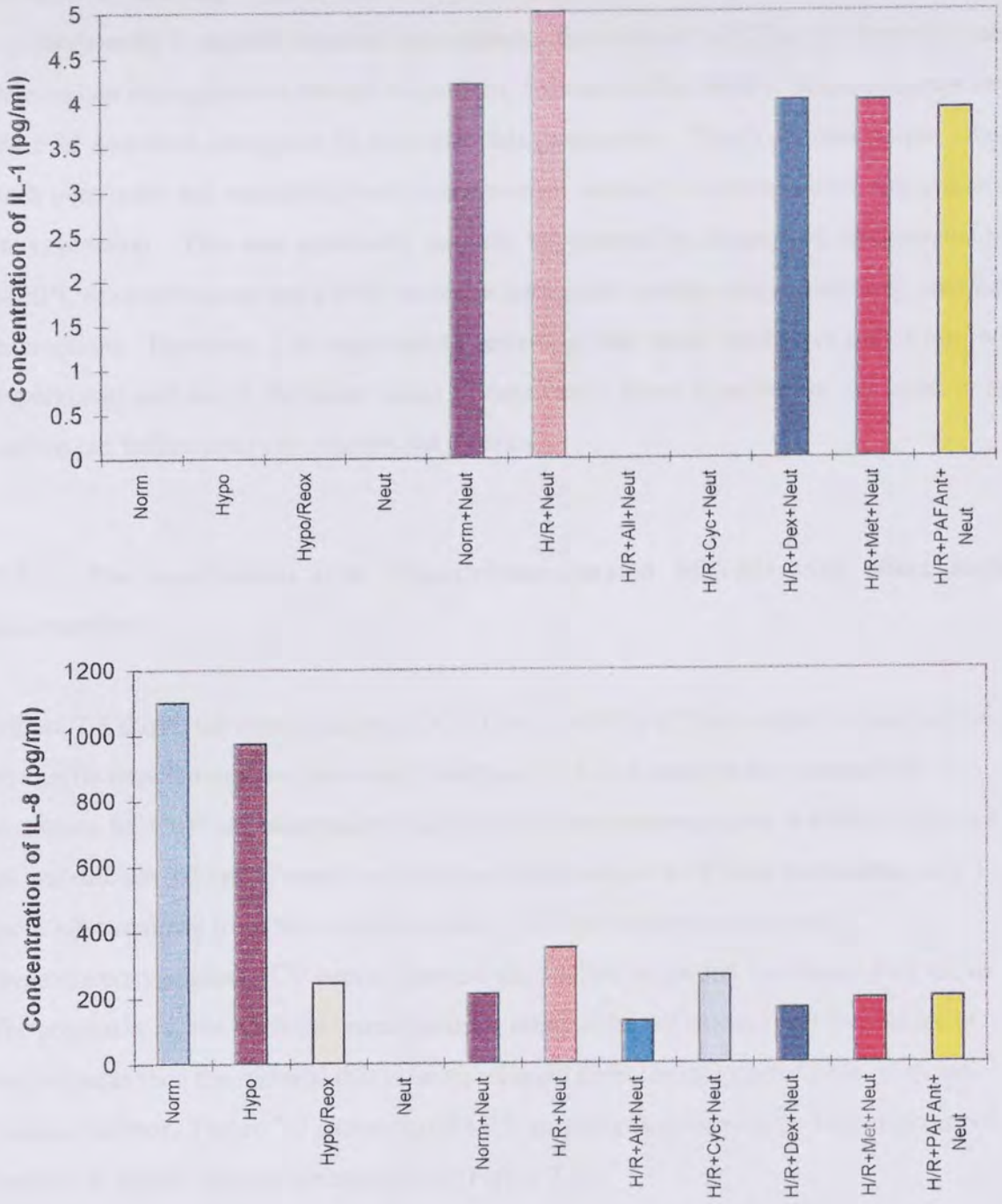
The supernatant of ECV304 cells exposed to hypoxia for 4 hours and reoxygenation for up to 8 hours were assessed for IL-1, IL-8 and TNFα using R+D ELISA's. The values given are means where n=2. An assay for LTB_4 demonstrated that none was present.

Figure 7.3 Effect of antioxidants, free radical scavengers and inhibitors on the IL-8 secreted by ECV304 cells exposed to Hypoxia/Reoxygenation.



ECV304 cells were exposed to 4 hours hypoxia and 1/2 reoxygenation in fresh medium in the presence of Allopurinol (20μM), Cycloheximide (5mg/ml), Dexamethosone (5μM), MMPI (1μM) or a PAF secretion antagonist (10-6 M) and the IL-8 secreted recorded. Values given are means where n=2. Assays for IL-1, LTB4 and TNFα were unable to detect any present in the supernatant.

Figure 7.4 Effect of antioxidants, free radical scavengers and inhibitors on the IL-1 and IL-8 secreted by ECV304 cells exposed to hypoxia with neutrophils present during the reoxygenation period.



