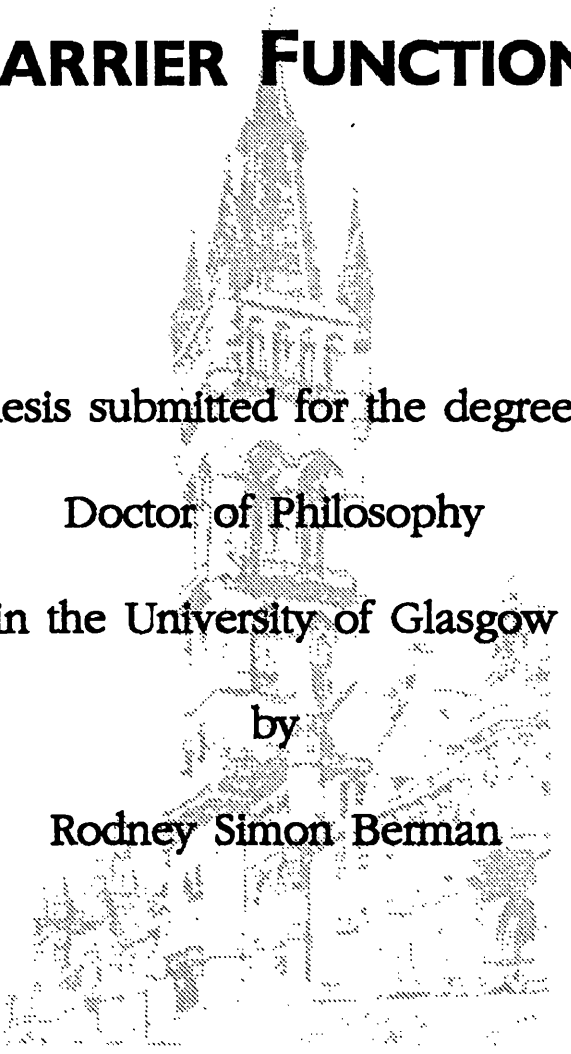
MODULATION OF ENDOTHELIAL BARRIER FUNCTION



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Statistical Analysis was performed using Minitab.

SUMMARY

A. Effects of the hypoxanthine-xanthine oxidase system and homocysteine on endothelial barrier function

1. Endothelial barrier function was assessed by use of an *in vitro* model in which the transfer of trypan blue-labelled albumin was measured across monolayers of bovine aortic endothelial cells (BAEC) grown on polycarbonate membranes.

2. Addition of either hypoxanthine (0.2 mM) or xanthine oxidase (20 mU ml^{-1}) alone to BAEC monolayers during a 90 minute incubation period was found to have no effect on the level of albumin transfer obtained, but a combination of both was found to significantly increase transfer.

3. The increase in albumin transfer induced by hypoxanthine and xanthine oxidase was abolished by catalase (3 U ml^{-1}), significantly reduced by allopurinol (4 mM), but unaffected by superoxide dismutase (6000 U ml^{-1}), the hydroxyl radical scavengers, mannitol (15 mM), dimethylthiourea (10 mM) and N-(2-mercaptopropionyl)-glycine (1 mM), overnight pretreatment with the iron chelator, deferoxamine (0.5 mM), ferric chloride (50 μM), an inhibitor of nitric oxide synthase, N^{ω}-nitro-L-arginine (L-NOARG), or the antioxidant, dithiothreitol (3 mM).

4. Addition of xanthine (0.2 mM) in combination with xanthine oxidase (20 mU ml^{-1}) generated a similar increase in albumin transfer across BAEC monolayers to that obtained using hypoxanthine in combination

with xanthine oxidase. The increase induced by xanthine and xanthine oxidase was similarly abolished by catalase (3 U ml^{-1}).

5. Hydrogen peroxide (0.1–30 mM) itself induced an increase in albumin transfer across monolayers of BAEC, exhibiting a biphasic concentration-response curve with peaks at around 0.1–0.3 mM and 10–30 mM. The increase in albumin transfer induced by 0.1 mM was abolished by 0.3 U ml^{-1} catalase, whilst that induced by 10 mM hydrogen peroxide was abolished by 3000 U ml^{-1} catalase.

6. Homocysteine (0.5 and 1.5 mM) was found to have no effect on the level of albumin transfer across BAEC monolayers which was obtained when it was added alone. However, when it was added in combination with copper sulphate (5 and 50 μM) which catalyses its oxidation to homcystine, a significant increase in albumin transfer was observed.

7. The increase in albumin transfer induced by the combination of homocysteine (1.5 mM) and copper sulphate (50 μM) was abolished by catalase (1 U ml^{-1}), but was unaffected by superoxide dismutase (6000 U ml^{-1}), mannitol (15 mM), dimethylthiourea (1 mM) or overnight pretreatment with deferoxamine (0.5 mM).

8. The data suggest that the endothelial barrier dysfunction induced by the combination of hypoxanthine and xanthine oxidase is likely mediated solely by the actions of hydrogen peroxide and not by superoxide anion, hydroxyl radical, peroxynitrite anion, nitric oxide or hypochlorous acid. Also, it was shown that xanthine and hypoxanthine may both equally well be used as substrates for xanthine oxidase in order to induce endothelial barrier dysfunction.

These findings further indicate that the endothelial barrier dysfunction which is associated with ischaemia-reperfusion injury could well be mediated by the hypoxanthine-xanthine oxidase system which is known to be activated in this condition.

9. The data also indicate that endothelial barrier dysfunction is induced by the copper-catalysed oxidation of homocysteine, rather than by a direct action of homocysteine itself. This dysfunction is also likely mediated solely by hydrogen peroxide and not by superoxide anion or hydroxyl radical. This ability of homocysteine to induce endothelial barrier dysfunction in the presence of copper may contribute to the atherogenic actions of homocysteine observed in sufferers of homocystinuria.

B. Effects of lipopolysaccharide (LPS) on endothelial barrier function

10. Following 24 hours' incubation, after which the transfer of albumin across monolayers of BAEC was measured, LPS (0.1–1000 ng ml^{-1}) was found to induce a concentration-dependent increase in albumin transfer.

11. The increase in albumin transfer induced by LPS (30 ng ml^{-1}) was found to develop with a biphasic time-course. An early, transient peak was observed which was maximal at around 2 hours. The level of albumin transfer then declined back towards basal levels before rising again, nearing a second maximum by 24 hours.

12. The increase in albumin transfer induced following 24 hours' incubation with LPS (30 ng ml^{-1}) was abolished by polymixin B

(10 μg ml^{-1}), enhanced by the nitric oxide synthase inhibitor, L-NMMA (2 mM), but unaffected by the nitric oxide synthase inhibitors, L-NOARG (100 μM) and L-NAME (500 μM), the cyclo-oxygenase inhibitor, flurbiprofen (30 μM), catalase (1000 U ml^{-1}) or by a 20 hour pretreatment with the glucocorticoid, dexamethasone (1 μM).

13. The increase in albumin transfer induced following 2 hours' incubation with LPS (30 ng ml^{-1}) was abolished by polymixin B (10 μg ml^{-1}), enhanced by L-NMMA (2 mM), but unaffected by L-NAME (500 μM) or by a 20 hour pretreatment with dexamethasone (1 μM).

14. Following 24 hours' incubation, human recombinant interferon-γ (IFN-γ) (0.75–75 U ml^{-1}) was found to have no effect on the level of albumin transfer obtained across monolayers of BAEC.