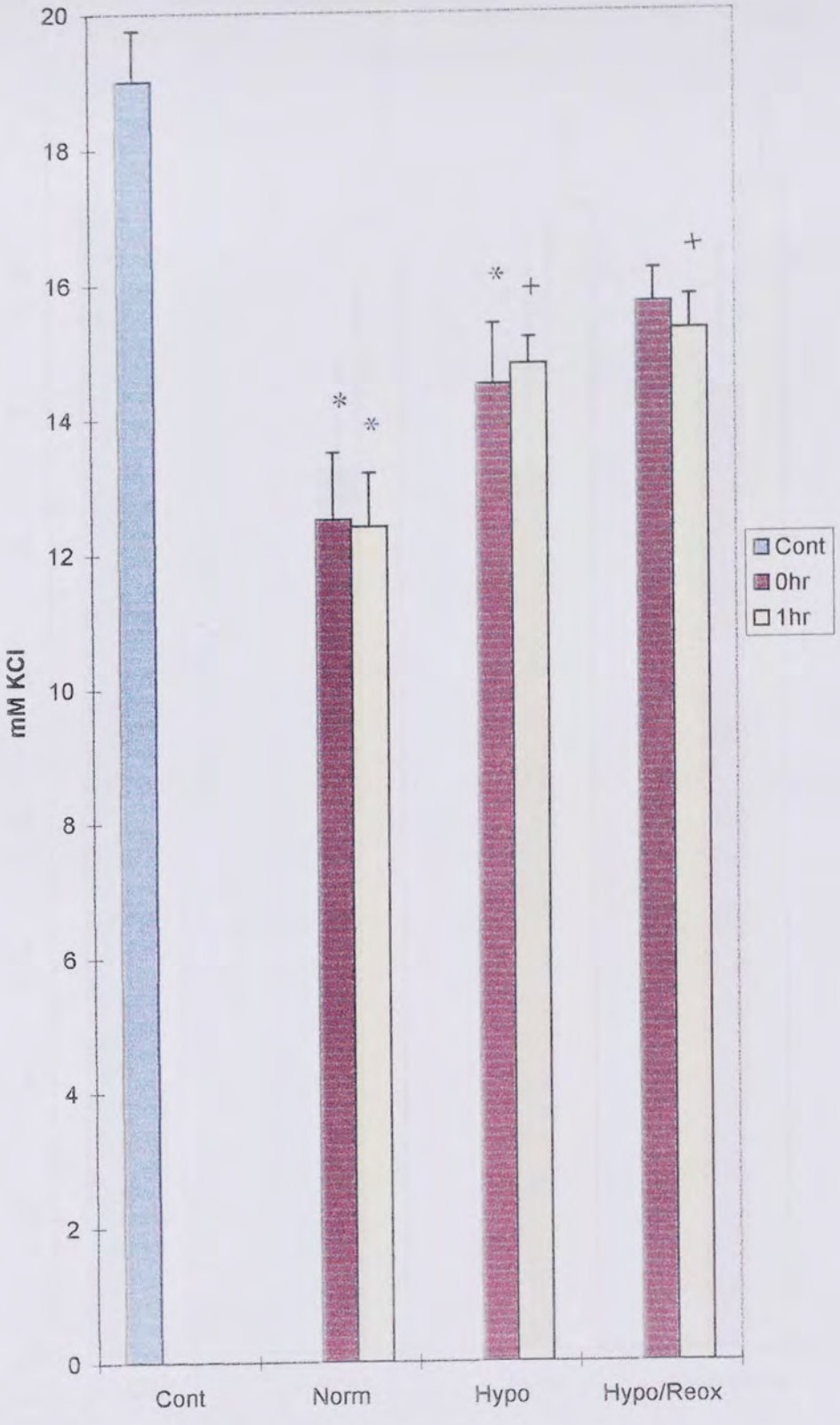
ECV304 cells were exposed to 4 hours hypoxia and 1/2 reoxygenation, with neutrophils present throughout the reoxygenation period, in the presence of Allopurinol (20μM), Cycloheximide (5mg/ml), Dexamethosone (5μM), MMPI (1μM) or a PAF secretion antagonist (10^{-6} M). The amount of IL -1 and IL-8 secreted was recorded. Values given are means where n=2. No TNFα was detected though small levels, 0.04pg/ml, of LTB_4 were recorded in the supernatant of hypoxic/reperfused cells if neutrophils were present.

types increased both IL-1 and LTB_4 production. There was no significant increase in IL-1 production following hypoxia and reoxygenation though and a protein synthesis inhibitor (cycloheximide 5 mg/ml) impaired this release. Low levels of LTB_4 (0.04pg/ml) were detected on reoxygenation though allopurinol, cycloheximide, MMPI, dexamethasone and the PAF secretion antagonist all abolished this production. The IL-8 total output when both neutrophil and endothelial cells were present seemed to increase after hypoxia and reoxygenation. This was apparently partially suppressed by allopurinol, cycloheximide, MMPI, dexamethasone and a PAF secretion antagonist perhaps suggesting very complex interactions. However, it is important to remember that these results are only from two experiments and are at the lower limits of detection. More experiments will need to be carried out before solid conclusions can be drawn.

7.2.3 The supernatant from hypoxic/reoxygenated ECV304 may effect aorta contractility

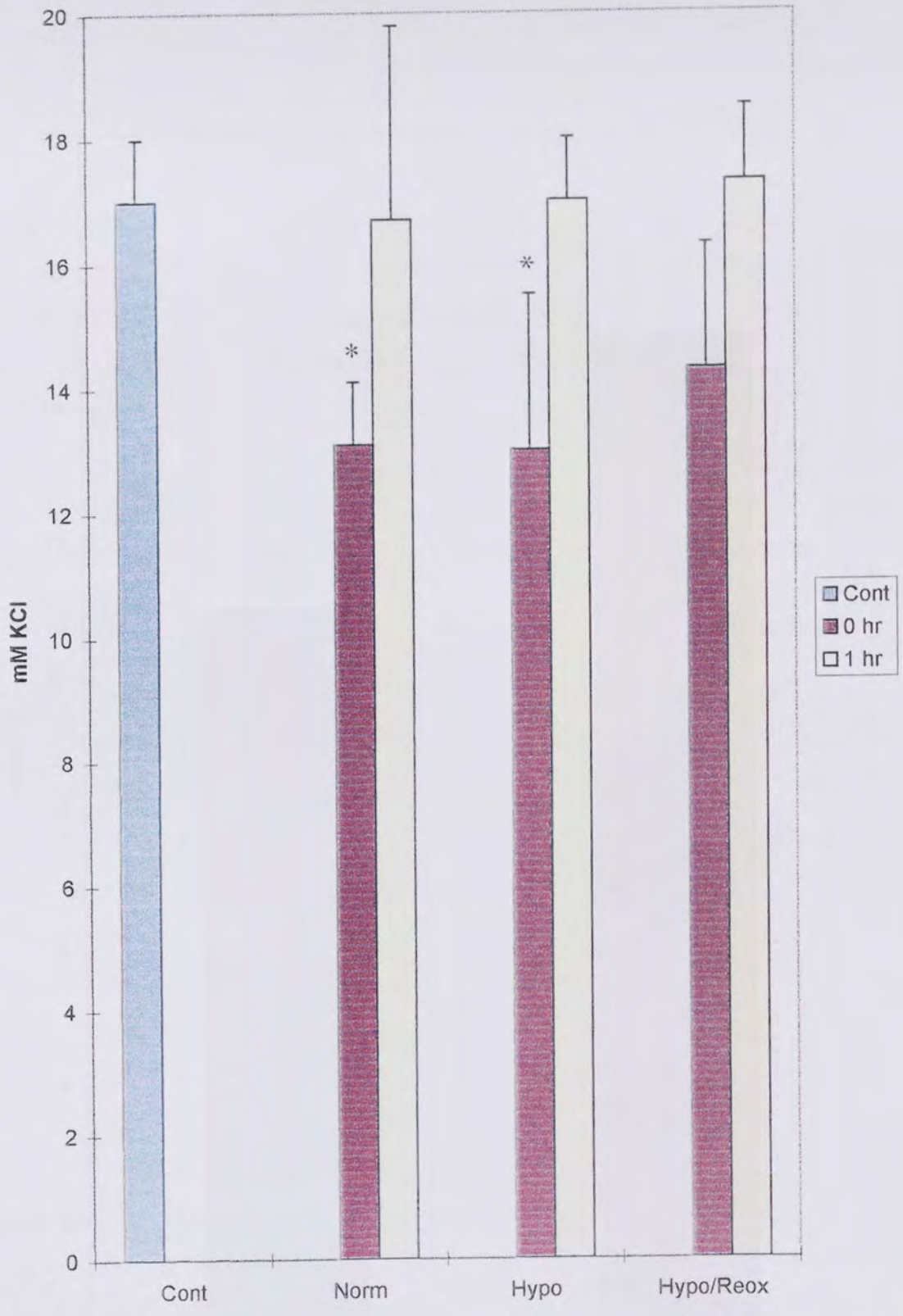
Figure 7.5 shows the concentration of KCl that gave 50% of the maximal contraction of the aortic muscle rings, as described in Section 2.1.3.5. Clearly in the presence of normoxic ECV304 cell supernatant the concentration-response curve is shifted to the left in low calcium indicating vasoconstriction at low doses of KCl, both immediately and 1 hour after removal from the endothelial cells. The curves for hypoxic and hypoxic/reoxygenated ECV supernatant are also shifted to the left but less so than those for normoxia. If the normoxic supernatant is taken to be the true control for this set of experiments then the material that is being released form the endothelial cells is an anti-vasoconstrictor. Figure 7.7 shows that PAF is an anti-vasoconstrictor. This effect is not evident at higher calcium concentrations (Figure 7.6).