15. The data show that LPS can induce endothelial barrier dysfunction through a direct action on the endothelium which develops with a biphasic time-course. Neither the increase induced by LPS after 2 hours' incubation, nor that obtained after 24 hours' incubation, is mediated by production of nitric oxide. Additionally, the increase induced by LPS after 24 hours' incubation is also not mediated by a cyclo-oxygenase product or hydrogen peroxide, and is unlikely to be mediated by IFN-γ. These findings may be of relevance to the profound vascular leakage observed in septic shock of which LPS is a common cause.

CONTENTS

LIST OF FIGURES

ABBREVIATIONS

5-HT 5-hydroxytryptamine
ADP ..... adenosine diphosphae
AIDS ..... acquired immuno-deficiency syndrome
ARDS ..... adult respiratory distress syndrome
ALLO ..... allopurinol
AMP ..... adenosine monophosphate
ATP ..... adenosine triphosphate
BAEC ..... bovine aortic endothelial cells
CAT ..... catalase
DAG ..... diacylglycerol
DEF ..... deferoxamine
DEX ..... dexamethasone
DMEM ..... Dulbecco's modification of Eagle's medium
DMTU 1,3-dimethyl-2-thiourea
DNA ..... deoxyribonucleic acid
DTT ..... dithiothreitol
EC ..... endothelial cells
EDRF ..... endothelium-derived relaxing factor
EDTA ..... ethylenediamene tetraacetic acid
F-met-leu-phe ..... formylmethionine-leucine-phenylalanine
FAD ..... flavin adenine dinucleotide (oxidised from)
FBP ..... flurbiprofen
FITC-dextran ..... fluorescein-isothiocyanate-labelled dextran
FMN ..... flavin mononucleotide (oxidised form)
GMP ..... guanosine monophosphate
HC ..... homocysteine
HEPES ..... N-2-hydoxyethylpiperazine-N´-2-ethanesulphonic acid

HX ..... hypoxanthine

IFN-γ ..... interferon-γ

IL-1 ..... interleukin-1

IL-2 ..... interleukin-2

IL-6 ..... interleukin-6

L-NIO ..... N^{ω}-iminoethyl-L-ornithine

L-NAME ..... N^{ω}-nitro-L-arginine methyl ester

L-NOARG ..... N^{ω}-nitro-L-arginine

L-NMMA ..... N^{ω}-monomethyl-L-arginine

LDL ..... low density lipoprotein

LPS ..... lipopolysaccharide

MAN ..... mannitol

MPG ..... N-(2-mercaptopropionyl)-glycine

NAD ..... nicotinamide adenine dinucleotide (oxidised from)

NADH ..... nicotinamide adenine dinucleotide (reduced from)

NADPH .... nicotinamide adenine dinucleotide phosphate (reduced from)

NANC ..... non-adrenergic, non-cholinergic

NSAID ..... non-steroidal anti-inflammatory drug

OAG ..... oleoyl acetyl glycerol

PAF ..... platelet activating factor

PMA ..... phorbol myristate acetate

PMB ..... polymixin B

SOD ..... superoxide dismutase

TNF-α ..... tumour necrosis factor-α

X ..... xanthine

XO ..... xanthine oxidase

PUBLICATIONS

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INTRODUCTION

CHAPTER 1

1.1 The vascular endothelium

The vascular endothelium comprises a single layer of cells, resting on a basement membrane, which lines the luminal side of every blood vessel within the body, as well as the chambers of the heart. It forms an interface between the blood and the underlying interstitium, thereby exerting a regulatory effect on both these components as well as controlling the passage of solutes, fluids and cells from the blood into the interstitial space.

The tone of the vascular smooth muscle is regulated by a number of vasoactive agents which are either released from cells into the bloodstream, from nerve terminals in the vessel wall or are secreted by the endothelium. Included in this last category are two vasodilator substances, prostacyclin (Moncada *et al.,* 1976) and endothelium-derived relaxing factor (EDRF) (Furchgott & Zawadzki, 1980), and the endothelins, a group of vaso-constrictor peptides (Yanagisawa *et al.,* 1988).

The endothelium also provides a non-thrombogenic lining to the blood vessel both due to the nature of the endothelial cell surface and through the release of EDRF and prostacyclin which inhibit platelet aggregation (Moncada *et al.,* 1976; Azuma *et al.,* 1986; Furlong *et al.,* 1987; Radomski *et al.,* 1987a). Additionally, EDRF has been shown to inhibit platelet adhesion (Radomski *et al.,* 1987b), whilst prostacyclin has been shown to inhibit the adhesion only of activated platelets (Fry *et al.,* 1980). Under certain circumstances, such as in response to tissue injury, the endothelium may also promote platelet adhesion and aggregation through the production of

collagen (Sage *et al.*, 1979), thrombospondin (Mosher *et al.*, 1982), fibronectin (Jaffe & Mosher, 1978) and von Willebrand factor (Jaffe *et al.*, 1973).

1.1.1 Endothelium-derived relaxing factor (EDRF)

EDRF was first described by Furchgott & Zawadzki (1980) who observed that acetylcholine-induced relaxation of pre-constricted rabbit aortic strips was dependent on the presence of the endothelium. They also showed using a "sandwich" preparation in which an endothelium-denuded transverse aortic strip was co-mounted with an endothelium-containing longitudinal strip (which due to its orientation contributed only negligibly to the contraction recorded), that following pre-constriction with phenylephrine, acetylcholine induced the release of a relaxant from the endothelium of the longitudinal strip which could relax the transverse strip. From these observations it was postulated that the endothelial cells were releasing a factor, in response to stimulation with acetylcholine, which can relax vascular smooth muscle. This factor was termed EDRF.

Subsequent research has shown that EDRF release may be induced by a wide variety of stimuli including bradykinin, ATP, thrombin, histamine, substance P and the calcium ionophore, A23187 (Furchgott, 1984). Endothelium-dependent relaxation of vascular smooth muscle has also been shown to be induced by hypoxia, increased blood flow and electrical stimulation (see Moncada *et al.*, 1988 for review).

Following on from the "sandwich" preparation described by Furchgott & Zawadzki (1980), cascade bioassay techniques have been used to confirm the humoral nature of EDRF. For instance, Griffith *et al.* (1984) showed that the perfusate passing through pre-constricted endothelium-containing

aortic segments stimulated with acetylcholine or A23187 could subsequently relax segments of endothelium-denuded coronary artery. This study also revealed that EDRF had a biological half-life of around 6 seconds.

EDRF has also been shown to be an inhibitor of platelet aggregation (Azuma *et al.*, 1986) and adhesion (Radomski *et al.*, 1987b), and a rise in cellular levels of cyclic GMP following stimulation of soluble guanylate cyclase is associated with both endothelium-dependent inhibition of platelet aggregation and endothelium-dependent vascular relaxation (Rapoport & Murad, 1983; Busse, 1987). Moreover, selective inhibitors of cyclic GMP phosphodiesterase, such as M&B 22948 (Zaprinast) and MY 5445, have been shown to potentiate endothelium-dependent relaxation (Martin *et al.*, 1986a), as well as the actions of EDRF on smooth muscle (Kukovetz *et al.*, 1982) and platelets (Radomski *et al.*, 1987b).

The breakdown of EDRF has been shown to be inhibited by superoxide dismutase (Gryglewski *et al.*, 1986) thus suggesting that it is destroyed by superoxide anion ($\cdot O_2^-$). Superoxide dismutase also prevents the action of various EDRF inhibitors, such as pyrogallol, dithiothreitol and hydroquinone, suggesting that these act by generating $\cdot O_2^-$ (Moncada *et al.*, 1986). Other inhibitors of EDRF include haemoglobin, which acts by binding the EDRF molecule (Martin *et al.*, 1986b), and methylene blue which inhibits soluble guanylate cyclase (Gruetter *et al.*, 1981).

1.1.2 The L-arginine-nitric oxide pathway

In 1986, both Furchgott and Ignarro independently suggested that EDRF might be nitric oxide or a nitric oxide-like substance (see Furchgott, 1988; Ignarro *et al.*, 1988). Evidence that this is in fact the case was subsequently provided by Palmer *et al.* (1987). Using a cascade bioassay,

they showed that addition of exogenous nitric oxide mimicked the ability of EDRF, released from porcine aortic endothelial cells grown on microcarrier beads, to relax spiral strips of endothelium-denuded rabbit aorta. Both nitric oxide and EDRF were found, using this system, to have similar biological half-lives of around 3 seconds, and, like EDRF, nitric oxide was found to be inactivated by $\cdot O_2^-$. The most compelling evidence, however, was obtained using a chemiluminescence assay which directly demonstrated that bradykinin induced the release of nitric oxide from cultured endothelial cells in quantities similar to those required to cause a relaxation using the cascade bioassay. Other work, using spectrophotometry to measure the formation of nitric oxide-bound haemoglobin from deoxyhaemoglobin (Ignarro *et al.*, 1987b) or the diazotisation of sulphanilic acid (Ignarro *et al.*, 1987a), confirmed the identification of EDRF as either nitric oxide or a labile nitroso compound.

Further work, using mass spectrometry, revealed that the nitric oxide produced by both macrophages (Iyengar *et al.*, 1987) and endothelial cells (Palmer *et al.*, 1988a) originates from one of the two terminal guanadino nitrogen atoms of L-arginine with L-citrulline being formed as a co-product (see Figure 1.1). Also, the formation of L-citrulline from L-arginine in macrophages (Hibbs, Jr. *et al.*, 1987), and of nitric oxide from L-arginine in cultured endothelium (Palmer *et al.*, 1988b), was found to be inhibited by the L-arginine analogue, N^{ω}-monomethyl-L-arginine (L-NMMA).

The enzyme, nitric oxide synthase, which synthesises nitric oxide from L-arginine in endothelial cells, was subsequently studied using endothelial homogenates and characterised (Palmer & Moncada, 1989). It was found to be both NADPH- and Ca^{2+}-dependent (Mayer *et al.*, 1989; Mülsch *et al.*, 1989). Also, its activity was shown to be inhibited by calmodulin-

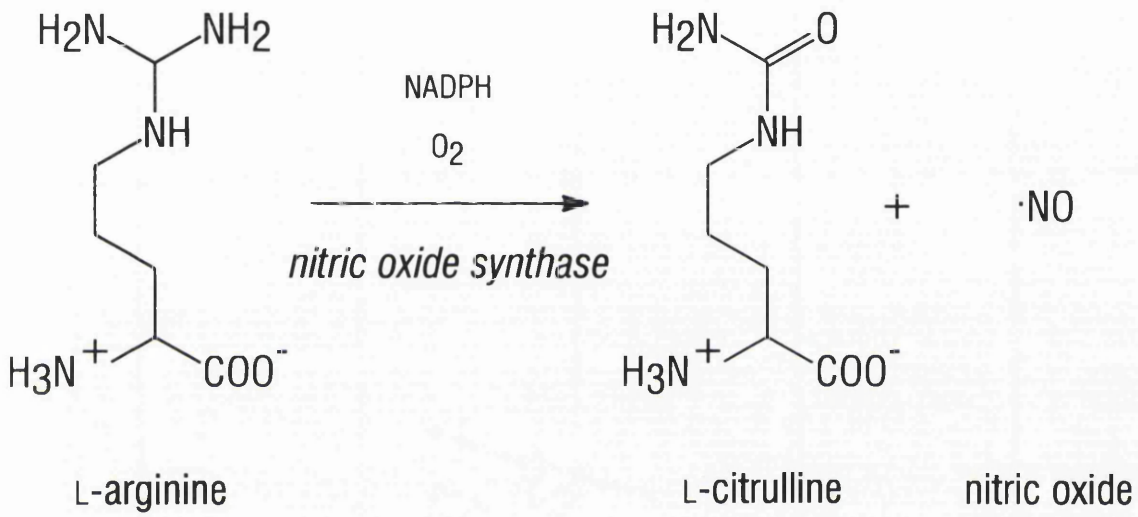


Figure 1.1 The nitric oxide synthase-catalysed formation of nitric oxide and L-citrulline from L-arginine.

binding peptides and antagonists suggesting that its Ca^{2+}-dependence is mediated by calmodulin (Busse & Mülsch, 1990a).

In addition to L-NMMA, other analogues of L-arginine have been shown to inhibit the synthesis of nitric oxide in endothelial cells including N^{ω}-nitro-L-arginine (L-NOARG), its more soluble, methyl ester form, N^{ω}-nitro-L-arginine methyl ester (L-NAME), and N^{ω}-iminoethyl-L-arginine (L-NIO) (Moore *et al.*, 1990; Rees *et al.*, 1990).

1.1.3 Nitric oxide synthase isoforms

In addition to vascular endothelial cells, nitric oxide synthases have been found to be present in a variety of cell types. Based on their mechanisms of regulation, two main isoforms have been shown to exist—termed *constitutive* and *inducible*, respectively. The constitutive enzyme binds calmodulin in a reversible, Ca^{2+}-dependent manner and therefore may be activated by agonists which elevate intracellular Ca^{2+} levels. The inducible enzyme was originally believed to be both Ca^{2+}- and calmodulin-independent but has recently been shown to bind calmodulin so tightly as to effectively be irreversibly bound (Cho *et al.*, 1992). Thus, its activity is unaffected by exogenous levels of both Ca^{2+} and calmodulin, and instead has been shown to be regulated by the induction of transcription (Xie *et al.*, 1992) and the availability of co-factors. For instance, it has been shown that induction of an inducible nitric oxide synthase may be accompanied by induction of GTP cyclohydrolase, an enzyme required for the formation of tetrahydrobiopterin, a necessary co-factor for nitric oxide synthesis (Hattori & Gross, 1993).

In addition to tetrahydrobiopterin and Ca^{2+}/calmodulin, binding sites for various cofactors required for nitric oxide synthesis have been identified

in both constitutive and inducible nitric oxide synthases, including NADPH, FAD, FMN and iron protoporphyrin IX (a haeme-containing group) (see Marletta, 1993 for review). The conversion of L-arginine to nitric oxide and L-citrulline involves a 5-electron oxidation and it is thought that NADPH, FAD, FMN and iron protoporphyrin IX are involved in the electron transfer process. Calcium and calmodulin play a regulatory role, whilst tetrahydrobiopterin is thought to be involved in stabilising the enzyme.

Constitutive nitric oxide synthases have now been shown to be present in a variety of cell types including endothelial cells, certain central neurones, certain peripheral non-adrenergic, non-cholinergic (NANC) neurones, blood neutrophils, mast cells, platelets, adrenal medullary cells, pancreatic β cells and astrocytes. Inducible nitric oxide synthases have been shown to be present following immunological stimulation in, amongst others, macrophages, Kupffer cells, hepatocytes, vascular smooth muscle cells, endothelial cells, inflammatory neutrophils, fibroblasts, astrocytes and mesangial cells (see Nathan, 1992 for review).