Figure 7.5 Effect of the supernatant from hypoxic/reoxygenated ECV304 cells on the contractility of rat aorta (EC50) in 0.6mM Ca++.



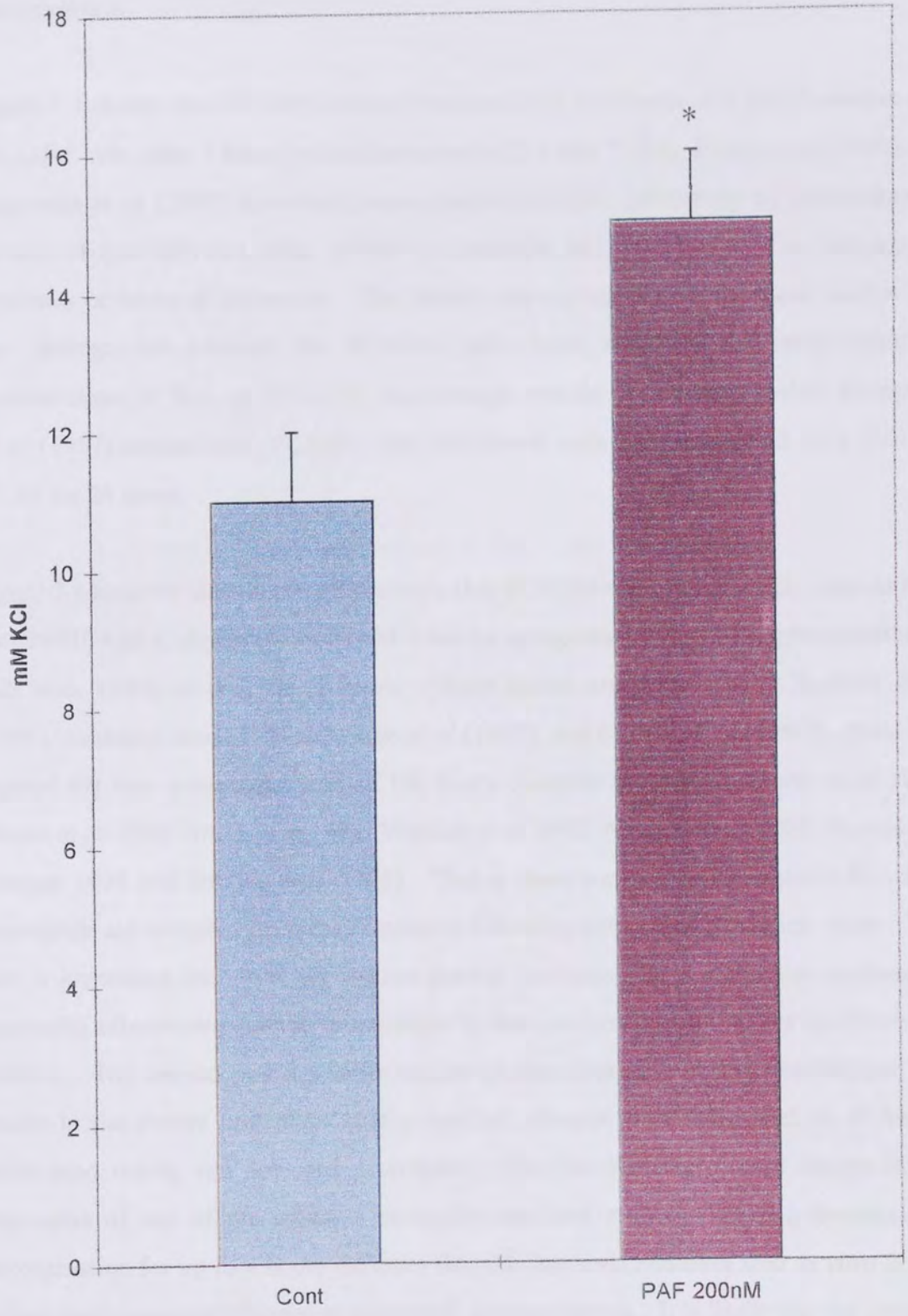
ECV304 cells were exposed to hypoxia for 4 hours and reoxygenated for 30mins. Rat aorta was then exposed to the supernatant from these cells and its ability, in 0.6mM Ca++, to contract to KCl recorded immediately and after 1 hour. Values given are means +/- SEM where n is atleast 4. * indicates a significant difference from control ($p<0.05$) and + indicates a significant difference from normoxic supernatant.

Figure 7.6 Effect of the supernatant from hypoxic/reoxygenated ECV304 cells on the contractility (EC50) of rat aorta in 2.5mM Ca++.



ECV304 cells were exposed to hypoxia for 4 hours and reoxygenated for 30mins. Rat aorta was then exposed to the supernatant from these cells and its ability, in 2.5mM Ca++, to contract to KCl recorded immediately and after 1 hour. Values given are means +/- SEM where n is atleast 4. * indicates a significant difference from control ($p<0.05$).

Figure 7.7 Effect of PAF on the contractility (EC50) of rat aorta in 0.6mM Ca++.



Rat aorta was exposed to PAF and its ability, in 0.6mM Ca++, to contract to KCl recorded. Values given are means +/- SEM where n=4. * indicates a significant difference from control ($p<0.05$).