The constitutive enzyme has been purified in both brain (Bredt & Snyder, 1990; Mayer *et al.*, 1990; Schmidt & Murad, 1991) and vascular endothelial cells (Pollock *et al.*, 1991), whilst the inducible enzyme has been purified in macrophages (Stuehr *et al.*, 1991; Hevel *et al.*, 1991). Both the constitutive nitric oxide synthase found in brain and the inducible nitric oxide synthase found in macrophages have been shown to be cytosolic enzymes, whilst the constitutive enzyme purified from vascular endothelium was found to be membrane-bound.

At present, therefore, there would appear to be at least three distinct isoforms of nitric oxide synthase—two constitutive and one inducible. Additionally, at least three nitric oxide synthase genes have so far been

identified (Nathan, 1992), with at least one of these giving rise to enzyme isoforms of different molecular weights through alternative splicing.

1.2 Endothelial barrier function

The vascular endothelium acts as an important barrier between the components of the blood and the underlying tissues, thereby fulfilling a key homeostatic role within the body. The ability of molecules to cross this barrier is determined both by their molecular size and charge. Smaller, non-polar molecules are more easily able to cross than either charged molecules or those of high molecular weight.

Classically, the endothelium was regarded to have a fixed hydraulic conductivity. Only physical properties such as mass and charge were believed to affect its permeability to different molecules, with the endothelium itself playing a passive role (Starling, 1896). A pore theory was subsequently put forward which suggested that passive pores existed in the endothelium to allow molecules to cross (Pappenheimer, 1953). These pores existed either running across the endothelial cells themselves or passing through the junctions between them. Additionally, there were believed to be two distinct sizes of pore—termed small and large pores respectively—with there being substantially greater numbers of small pores thus explaining why small molecules could more easily cross than large molecules (Grotte, 1956).

This classical view likened the endothelial barrier to that of a semi-permeable membrane and, indeed, filtrates of plasma through artificial semi-permeable membranes with pores of a certain size have been found to be comparable to capillary filtrates (Grega, 1986a). This model, however, cannot take

account of the significantly increased protein efflux, across the endothelial barrier from the blood to the interstitium, which is observed in inflammation.

Evidence that the endothelial cells themselves could influence permeability followed primarily from observations that the presence of various mediators could enhance the transport of macromolecules across the endothelial barrier as compared to that obtained under basal conditions (Grega, 1986b). These findings conflicted with the idea of the endothelial barrier as being one which was passive and not under physiological regulation and suggested that the endothelial cells were, in fact, active functional units which were involved in the regulation of endothelial permeability.

Two possible routes exist by which molecules can cross the endothelial barrier—*intracellular* (through the cells) and *paracellular* (between the cells)—with evidence having been shown for the existence of both routes. Debate, however, still surrounds the mechanism by which macromolecules cross the endothelium with some groups arguing that an extensive intracellular system of vesicular transport is the most important (Simionescu *et al.*, 1987), whilst others argue that such transport occurs mainly by a paracellular route through pores between endothelial cells (Rippe & Haraldsen, 1987).

1.2.1 Intracellular routes of transendothelial passage

Intracellular routes across the endothelial barrier have been shown to exist in various forms. Several studies using tracer molecules have revealed numerous plasmalemmal vesicles within the endothelial cell and it has been hypothesised that such vesicles form the basis of transport mechanisms across the endothelial barrier. However, since these studies are based on the use of electron microscopy, they only reveal the state of

the endothelium at any one fixed moment in time and hence three different hypotheses have been put forward as to exactly how these vesicles may contribute to transendothelial passage (shown diagrammatically in Figure 1.2).

The first hypothesis to be put forward has been referred to as the "shuttle" hypothesis (Palade, 1960). According to this hypothesis, vesicles containing molecules to be transported bud off from the plasmalemma on one side of the endothelial cell, traverse the cell cytoplasm and then fuse with the plasmalemma on the opposite side of the cell to release their contents. These vesicles may either form *de novo,* when required, by plasmalemmal invagination or, alternatively, may be more permanent structures which maintain a stable presence on fusing with the plasmalemma and can again bud off to become free cytoplasmic vesicles.

The second hypothesis of transendothelial vesicular transport has been referred to as a fusion-fission process (Clough & Michel, 1981). According to this hypothesis, vesicles do not traverse fully across the endothelial cell, but instead only move a short distance to fuse with neighbouring vesicles. This enables each vesicle to transfer its contents into its neighbour which subsequently buds off from it to fuse with another vesicle. This process continues until the plasmalemma on the opposite side of the endothelial cell is reached, whereupon any molecules which had entered the original vesicle are released. In a variant of this hypothesis, groups of vesicles may be fused together forming clusters that are separated from each other by diaphragms. These diaphragms form and reform on a cyclical basis, allowing molecules to progress across the cell in stages as they progress from one cluster to the next.

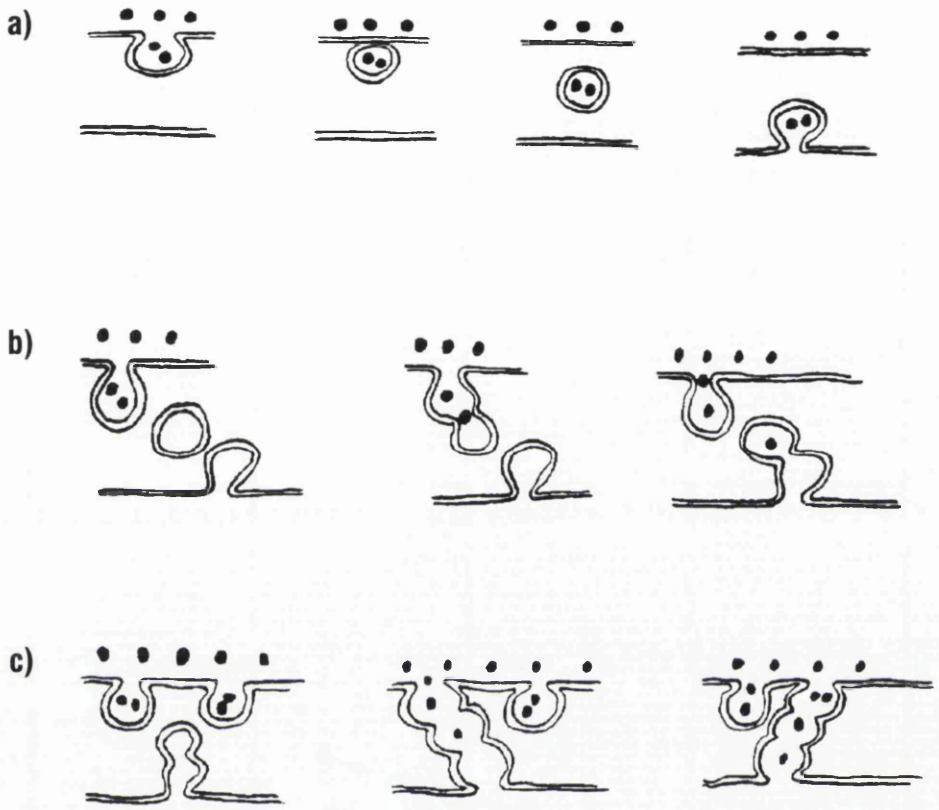


Figure 1.2 Diagrammatic representation of the three proposed hypotheses of transendothelial vesicular transport mechanisms. Reproduced from Michel, 1992. **a)** the "shuttle" hypothesis. **b)** the "fusion-fission" hypothesis. **c)** transendothelial vesicular channels.

In the third hypothesis, vesicles form fused structures which create channels across the endothelium, running from the luminal surface through to the abluminal surface (Simionescu *et al.*, 1975). Thus, molecules can easily traverse the endothelium from one side to the other by passing along these channels. These channels may be permanent or, alternatively, may be transient in their existence, but with their numbers at any one time in a given area remaining constant.

1.2.2 Paracellular routes of transendothelial passage

The first evidence for a paracellular route of transendothelial passage was provided by Majno & Palade (1961) from electron microscopic studies of rat cremaster muscle. Using tracer molecules of colloidal HgS given by intravenous injection, they observed the formation of intercellular gaps on subsequent administration of the inflammatory mediators, histamine and 5-HT. Further investigation revealed that these intercellular gaps were confined to the venous side of the circulation and occurred predominately at the post-capillary venule (Majno *et al.*, 1961). Later work showed that the formation of intercellular gaps was accompanied by deformations in the endothelial cell nuclei and this was regarded as a sign of cell contraction (Majno *et al.*, 1969). This lead to the hypothesis that inflammatory mediators induce an increase in endothelial permeability by causing the contraction of individual endothelial cells away from each other, thereby leading to intercellular gap formation.

Studies of the effects of mediators on endothelial permeability have also been shown using hamster cheek pouch preparations (Duling, 1973; Svensjö *et al.*, 1978). In this model, fluorescein-isothiocyanate-labelled dextran (FITC-dextran) is injected intravenously into hamsters prior to the administration of mediators. Intravital light microscopy is then used to

identify sites of vascular leakage, shown by the leakage of the fluorescent FITC-dextran, which form at post-capillary venules in the cheek pouch. Using such a model, Svensjö & Grega (1986) demonstrated the ability of various inflammatory mediators, including leukotrienes (C_4, D_4, E_4 and B_4), bradykinin, histamine, 5-HT, substance P, complement 3a, complement 5a, PAF and prostaglandins (E_1, E_2 and $F_{2\alpha}$), to increase permeability at the post-capillary venule through the formation of intercellular gaps.

Venular endothelial cells have been found to possess receptors for a wide variety of inflammatory mediators (Buonassisi & Venter, 1976). For instance, histamine H_1 receptors are found in large numbers in venular endothelial cells where they are localised in close proximity both to cell junctions and to contractile proteins (Heltianu *et al.,* 1982). Also, it has been shown that vascular leakage may be stimulated by selective H_1 agonists, whilst histamine-induced vascular leakage may be blocked by H_1 antagonists (Guth & Hirabayashi, 1983) confirming that the actions of histamine on endothelial barrier function are receptor-mediated.

1.2.3 Role of contractile proteins in intercellular gap formation

The hypothesis that inflammatory mediators cause the formation of intercellular gaps through cell contraction was lent further weight by the identification of contractile proteins, such as actin, myosin, tropomyosin, α-actinin and vinculin, within endothelial cells (Becker & Nachman, 1973; Drenckhahn, 1983; Gabbiani *et al.,* 1983; Drenckhahn & Wagner, 1986). Although the presence of contractile proteins does not necessarily indicate a contractile function, the distribution of contractile proteins within venular endothelial cells was found to be different from that found in arterial endothelial cells and this is therefore consistent with the fact that inflammatory mediators only increase permeability on the venular side of

the circulation (Grega, 1986a). This lack of activity may, however, also be due to a lack of receptors for inflammatory mediators in arterial endothelial cells.

Further confirmation that endothelial cell contractile proteins are involved in the development of an increase in permeability was shown by Shasby *et al.* (1982). In their study using porcine pulmonary artery endothelial cells, two microfilament disrupting agents, cytochalasin B and cytochalasin D, were shown to induce an increase in endothelial monolayer permeability to albumin. Microscopic studies confirmed that these agents were causing a disruption of microfilament bundles and revealed a retraction of the cell cytoplasm consistent with cell contraction. Furthermore, on addition of histamine to monolayers of human umbilical vein endothelial cells, Rotrosen & Gallin (1986) have reported a decrease in cytoplasmic F-actin content. More recently, using an *in vitro* model of endothelial permeability utilising monolayers of calf pulmonary artery endothelial cells, Phillips *et al.* (1989) demonstrated that thrombin-induced increases in permeability were inhibited in the presence of phallacidin, an actin-stabilising agent which prevents its activation.

1.2.4 Role of calcium (Ca^{2+}) in intercellular gap formation

Many studies have suggested a requirement for Ca^{2+} to facilitate endothelial cell contraction in intercellular gap formation. The first evidence was shown by Liddell & Simpson (1980) who found that histamine-induced gap formation between venular endothelial cells was abolished when EDTA was used to remove Ca^{2+} from the extracellular fluid. It has also been shown that the increase in venular permeability induced by histamine in the hamster cheek pouch can be blocked by the calcium channel blocker, verapamil (Mayhan & Joyner, 1984). Furthermore, using

the fluorescent Ca^{2+} probe, quin 2, Rotrosen & Gallin (1986) demonstrated that histamine causes both mobilisation of intracellular Ca^{2+} and influx of extracellular Ca^{2+} in human umbilical vein endothelial cells and this occurs over a similar concentration range to the histamine-induced permeability increase which can be observed using monolayers of these cells *in vitro*.

Evidence for Ca^{2+}-dependence has also been shown in thrombin-induced permeability increases. For instance, Lum *et al.* (1989) demonstrated both that thrombin induced an influx of Ca^{2+} into bovine pulmonary artery endothelial cells and induced an increase in albumin permeability across monolayers of these cells which was calcium-dependent. Evidence for this Ca^{2+}-dependence was that $LaCl_3$, which competes with Ca^{2+} for entry into cells, caused a reduction in the thrombin-induced permeability increase and, additionally, preloading the cells with quin 2 to buffer intracellular Ca^{2+}, induced a concentration-dependent inhibition.

Further evidence for a role of Ca^{2+} in mediating increases in endothelial permeability has been shown in various studies using the Ca^{2+} ionophore, A23187 which facilitates Ca^{2+} entry into endothelial cells. *In vivo*, A23187 has been shown to increase endothelial permeability in cerebral venules (Olesen & Crone, 1986; Olesen, 1987) whilst using *in vitro* models, it has been shown to increase permeability of monolayers of porcine pulmonary artery (Shasby & Shasby, 1986), porcine aortic (Gudgeon & Martin, 1989) and human umbilical vein (Yamada *et al.*, 1990a) endothelial cells.

In contrast to these findings, research in this laboratory has suggested a dissociation of the effects thrombin on endothelial permeability from its effects on cytosolic calcium levels (Buchan & Martin, 1992). In this study, thrombin was found to induce similar increases in intracellular calcium levels in both bovine pulmonary artery and bovine aortic endothelial cells.

However, thrombin was only found to be able to cause an increase in albumin permeability across monolayers of pulmonary artery endothelial cells, having no effect on the permeability of monolayers of aortic endothelial cells. Moreover, the permeability increase induced by thrombin was found to be inhibited by atriopeptin II and 8 bromo cyclic GMP, which had no effect on intracellular calcium levels, and also by forskolin which in fact enhanced these levels. Buchan & Martin therefore proposed that the effects of thrombin on endothelial barrier function are not mediated by Ca^{2+} and may instead be mediated by other second messengers such as cyclic nucleotides and protein kinase C.