7.3 Discussion

The purpose of this chapter was to provide a preliminary examination of the adhesion molecules expressed and factor (s) produced in our *in vitro* model following hypoxia and reoxygenation.

Figure 7.1 shows that ECV304 cells only express very low levels of E and P-selectin and VCAM-1 even after 4 hours preincubation with IL-1 and TNFα. Pigott *et al* (1992) and Majewska *et al* (1997) have both shown that in HUVEC (effectively an untransformed version of our ECV304 cells) ICAM-1, E-selectin and VCAM-1 can be induced by cytokines or bacterial endotoxin. The blatant contradictions between these studies and our findings are because the ECV304 cells were not incubated with optimum concentrations of IL-1 or TNFα for long enough periods. For example when Majewska *et al* (1997) upregulated VCAM-1 the endothelial cells were incubated with IL-1 or TNFα for 24 hours.

Figure 7.1 however does show conclusively that ECV304 cells constitutively express high levels of ICAM-1. Furthermore ICAM-1 can be upregulated by incubating the endothelial cells with TNFα or IL-1 for 4 hours. These results are supported by Bochner *et al* (1991), Seekamp *et al* (1993), Sorkine *et al* (1995), and Colletti *et al* (1996). They also support the two stage hypothesis of I/R injury (Gamble *et al* 1985, Smith *et al* 1988, Wayne *et al* 1989, Bruce *et al* 1991, Yoshida *et al* 1992, Arnould *et al* 1993, Kurose and Granger 1994 and Sorkine *et al* 1995). That is there are two basic pathways by which neutrophils are recruited from the circulation following ischaemic/reperfusion injury. The first is immediate and does not require protein synthesis. It is due to an increase in neutrophil adhesiveness for the endothelium by fast conformational changes in LFA-1 and MAC-1. The second pathway does require protein synthesis by the endothelium and results in the slower upregulation of a host of adhesion molecules, such as ICAM-1, which bind resting and activated neutrophils. The fact that there is no change in the expression of any of the adhesion molecules we have studied following hypoxia and reoxygenation for up to 6 hours indicates that the increased adhesion seen *in vitro* is due to fast conformational changes in neutrophil counter ligands. It is likely that the counter ligands for ICAM-1 are involved, LFA-1 and MAC-1, as ICAM-1 is the only adhesion molecule expressed in large enough quantities to bind to the neutrophils. Use of an anti-ICAM-1 monoclonal antibody also attenuated adhesion (Chapter 3).

Our results however do not show the later upregulation of adhesion molecules on the ECV304 cell surface. The explanation for this became apparent in the set of experiments which tried to characterize the factor (s) produced by the endothelial cells following hypoxia/reoxygenation (Figure 7.2).

The only cytokine produced immediately after hypoxia was IL-8. TNFα was secreted by the ECV304 cells 2 hours post hypoxia while IL-1 was detectable after 4 hours reoxygenation. At these time intervals only low levels of the cytokines were present (30pg/ml and 15pg/ml respectively). As previously shown (Figure 7.1) preincubation with IL-1(100pg/ml) and TNFα (5IU/ml) for 4 hours is required to increase ICAM-1 expression. By examining longer reoxygenation periods we may have seen a change but it is likely that in our *in vitro* model the IL-1 and TNFα secreted by the ECV304 cells was simply not there at high enough concentrations for ICAM-1 upregulation.

Furthermore, in the presence of neutrophils, IL-1 was detected earlier and at higher concentrations in the normoxic, hypoxic and hypoxic/reoxygenated supernatant (Figure 7.4). That is the neutrophils were also releasing IL-1 in this *in vitro* model. Obviously *in vivo* both neutrophils, endothelial cells and a host of other cells are present following ischaemic/reperfusion injury and the enhanced IL-1 released could contribute to the second stage, upregulation of ICAM-1 as illustrated by Tamiya *et al* (1994) Farhood *et al* (1995), Nakano *et al* (1995), Oshiro *et al* (1995) Vollmar *et al* (1995) and Soriano *et al* (1996).

The large amounts of IL-8 produced by the endothelial cells in response to hypoxia/reoxygenation were reduced, from 500 pg/hr compared to 275 pg/hr during normoxia, by pre-treatment with allopurinol, the xanthine oxidase inhibitor (Figure 7.3). This suggests a role for the free radicals produced following ischaemia in provoking IL-8 secretion. IL-1 production, by hypoxic/reoxygenated ECV304 cells and neutrophils, was also attenuated by allopurinol pre-treatment (Figure 7.4). Furthermore IL-1 production was inhibited by pre-treating the ECV304 cells with cycloheximide. This result is supported by Obrig *et al* (1971) who demonstrated that cycloheximide inhibits peptide synthesis and therefore IL-1 turnover. Although these studies only contained two separate observations the trends suggest that dexamethosone, an inhibitor of arachidonate metabolism that may block a variety of early activation responses by

interfering with nuclear transcription factors, a MMPI that blocks TNFα processing, and a PAF secretion antagonist all partially curtail IL-1 and IL-8 production. Although these inhibitors do not work directly by blocking IL-1 and IL-8 production they highlight how complex interacting networks of cytokines may be involved in their production. Similar results were found by Seekamp *et al* (1993) who found a close correlation and interaction between TNFα, IL-1 and IL-6 production in ischaemic/reperfusion injury in the lung.

These results (Figure 7.2-5) provide strong evidence for the involvement of IL-8 in ischaemic/reperfusion injury. There is also a large body of evidence suggesting that IL-8 plays a causative role in acute inflammation by recruiting and activating neutrophils (Harada *et al* 1994). However, in our *in vitro* models using reconstituted IL-8 we were unable to demonstrate that IL-8 (1-1000pg/ml) stimulates either neutrophil migration or activation (Chapter 4 and 5). The concentrations secreted by the hypoxic/reoxygenated ECV cultures are well within the concentration ranges employed for these assays. One possible explanation for these findings could be the instability of the IL-8 used, though it was used as recommended and did promote a significant increase in neutrophil adhesion (Chapter 3). To resolve these anomalies further studies will need to be performed.