1.2.5 Role of protein kinase C in the regulation of endothelial barrier function

Various studies have indicated a role for protein kinase C in regulating endothelial barrier function. The phorbol ester, phorbol myristate acetate (PMA) which activates protein kinase C has been shown *in vivo* to increase the endothelial permeability of cerebral venules (Olesen, 1987) whilst *in vitro* it has been shown to increase the permeability of monolayers of porcine aortic (Gudgeon & Martin, 1989), porcine pulmonary artery (Shasby *et al.*, 1983), bovine aortic (Oliver, 1990; Buchan & Martin, 1992) and bovine pulmonary artery (Lynch *et al.*, 1990; Buchan & Martin, 1992) endothelial cells. Buchan & Martin (1992) additionally showed that these permeability increases induced by PMA occurred without an accompanying increase in intracellular Ca^{2+} levels thereby providing further evidence for a dissociation of Ca^{2+} from a role as a necessary mediator of permeability increases.

In contrast, using human umbilical vein endothelial cells, Yamada *et al.* (1990a) found that both PMA and another phorbol ester which activates

protein kinase C, oleoyl acetyl glycerol (OAG), caused a decrease in basal monolayer permeability, whilst Morel *et al.* (1990) showed that PMA prevented endothelial cell contraction induced by angiotensin II in cultured bovine pulmonary microvessel endothelial cells.

Evidence however exists that protein kinase C mediates the permeability increases induced by various agents. Lynch *et al.* (1990) showed that H7, a protein kinase C inhibitor, prevented the increases in albumin permeability of bovine pulmonary artery endothelial cell monolayers induced by PMA, thrombin and phospholipase C which is believed to activate protein kinase C through the formation of diacylglycerol (DAG).

1.2.6 Role of cyclic nucleotides in the regulation of endothelial barrier function

Cyclic AMP has been shown to have a regulatory effect on the permeability of epithelium in which it has been demonstrated to enhance tight junction integrity (Duffey *et al.*, 1981). Additionally, β-adrenoceptors agonists, which stimulate adenylate cyclase causing cyclic AMP production, have been shown *in vivo* to inhibit the endothelial permeability increases induced by various inflammatory mediators in the hamster cheek pouch (Svensjö *et al.*, 1979; Svensjö & Grega, 1986). Also, β-adrenoceptors agonists have been shown *in vitro* to inhibit both the PAF- (Grigorian & Ryan, 1987) and thrombin- (Minnear *et al.*, 1989) induced permeability increases in monolayers of bovine pulmonary artery endothelial cells, to inhibit the PMA-induced permeability increase in monolayers of porcine aortic endothelial cells (Gudgeon & Martin, 1989) and to cause the relaxation of bovine pulmonary microvessel endothelial cells (Morel *et al.*, 1990).

More direct evidence for a role of cyclic AMP in inhibiting increases in endothelial permeability has been obtained both using forskolin, which directly activates adenylate cyclase, and from the effects of addition of stable analogues of cyclic AMP, such as dibutyryl cyclic AMP and 8 bromo cyclic AMP. Forskolin has been shown to inhibit the permeability increase induced by PMA in monolayers of porcine aortic (Gudgeon & Martin, 1989), as well as bovine pulmonary artery and bovine aortic (Buchan & Martin, 1992) endothelial cells, and also to inhibit the permeability increase induced by thrombin in monolayers of bovine pulmonary artery endothelial cells (Buchan & Martin, 1992). Additionally, it has been shown, in itself, to decrease the permeability of monolayers of bovine pulmonary artery (Stelzner *et al.,* 1989) and human umbilical vein (Yamada *et al.,* 1990a) endothelial cells.

Dibutyryl cyclic AMP has also been shown to decrease the basal permeability of monolayers of bovine pulmonary artery (Stelzner *et al.,* 1989) and human umbilical vein (Yamada *et al.,* 1990a) endothelial cells and to inhibit the permeability increase induced by PMA in monolayers of porcine aortic endothelial cells (Gudgeon & Martin, 1989). In addition, 8 bromo cyclic AMP has been shown to decrease the basal permeability of monolayers of bovine aortic endothelial cells (Oliver, 1990).

Cyclic GMP has also been shown to exhibit a regulatory effect on endothelial permeability. The membrane-permeant cyclic GMP analogues, 8 bromo cyclic GMP and dibutyryl cyclic GMP, were found to decrease basal permeability in monolayers of human umbilical vein endothelial cells (Yamada *et al.,* 1990a) and, additionally, 8 bromo cyclic GMP was found to inhibit the thrombin-induced permeability increase in monolayers of bovine pulmonary artery cells (Buchan & Martin, 1992). In contrast to agents which elevate cell cyclic AMP levels, however, 8 bromo cyclic GMP

was found to have no effect on the permeability increases induced by PMA in monolayers of porcine aortic endothelial cells (Gudgeon & Martin, 1989), or in monolayers of either bovine aortic or bovine pulmonary artery endothelial cells (Buchan & Martin, 1992).

1.2.7 Role of endothelial barrier function in the inflammatory process

A decrease in endothelial barrier function is a fundamental aspect of the inflammatory process, the mechanism by which the body responds both to microbial infection and to cell or tissue injury. This decrease is important since it facilitates the entry, to a site of injury, of cells and mediators produced by the body to fight infection and to deal with tissue injury.

The first observable effects of the inflammatory process are those of haemodynamic changes which result initially in a substantial increase in blood flow to a site of injury. This is followed by a reduction in blood flow, a decrease in endothelial barrier function and the exudation of fluid to produce oedema. The initial increase in blood flow is brought about by the release of vasodilators some of which have also been shown to increase oedema formation, including histamine (Marciniak *et al.,* 1978), prostacyclin (Rampart & Williams, 1986), prostaglandin E_2 and PAF (Svensjö & Grega, 1986), and these may therefore initiate the decrease in endothelial barrier function. Histamine and PAF, as well as leukotriene B_4, have also been shown to contribute towards the attraction, migration and activation of neutrophils and these contribute to the later, more sustained phase of decreased endothelial barrier function (Kubes, 1993).

The decrease in endothelial barrier function at the site of inflammation enables a variety of mediators to enter the interstitial space from the bloodstream. These include components of the complement, coagulation, fibrinolytic and kinin systems. Many of these mediators themselves contribute to the decrease in barrier function including bradykinin, which can act directly on the endothelium (Svensjö *et al.,* 1979), as well as complement 3a and complement 5a. Both of these complement components act indirectly to decrease barrier function through the release of histamine, whilst complement 5a additionally does so by acting as a chemoattractant for leucocytes including neutrophils.

Many cell types which are normally only present in the blood gain access to tissues during inflammation as a result of the decrease in endothelial barrier function. Polymorphonuclear leucocytes, including neutrophils, eosinophils and basophils, are the first leucocytes to appear at the site of inflammation from the bloodstream. Neutrophils and eosinophils attack invading organisms, which they can engulf and digest, and neutrophils can also release damaging reactive oxygen species (Martin, 1984; Ward *et al.,* 1983) which can further impair endothelial barrier function (Shasby *et al.,* 1983). Monocytes/macrophages enter the area of inflammation several hours later. They can also engulf micro-organisms as well as dead cells and tissue debris, and play a role in the repair process. As well as enzymes, they can secrete complement components, eicosanoids and also cytokines such as interleukin-1 and interferon-γ, both of which have also been shown to impair endothelial barrier function (Campbell *et al.,* 1992; Burke-Gaffney & Keenan, 1993b; Burke-Gaffney & Keenan, 1993a).