The final set of results in this chapter show that factors produced by the ECV304 cells following hypoxia/reoxygenation may be responsible for impaired cardiac contractility and the "no-reflow" phenomenon possible seen as a consequence of arteriolar constriction (Heyndrickx *et al* 1975) (Figure 7.5 and 7.6). The results are interesting in that interpretation depends on what is considered the true control. Clearly the concentration-response curves for normoxic supernatant are shifted to the left in low calcium indicating vasoconstriction at low doses of KCl. The curves for hypoxia and hypoxia/reoxygenation are also shifted to the left but less than those for normoxia. If the true control is considered to be the response in normoxic supernatant the material released from the endothelial cells during hypoxia and reoxygenation is an anti-vasoconstrictor or relaxant. If the material acts in the same way on aortic smooth muscle it might account for the impaired contractility seen during stunning, as described by Heyndrickx *et al* (1975) and Braunwald and Kloner (1985). However, it could not account for impaired cardiac contractility or "no-reflow" as a consequence of arteriolar constriction (Ames *et al* 1968). Two possibilities that may fill this anti-vasoconstrictor role are TNFα and PAF (Lefer *et al* 1984). Certainly our results show that PAF significantly prevents contraction (Figure 7.7).

From this chapter we have been able to conclude that the main factor still implicated in our hypoxic/reoxygenation models, as demonstrated by using a secretion antagonist on neutrophil adhesion, migration and activation following hypoxia/reoxygenation (Chapters 3,4 and 5) is PAF. This hypothesis is supported by several other lines of evidence; PAF levels rise in tissue exposed to I/R (Filep *et al* 1989), PAF receptor antagonists attenuate the microvascular alterations elicited by I/R (Kubes *et al* 1990) and the local administration of PAF elicits most of the microvascular alterations elicited by I/R injury (Kurose and Granger 1994).

Again it is important to remember that this assay and the other experiments in this chapter are only initial observations made in an attempted to unravel the nature of the relationship between neutrophils and ischaemic/reperfusion injury. In so doing they have raised a multitude of questions and opened up many possible avenues of investigation. By pursuing one or more of these lines of investigation it is hoped that one would be closer to understanding the precise role of neutrophils in the pathogenesis of reperfusion injury.

8 GENERAL DISCUSSION

The overall purpose of this thesis was to address the role of the neutrophil in the hypoxia and reoxygenation associated with ischaemic/reperfusion injury by means of *in vitro* model systems. Thus we examined the three potential facets of the injurious process i.e., the initial adhesion of neutrophils to the endothelial surface, their migration in response to released chemoattractants and their capacity to generate an inappropriate oxidative burst.

Initially we developed an *in vitro* model to study neutrophil adhesion following hypoxia and reoxygenation. This was achieved by exposing endothelial cells cultured in 96 well plates to 4 hours hypoxia and adding neutrophils to the endothelium after varying periods of reoxygenation. Although earlier studies have used hypoxic periods as short as 30 mins 4 hours was chosen as neutrophil adhesion is not further enhanced by additional hypoxia (Budd *et al* 1989). Using this model we were able to show that preincubating the ECV304 cells with known physiological stimulants IL-1 and TNFα for 4 hours maximally increased neutrophil adhesion ($p<0.05$). These results show that our model is physiologically viable and is consistent with other studies, such as Bruce *et al* (1991), that demonstrate that IL-1 up-regulates ICAM-1 and E selectin. This model also shows conclusively that there is a significant ($p<0.05$) increase in neutrophil adhesion to ECV304 cells following 4 hours hypoxia and 30 mins reoxygenation (Figure 3.3). This effect was transient and persisted for only 30 mins after reoxygenation was initiated. It was no longer evident at 1, 2 and 4 hours.

An inherent problem with this and all other *in vivo* and *in vitro* assays is the variability in neutrophil adhesion not only between different individuals but also between the same individual on different days. Yoshida *et al* (1992) using ^{51}Cr-labeled neutrophils found mean basal adhesion to HUVEC varied from 8-25% in different experiments using 4 subjects on each occasion. This variation was also seen in our assays. Therefore, to control for it, experiments were carried out on the same day using 4 different individuals, and preferably on the same plate of cultured endothelial cells. Furthermore a two way anova, which accounts for variation between paired data, was used to analyse the results.

The increased neutrophil adhesion demonstrable following hypoxia and reoxygenation is the direct result of the production of reactive oxygen species by the endothelial cells, as

shown in Figure 3.5. Allopurinol, an inhibitor of xanthine oxidase, SOD, a superoxide scavenger, and catalase, an antioxidant that catalyzes the reduction of H_2O_2 to H_2O and O_2 respectively, all significantly reduced the enhanced neutrophil adhesion following hypoxia/reoxygenation.

ICAM-1 is clearly one participant in this enhanced adhesion since the use of a mouse anti-human ICAM-1 monoclonal antibody completely eliminated the increased neutrophil adhesion observed following hypoxia (Figure 3.13). Not only did it abolish the increased neutrophil adhesion seen after hypoxic and reoxygenation but it also significantly decreased normoxic adhesion ($p<0.05$). We have confirmed this role by FACs analysis (Figure 7.1). Of the limited range of receptors assessed ICAM-1 was the only adhesion molecule expressed at high concentrations on the endothelial cell surface. E-selectin, P-selectin and VCAM-1 are not constitutively expressed. These results are consistent with the hypothesis that in our static model using ECV304, which fail to express selectins, the increased adhesion seen following hypoxia and reoxygenation is due to rapid conformational changes in adhesion molecules, such as LFA-1, on the neutrophil which increase its affinity for ICAM-1 (Oliver *et al* 1991). In Figure 3.15 we were able to demonstrate that a soluble factor (s) is released from the endothelial cells following hypoxia and reoxygenation. We believe that it is this factor (s) that is responsible for fast conformational changes in the neutrophil's adhesion molecules and subsequent increased affinity for the ECV304 cells in our adhesion model.

To establish what possible factors initiated by the ROS could be responsible for this increased neutrophil adhesion post hypoxia cycloheximide and dexamethasone were used. Preincubation of ECV with an inhibitor of protein synthesis (cycloheximide) (Figure 3.9) or an inhibitor of eicosanoid production (dexamethasone) (Figure 3.10) prior to hypoxia both reduced subsequent neutrophil adhesion. These results suggest that indeed factors released by the ECV cells are responsible for the enhanced neutrophil adhesion following hypoxia and reoxygenation.

Two possible candidates could be PAF and TNFα since both a PAF secretion antagonist and a MMPI, an inhibitor of TNFα processing and release, attenuated neutrophil adhesion following hypoxia and reoxygenation (Figure 3.11 and 3.12). In addition analysis of the supernatant from ECV304 cells following hypoxia and reoxygenation by ELISA (Figure 7.2 and 7.4) showed that TNFα was detectable in the supernatant 2 hours after

reoxygenation. This does not exclude it as a factor involved in the increased neutrophil adhesion post hypoxia as small undetectable amounts may be able to cause rapid conformational changes in neutrophil adhesion molecules or bound TNFα could possibly elicit the same response.

The only cytokine detected, by ELISA, in measurably larger amounts following hypoxia was IL-8 (Figure 7.2 and 7.3). We have been able to illustrate that IL-8 heightens neutrophil adhesion to naive ECV cells (Figure 3.16). However since decreased neutrophil adhesion was seen using both cycloheximide and dexamethasone, implicating the involvement of more than one factor, it would have been pertinent to try combinations of the possible cytokines involved in an attempt to reproduce post hypoxia adhesion.

Even so we have thus ear-marked PAF, TNFα and IL-8 as possible factor (s) released by endothelial cells following hypoxia and reoxygenation that could potentiate neutrophil adhesion. Indeed other authors with different systems have also implicated IL-1 (Bevilacaque *et al* 1985 and Seekamp *et al* 1993), IL-8 (Sekido *et al* 1993, Hisama *et al* 1995, and Ivey *et al* 1995), PAF (Milhoan *et al* 1992, Arnould *et al* 1993, Kurose and Granger 1994 and Kubes *et al* 1995) and TNFα (Seekamp *et al* 1993 and Adams *et al* 1995). Furthermore Rainger *et al* (1997) have clearly shown that PAF and IL-8 act together to promote activation and migration of neutrophils after hypoxia and reoxygenation.

When we turned our attention to the next facet of this injurious process the best artificial neutrophil chemoattractant was HIZAS, presumably due to the activation of the complement cascade (figure 4.1). However, the supernatant from hypoxic or hypoxic/reoxygenated ECV cells was even better (Figure 4.4). The potency of the factor (s) produced was unexpected since enough was produced in only 30 minutes of reoxygenation to attract more neutrophils than optimum concentrations of HIZAS. These studies unequivocally demonstrate that a powerful neutrophil chemoattractant (s) is produced following hypoxia.

This factor (s) could not have been complement in our experimental system since no obvious source for components existed. Furthermore we cannot stipulate that IL-8 has any role since it cannot produce migration through naked filters as used in our studies (Figure 4.10). Although others (Welbourn *et al* 1991) have suggested a role for LTB_4 we

have been unable to demonstrate migration in response to this agent which possibly is only active if neutrophils are pre primed as artefacts of isolation or other agents (Figure 4.11). Furthermore analysis of the supernatant from hypoxic/reoxygenated ECV304 cells showed conclusively that LTB_4 was not present in our model (Figure 7.2).

The most likely candidate may well be PAF since we did find a moderate reduction in the release of chemoattractants by the hypoxic/reoxygenated endothelial cells in the presence of a PAF secretion antagonist (Figure 4.6) Perhaps high concentrations of antagonist may have been more effective. Certainly PAF at high concentrations has the capacity to stimulate migration in our experimental system (Figure 4.12). However, whatever is produced by the hypoxic/reoxygenated endothelial cells a necessary prelude to this is xanthine oxidase activation, and presumably the generation of ROS (Figure 4.5).

To complete the third facet of the neutrophil's involvement in ischaemic/reperfusion injury this thesis demonstrates unequivocally that following adhesion and migration neutrophil activation can occur. Although several studies have now implicated neutrophil activation as being central to the genesis of tissue reperfusion injury and posttraumatic multiple organ failure (MOF), in human subjects data is scarce (Anderson 1995).

Presence of soluble priming factors and contact with ECV cells will induce neutrophils to release ROS which undoubtedly would be damaging. Both isolated purified neutrophils and those contained in whole blood gave this response (Figure 5.1 and 5.2). Allopurinol, a xanthine oxidase inhibitor, attenuates this activation highlighting that it is the direct consequence of ROS generation and therefore hypoxia/reoxygenation. One hour after the endothelial cells were reoxygenated neutrophil activation was diminishing towards normoxic levels (Figure 5.3).

Attempts to identify possible soluble factor (s) responsible for this activation showed that PAF, TNFα and LTB_4 are all able to prime neutrophils to respond to subsequent stimuli (Figure 5.11-5.14). Because analysis of the supernatant from hypoxic/reoxygenated ECV cells (Figure 7.4) showed that no TNFα and only low levels of LTB_4 were present at this time period we concluded that PAF is involved in neutrophil activation. Furthermore PAF has been implicated in both neutrophil adhesion and migration and by using a PAF secretion antagonist we were able to attenuate the increased ROS generation following hypoxia/reperfusion (Figure 5.5). However, PAF can not directly activate the neutrophils;

it either primes the neutrophils to respond to other factors or PAF plus binding to the ECV cells produces the required dual signal. Certainly in this set of experiments we have been able to reveal that both neutrophil adhesion and the presence of soluble factor (s) are required for neutrophil activation (Figure 5.15-5.17). This phenomenon may account for the accumulation of neutrophils in areas other than the hypoxic site, such as the lungs (Schmeling *et al* 1989). The very low shear forces in slow flow microvasculature is thought to allow tethering rather than rolling of the primed neutrophil. Certainly reperfused gut elaborates PAF that attracts and primes neutrophils (Kim *et al* 1995).

The thesis has shown that neutrophil adherence to ECV304 cells is significantly increased following hypoxia and reoxygenation. Isolated neutrophils will also preferentially migrate into the supernatant from hypoxic/reoxygenated ECV304 cells and the presence of factor (s) in the supernatant along with adhesion molecules on the ECV304 cell surface will cause the neutrophil to produce an oxidative burst.

A problem inherent throughout these investigations however has been the variation seen between the neutrophils from different individual's and even the same individual on different days. To try and eliminate this variation, also widely seen in other *in vitro* and *in vivo* models, and give more consistency to our results we tried to use HL60 cells differentiated towards neutrophil function. HL60's have not been previously used in models of ischaemic/reperfusion injury. HL60 can be induced by 1.25% DMSO to differentiate along a neutrophil like line (Section 6.1). HL60's are certainly easier to use than freshly isolated neutrophils. Using HL60 cells differentiated in this way we were able to achieve partial success. HL60's will adhere to endothelial cells following hypoxia/reoxygenation and migrate towards hypoxic/reoxygenated supernatant (Figure 6.2 and 6.3). Unfortunately they failed to fulfill the final feature of an *in vitro* model of ischaemia/reperfusion. No ROS could be elicited. One possible reason for this partial success may have been the concentration of DMSO used. It may be possible that future studies could manipulate this final facet and provide another *in vitro* model to study ischaemic/reperfusion injury.

Another explanation for the large variations seen in normoxic adhesion and migration could be the endothelial cells used. Although use of ECV rather than HUVEC did not eliminate this wide variation, as originally hypothesized, from experiment to experiment they were easier to handle and maintain. The umbilical vein endothelium however is not

one normally implicated as a site for ischaemic/reperfusion injury *in vivo*. Primary cultures of endothelial cells from gut, heart and lungs should certainly be attempted and compared to HUVEC and our ECV model system in an attempt to reduce this variation.