1.2.8 *In vitro* techniques for the assessment of endothelial barrier function

Over the last 10–15 years, various *in vitro* models have been developed to determine the effects of exogenously added agents on endothelial barrier function. Such *in vitro* techniques afford many advantages over *in vivo* techniques. For instance, they enable the effects of agents to be tested directly on endothelial cells themselves, whilst eliminating any potentially conflicting and complicating effects these agents may simultaneously be having on other cell types. Also, other physiological effects of the agents being studied which may indirectly affect endothelial barrier function, such as those on blood pressure and blood flow, are circumvented, thereby ensuring only direct actions on barrier function are observed.

In vitro techniques also enable the employment of more precise control over drug concentrations used and allow hydrostatic gradients across the endothelial barrier to be more carefully regulated, thereby facilitating a greater degree of reproducibility in experimental conditions than that afforded by *in vivo* models. Potentially, *in vitro* models also allow a more rapid throughput of experimental testing and may, through the use of cell culture, reduce the cost in animal life required.

The first *in vitro* models employing monolayers of cultured endothelium were established to investigate the migration of other cell types through these monolayers. Using a model system previously established to investigate the adherence of granulocytes (Beesley *et al.,* 1978), the migration of granulocytes through monolayers of porcine endothelial cells isolated from the post-caval vein, was studied (Beesley *et al.,* 1979). Granulocytes were added in suspension to endothelial monolayers cultured both on glass

coverslips and in plastic wells. After an incubation period involving continuous agitation, the cell preparations were appropriately stained and both light and electron microscopy were then used to visualise the extent to which granulocytes had migrated across the monolayers.

In 1981, Taylor *et al.* demonstrated the use of a two-chamber system both to study the migration of neutrophils across monolayers of bovine aortic endothelial cells and to measure the permeability of these monolayers to albumin. Cell monolayers were first grown on polycarbonate micropore filters. Each polycarbonate filter was placed on top of a nitrocellulose filter to which it was sealed using a silicone ring, and a chamber was constructed around these filters to yield an upper compartment above the cell monolayers and a lower compartment below, these representing the luminal and abluminal sides of the endothelium *in vivo*, respectively. Either ^{51}Cr-labelled neutrophils or ^{125}I-labelled albumin was placed above the monolayer which was then incubated at 37°C. After an appropriate incubation period, samples were removed from each upper and lower chamber and counted for radioactivity to assess the extent to which the neutrophils or albumin had crossed the endothelial monolayer. Using this model, endothelial monolayers were found to restrict the passage of both albumin and neutrophils compared to that obtained in the absence of monolayers, and it was found that addition of the chemoattractant F-met-leu-phe to the lower chamber could stimulate neutrophil migration. Thus, the model was concluded to be a useful one for studying both neutrophil migration and the transfer of albumin across monolayers of cultured endothelial cells.

This model as first established by Taylor *et al.* has formed the basis of many subsequently developed models to assess endothelial barrier function *in vitro*. Such models are all based on the principle of a two-chamber

system in which monolayers of endothelial cells cultured on polycarbonate membranes form the junction between the two chambers. Moreover, they have been used extensively to study the effects of various agents on the albumin permeability of endothelial monolayers of various tissues including porcine pulmonary artery (Shasby *et al.*, 1982; Wilson *et al.*, 1990), human umbilical vein (Rotrosen & Gallin, 1986; Casnocha *et al.*, 1989), porcine aorta (Gudgeon & Martin, 1989), bovine pulmonary artery (Stelzner *et al.*, 1989; Phillips *et al.*, 1989; Lum *et al.*, 1989), bovine aorta (Baron *et al.*, 1989), ovine pulmonary artery (Bechard *et al.*, 1990) and rat coronary microvessels (Watanabe *et al.*, 1991). In these studies albumin was either radiolabelled, or labelled with a dye such as trypan-blue (Rotrosen & Gallin, 1986) which facilitates quantification of albumin transfer by a colourometric assay. Alternatively, albumin has been quantified by its reaction with bromcresol green to yield a product detectable by measuring its absorbance at 630 nm (Shasby *et al.*, 1982).

Albumin is widely used as a marker of endothelial barrier function since it is present in the blood and is regarded as a major determinant of endothelial hydraulic conductivity (McCandless *et al.*, 1991). Other tracer molecules have also been used to determine endothelial barrier function in similar two-chamber models including water (Baetschler & Brune, 1983; Suttorp *et al.*, 1988; Malik *et al.*, 1989), horseradish peroxidase (Furie *et al.*, 1984), low density lipoprotein (Territo *et al.*, 1984; Hennig *et al.*, 1985), inulin (Brett *et al.*, 1989; Ogawa *et al.*, 1992), sorbitol (Brett *et al.*, 1989; Ogawa *et al.*, 1992) and culture medium (Schnittler *et al.*, 1990). Beynon *et al.* (1993) demonstrated a model in which the passage of ^{125}I-labelled anti-FITC antibody across endothelial monolayers was measured, having firstly coated the polycarbonate membranes on which the monolayers were grown with FITC-labelled fibronectin.

In the majority of these studies, macromolecular transfer is measured in the absence of a hydrostatic gradient, although some workers have conducted experiments under a specifically determined hydrostatic gradient (Baetschler & Brune, 1983; Suttorp *et al.*, 1988; Suttorp *et al.*, 1991). Suttorp *et al.* (1988) showed that maintaining a constant hydrostatic gradient across porcine pulmonary artery endothelial monolayers produced a slowly developing decrease in the basal permeability of the monolayers to both water and albumin which stabilised after a period of approximately 150 minutes. They suggested that this represented a "sealing" of the monolayers and argued that the use of a hydrostatic gradient represented a more physiologically relevant situation since hydrostatic gradients exist *in vivo*.

Endothelial barrier function has also been studied more simplistically by using microscopy to visualise morphological changes and the formation of intercellular gaps in endothelial cells monolayers exposed to various agents (Antonov *et al.*, 1986). Additionally, fluorescence microscopy has been used to visualise appropriately stained cytoskeletal components of cultured endothelial cells and to observe the responses obtained on the organisation of these components following the addition of agents which affect endothelial barrier function (Morel *et al.*, 1990; Camussi *et al.*, 1991).

Another method of assessing barrier properties of cultured endothelial monolayers is to measure the electrical resistance across them. Furie *et al.* (1984) demonstrated that after a sufficient growth period, monolayers of bovine microvascular endothelial cells generated an electrical resistance above basal levels obtained in the absence of cells, and suggested that the level of electrical resistance across a monolayer could be taken as an indicator of its integrity. Electrical resistance measurements have been used to assess the effects of hydrogen peroxide on endothelial barrier function (Yamada *et al.*, 1990b) and to detect shape changes in endothelial

cells in response to thrombin (Tiruppathi *et al.*, 1992). They have also been used to establish monolayer integrity prior to utilising monolayers for macromolecular transfer studies (Territo *et al.*, 1984) and have been used to correlate with macromolecular transfer studies (Yamada *et al.*, 1990a).

A variant on the two-chamber systems used in macromolecular transport studies is the culture of endothelial cells on microcarrier beads. In a model described by Boiadjieva *et al.* (1984), the passage of trypan blue was measured across confluent bovine aortic and bovine pulmonary artery endothelial cells grown on microcarrier beads. Cells on microcarrier beads were suspended in buffer containing trypan blue, agitated and sampled at intervals. The extent to which the trypan blue had crossed the endothelial cells and entered the underlying microcarrier beads was then determined as an indicator of the barrier function of the endothelium. Killackey *et al.* (1986) described a similar system in which Evans blue was used as an indicator of the barrier function of human umbilical vein endothelial cells.