It should also be noted that ECV304 cells do not express the selectins usually associated with endothelial cells. *In vivo* these are required for neutrophil recruitment from the circulation. As the models used in this thesis are static they do not rely on this interaction for neutrophil adhesion. However, it would certainly be a logical progression to use endothelial cells which express integrins in subsequent work, especially if examining the situation under flow.

Despite the vast body of experimental evidence contained within this thesis suggesting a role for neutrophils in ischaemia/reperfusion injury, limited clinical data is available. This is because while our experimental systems are relatively easy to define and manipulate the clinical situation is far more complex. Certainly ethical considerations would not allow you to inflict ischaemia on a patient and carry out many of the studies done *in vitro*. *In vitro* model systems like ours are therefore vital in gaining an understanding of events. The next step with our systems is to try and model the situation *in vitro* more closely. These studies could then provide a rationale for designing clinically applicable interventions directed at inhibiting neutrophil mediated damage.

One factor which certainly will be present *in vivo* and which has not been reproduced in any of the *in vitro* model systems is complement. Furthermore although numerous studies have looked at the role of neutrophils in ischaemic/reperfusion injury other cell types have been largely ignored as they do not appear at the site of injury until 1-2 hours after reperfusion. Neutrophils arrive within minutes (Mullane *et al* 1983). The role of these cell types, however, can not be disregarded as monocytes/macrophages, like neutrophils, secrete factors such as IL-1 which are potentially cytotoxic or may alter endothelial function (Harlan 1985). Subsequent work should certainly address these factors by adding complement and other cell types to our model systems.

All the *in vitro* systems we have developed have been performed under static conditions. Indeed it is hard to see how neutrophil activation could be studied in any other way. Static assays have also been widely used because they are easy to perform, yield much information, and our studies have certainly highlighted sites for pharmacological

intervention. However, they are clearly poor models of the situation in flowing blood, on reperfusion, when stress may fluctuate. In conjunction the morphological appearance of endothelium grown under static conditions and factors released is known to be modified after exposure to mechanical stress (Frangos *et al* 1985 and Yoshizumi *et al* 1989). Thus a logical progression would be to study neutrophil adhesion and migration, following hypoxia and reoxygenation, under conditions of flow. Several models now exist that could be easily adapted (Cook *et al* 1992).

The therapeutic options for limiting ischaemia/reperfusion injury suggested by our work include inhibition of oxygen radical formation (Simpson *et al* 1987 and Paulluy *et al* 1990), pharmacological prevention of neutrophil activation and chemotaxis (Firestein *et al* 1995 and Derian *et al* 1995) and also the use of monoclonal antibodies (MAb) which may block neutrophil-endothelial adhesion, (Smith *et al* 1988). Listed in more detail are a number of interventions that may attenuate ischaemia/reperfusion injury by directly or indirectly effecting the neutrophil.

8.1 Antioxidants

We have implicated oxygen-derived free radicals in ischaemic reperfusion injury. Certainly the use of antioxidants and free radicals scavengers abrogated the increased neutrophil adhesion, migration and activation associated with hypoxia and reoxygenation (Chapters 3, 4 and 5). The available evidence also suggests that antioxidants can attenuate microvascular injury as discussed (Opie 1989 and Kloner *et al* 1989). Hence it is our recommendation that antioxidants could be used if high enough levels can be achieved *in vivo* to prevent the liberation of ROS.

8.2 Neutrophil depletion

Experimental neutrophil depletion can be achieved by several methods, e.g., chemotherapy (Freed *et al* 1989), administration of neutrophil antibodies (Galinanes *et al* 1993) , or coronary perfusion through leukocyte-depleting filters (Juneau *et al* 1993). However, this method of ameliorating ischaemic/reperfusion injury has many limitations and as such we would not recommend it for therapeutic use. It may depress other blood cell lines in addition to neutrophils, neutrophil antiserum may

activate complement and cause alterations of the immunological network, which can potentially influence perfusion injury; and leukocyte filters require complex instrumentation, activate complement, release adenosine and other mediators from blood cells.

8.3 Inhibition of neutrophil adhesion

Our findings suggest that by preventing neutrophil adhesion, following ischaemia and reoxygenation, you may reduce production of factors which could injure (Figure 3.13). This finding is supported by many other investigators such as Simpson *et al* 1990 who illustrated that administration of a monoclonal antibody to CD11b/CD18 before reperfusion may induce sustained reduction in myocardial infarct size and neutrophil accumulation in a dog model. Similar beneficial effects have also been observed in cat models (Ma *et al* 1991). Soluble ligands to adhesion molecules (or soluble adhesion molecules per se) may also have the potential to attenuate postischaemic neutrophil endothelial interactions. Indeed, a sialyl Lewis^{x} containing oligosaccharide was shown to attenuate myocardial injury and preserve global cardiac performance after myocardial ischaemia and reperfusion in a feline model (Buerke *et al* 1994). Interestingly, a member of the new anti-inflammatory agents (leumedins), which inhibit neutrophil upregulation of CD11b/CD18, has been shown to decrease neutrophil accumulation, protect against mechanical dysfunction and attenuate the increase in myocardial vascular resistance after reperfusion of *in situ* neonatal piglet hearts subjected to hypothermic global ischaemia (Wilson *et al* 1993). However, it must be appreciated that by blocking neutrophil adhesion at sites of ischaemic/reperfusion injury adhesion at all other sites will also be prevented perhaps leaving the patient severally immunocompromised.

8.4 Inhibition of complement activation

Although complement was not available in our systems further studies should clearly be carried out with its presence, as the literature cites its importance in provoking ischaemic/reperfusion injury. Complement depletion by the administration of cobra

venom factor, which activates the alternative pathway, can reduce complement localization, neutrophil accumulation, and tissue necrosis in models of ischaemic reperfusion injury (Maroko *et al* 1978 and Crawford *et al* 1988). A recombinant soluble complement receptor type has been shown to block complement activation in human serum (Wiesman *et al* 1990). This agent induced sustained reductions in myocardial infarct size in rats and reduced neutrophil-dependent contractile dysfunction and oxygen free radical generation in isolated rat hearts subjected to global ischaemia and reperfusion (Shandelya *et al* 1992).

8.5 Inhibition of arachidonic acid metabolism of PAF

We have been able to show that factor (s) produced following hypoxia/reoxygenation may be responsible for neutrophil adhesion, migration and activation. By blocking these factors it may be possible to ameliorate damage. Lipoxygenase inhibitors (many of which are potent antioxidants) (Shappell *et al* 1990) have been shown to inhibit neutrophil function *in vitro*, and the 5-lipoxygenase inhibitor 5-aminosalicylic acid can attenuate the tissue damage after a reversible ischaemic period (Hansen *et al* 1995). Furthermore BW755C, a mixed cyclooxygenase-lipoxygenase inhibitor, *in vitro* inhibits neutrophil oxidative metabolism, degranulation and LTB_4 production but only has a minimal influence on neutrophil chemotaxis (Amsterdam *et al* 1993).

The effect of cyclooxygenase inhibitors on ischaemic reperfusion injury is unclear. Ibuprofen can reduce myocardial infarct size and neutrophil accumulation but treatment with other cyclooxygenase inhibitors has not shown beneficial effects (Jugdutt *et al* 1979 and Bonow *et al* 1981), though some of these agents may inhibit neutrophil function *in vitro* (Kaplan *et al* 1984). Recent evidence, however, has demonstrated that PAF antagonists can reduce tissue necrosis and injury after ischaemia and reperfusion to attenuate myocardial stunning after brief periods of coronary occlusion (Maruyama *et al* 1990 and Ma *et al* 1992).

We feel that *in vivo* it is extremely important to block specific factor (s) and not to use agents that have wide reaching implications and therefore side effects. Although our results show that cycloheximide prevents neutrophil adhesion post hypoxia, and therefore the two subsequent facets of the injurious process, it is obviously not

beneficial to block protein synthesis in a patient. To be able to be more specific we need to have a fuller understanding of the magnitude and chronology of any factors produced. Then by simply blocking the initial factor produced it will be possible to prevent generation of all subsequent factors that promote neutrophil adhesion, migration and activation.

8.6 Inhibition of proteases

Our results show that neutrophils become activated following periods of hypoxia and reoxygenation. By inhibiting the injurious material, such as proteases, it is theoretically possible to prevent damage. In rats subjected to permanent coronary occlusion, myocardial proteolysis is apparently primarily mediated by cathepsins and calcium-activated neutral proteases, but proteolysis is not significantly increased during the first 24 hours of occlusion (Bolli *et al* 1983). However, in this model myocardial infarct size was not reduced by a combination of protease inhibitors that almost completely suppressed proteolysis. These results show that dysfunction goes beyond protease production suggesting that its inhibition would not completely attenuate injury. As such it would not provide a realistic therapeutic option.

8.7 Dapsone

Dapsone, an anti-inflammatory sulphone, is frequently used in cutaneous disease characterized by immune complex deposition and accumulation of neutrophils. We have been able to show that the heightened neutrophil adhesion observed after hypoxia and reoxygenation is significantly lowered following treatment of the neutrophils with dapsone ($p<0.05$). Furthermore dapsone attenuated neutrophil migration towards hypoxic/reoxygenated ECV supernatant and production of an oxidative burst (Figure 4.7 and 5.7). These findings highlight a perhaps new novel way of preventing ischaemic/reperfusion injury.

Further possibilities for preventing neutrophil dysfunction post hypoxia, not investigated in this thesis due to time constraints, are;

8.8 Enhancement of nitric oxide availability

The mechanism by which superoxide mediates neutrophil adhesion is not clear. However, it has been suggested that superoxide may interact with and inactivate an endothelial cell-derived anti-adhesive substance. Nitric oxide (NO) is a biologically active substance produced by endothelial cells, and it is inactivated by superoxide (Gryglewski *et al* 1986 and Rubanyl and Vanhoutte 1986). NO has been shown to inhibit neutrophil aggregation *in vitro* (McCall *et al* 1988). Superfusion of cat mesentery with an NO inhibitor results in a large increase in the number of adherent neutrophils. Based on these observations one would predict that decreased NO production or increased NO inactivation would promote neutrophil adherence, and superoxide dismutase should be anti-adhesive. Indeed, Kubes *et al* (1991) have shown that superoxide dismutase attenuates neutrophil adhesion in mesenteric venules while others have shown that NO affords protection against intestinal reperfusion injury (Gryglewski *et al* 1986). These observations are consistent with the hypothesis that the beneficial actions of superoxide dismutase may in part be attributed to its ability to prevent NO inactivation.

8.9 Adenosine

In addition to its well known cardiovascular effects (Balardinelli *et al* 1989) adenosine is a strong inhibitor of neutrophil respiratory burst (Cronstein *et al* 1983) and prevents neutrophil adherence and cytotoxicity to endothelial cells *in vitro* (Cronstein *et al* 1986). Myocardial adenosine release increases in response to ischaemia (Balardinelli *et al* 1989) and adenosine thus acts as an endogenous modulator of the inflammatory process (Engler 1991). In agreement with this hypothesis, administration of adenosine can reduce myocardial infarct size, postischaemic neutrophil accumulation, endothelial damage and contractile dysfunction (Babbitt *et al* 1989), and AICA-riboside, an agent that increases adenosine release from energy-deprived cells, may decrease neutrophil accumulation and increase collateral blood flow to ischaemic myocardium (Engler 1987 and Gruber *et al* 1989).

8.11 Pentoxifylline

Pentoxifyllin, a methylxanthine used in the treatment of peripheral occlusive disease, inhibits neutrophil activation *in vitro* (Currie *et al* 1990 and Freyburger *et al* 1990) and reduces neutrophil-dependent vascular injury in experimental models of sepsis (Yonemaru *et al* 1991). Neutrophil inhibition by pentoxifyllin may be mediated by phosphodiesterase inhibition with increased intracellular levels of cAMP (Bessler *et al* 1986), and pentoxifylline and adenosine synergistically decrease neutrophil superoxide anion production (Thiel *et al* 1991). Recent evidence has indicated that pentoxifylline can decrease myocardial neutrophil accumulation and coronary endothelial injury after experimental ischaemia and reperfusion (Dauber *et al* 1992).

In summary this study has made a substantial contribution to understanding the nature of the relationship between neutrophils and ischaemic/reperfusion injury by following the three apparent facets of the injurious process. We have illustrated conclusively that neutrophil adhesion, migration and activation are all significantly augmented following hypoxia and reoxygenation. Furthermore our *in vitro* models have highlight areas where intervention may ameliorate injury. In so doing we have raised a multitude of questions and opened up many possible avenues of investigation. By pursuing one or more of these lines of investigation it is hoped that one would be closer to understanding the precise role of neutrophils in the pathogenesis of reperfusion injury.

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APPENDIX I

AAH Pharmaceuticals
Kingswindford
West Midlands
UK

Baxters Healthcare Ltd
Thetford
Norfolk
UK

Gibco Laboratories
Paisley
Scotland

NIBSC
Potters Bar
Hertfordshire
UK

Pharmacia Biotech
Sweden

Sigma Chemical Company Ltd
Poole
Dorset
UK