Over the past 10–15 years, *in vitro* models of the endothelial barrier have enabled a greater insight to be obtained into the many regulatory mechanisms which control the permeability of the endothelium. They are therefore valuable tools for investigating the actions of the many and various agents which have been observed to affect endothelial barrier function.

CHAPTER 2

2.1 Reactive oxygen species

Reactive oxygen species play a wide role in the pathogenesis of many clinical conditions, a selection of which is shown in Table 2.1. The term "reactive oxygen species" includes "oxygen-derived free radicals", such as superoxide anion and hydroxyl radicals, as well as other related species, such as hydrogen peroxide, hypochlorous acid and singlet oxygen, which are not in fact radicals but are nonetheless important damaging species. (The term "radical" is generally taken to refer to molecules which contain a single unpaired electron thus rendering them highly reactive.)

Reactive oxygen species also play an important physiological role. They are released, for example, by phagocytic cells (such as macrophages, monocytes, neutrophils and eosinophils) as part of the inflammatory response to kill invading micro-organisms (Halliwell, 1982; Ahnfelt-Ronne, 1991) and also have been implicated in contributing to the ageing process (Halliwell & Gutteridge, 1989). Certain cellular enzymes, such as ribonucleotide reductase additionally utilise reactive oxygen species in a catalytic role (Reichard & Ehrenberg, 1983).

Under normal conditions, the major source of "accidental" reactive oxygen species formation is from electron "leakage" from electron transport chains, whilst "leakage" of reactive oxygen species can also occur through the actions of phagocytes (see Cheeseman & Slater, 1993 for review). Reactive oxygen species can also be produced by the actions of flavin oxidases present in peroxisomes and through the autoxidation of various compounds including ascorbate, thiols (e.g. glutathione, cysteine, homocysteine),

Inflammatory-immune injury	**Red blood cells**	**Gastrointestinal tract**
Glomerulonephritis (idiopathic, membranous)	Phenylhydrazine	Endotoxic liver injury
Vasculitis (hepatitis B virus, drugs)	Primaquine, related drugs	Halogenated hydrocarbon liver injury (e.g. carbon tetrachloride, bromobenzene, halothane)
Autoimmune diseases	Lead poisoning	Diabetogenic action of alloxan
Rheumatoid arthritis	Protoporphyrin photoxidation	Pancreatitis
	Malaria	NSAID-induced gastrointestinal tract lesions
Ischaemia-reflow states	Sickle cell anaemia	Oral iron poisoning
Stroke/myocardial infarction/ arrythmias	Favism	
Organ transplantation	Fanconi's anaemia	**Brain/nervous system/ neuromuscular disorders**
Inflamed rheumatoid joint	Haemolytic anaemia of prematurity	Hyperbaric oxygn
Frostbite	**Lung**	Vitamin E deficiency
Dupuytren's contracture?	Cigarette smoke effects	Neurotoxins
	Emphysema	Parkinson's disease
Drug and toxin-induced reactions	Hyperoxia	Hypertensive cerebrovascular injury
Numerous	Bronchopulmonary dysplasia	Neuronal ceroid lipofuscinoses
	Oxidant pollutants (ozone, nitric oxide)	Allergic encephalomyelitis and other demyelinating diseases
Iron overload	ARDS (some forms)	Aluminium overload (Alzheimer's disease?)
Idiopathic haemochromatosis	Mineral dust pneumoconiosis	Potentiation of traumatic injury
Dietary iron overload (Bantu)	Asbestos carcinogenicity	Muscular dystrophy
Thalassaemia and other chronic anaemias treated with multiple blood transfusions	Bleomycin toxicity	Multiple sclerosis
	Sulphur dioxide toxicity	
Nutritional deficiencies (kwashiorkor)	Paraquat toxicity	**Heart and cardiovascular system**
	Skin	Alcohol cardiomyopathy
Alcoholism	Solar radiation	Keshan disease (selenium deficiency)
including alcohol-induced iron overload and alcoholic myopathy	Thermal injury	Atherosclerosis
	Porphyria	Adriamycin cardiotoxicity
Radiation injury	Hypericin, other photosensitizers	
Nuclear explosions	Contact dermatitis	**Kidney**
Accidental exposure		Autoimmune nephrotic syndromes
Radiotherapy	**Eye**	Aminoglycoside nephrotoxicity
Hypoxic cell sensitizers	Cataractogenesis	Heavy metal nephrotoxicity (Pb, Cd, Hg)
	Ocular haemorrhage	
Ageing	Degenerative retinal damage	
Disorders of premature ageing	Retinopathy of prematurity (retrolental fibroplasia)	
	Photic retinopathy	

ARDS—adult respiratory distress syndrome
NSAID—non-steroidal anti-inflammatory drug

Table 2.1 Clinical conditions in which the involvement of reactive oxygen species has been implicated. Reproduced from Halliwell & Gutteridge (1989).

adrenaline and flavin co-enzymes. Generally, this accidental production of reactive oxygen species is kept to a minimum both by the high efficiency of enzyme-mediated electron transfer processes and by keeping transition metal ions, which may catalyse the production of certain species (see below), tightly chelated. Since these precautions are not fully efficient, both enzymic and non-enzymic defence mechanisms also exist within cells to cope with normal low levels of reactive oxygen species production. When this production exceeds normal levels, however, such as in the presence of toxic agents or in certain disease conditions, cellular and tissue damage may occur.

2.1.1 Superoxide anion

Superoxide anion ($\cdot O_2^-$) is produced in many pathological states which cause alterations in metabolic pathways resulting in the incomplete reduction of oxygen (McCord, 1987). This reduction involves the transfer of a single electron as follows:

$$O_2 + e^- \rightarrow \cdot O_2^-$$

In itself, superoxide anion it is not believed to be widely toxic, but it is able both to initiate many radical chain reactions (Link & Riley, 1988) and to give rise to the production of other more damaging species such as hydrogen peroxide (see below). At low pH it will protonate to form the more reactive perhydroxyl radical ($\cdot HO_2$), but at physiological pH less than 1% of superoxide anion is found in this form (Cheeseman & Slater, 1993).

2.1.2 Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is formed spontaneously from superoxide anion by the dismutation reaction:

$$\cdot O_2^- + \cdot O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

This reaction will occur comparatively slowly on its own but in the presence of superoxide dismutase, it will occur substantially more quickly, resulting in the rapid removal of superoxide anion. Superoxide dismutase is present in a large number of cell types and acts as a natural defence against the production of superoxide anion.

Hydrogen peroxide is potentially more toxic than superoxide anion since, unlike superoxide, it is freely able to diffuse across cell membranes and can directly attack certain target components within the cell such as the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (Halliwell & Chirico, 1993). Its major significance as a cytotoxic agent is thought to lie in its ability to generate hydroxyl radicals in the presence of transition metals (see below).

The breakdown of hydrogen peroxide may be catalysed by the enzyme, catalase, according to the equation:

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

Like superoxide dismutase, catalase is also present in many cell types and acts as a natural defence against the production of hydrogen peroxide. Additionally, many cells possess the enzyme, glutathione peroxidase which also protects against hydrogen peroxide by catalysing its reaction with

reduced glutathione to form glutathione disulphide and water (Smith *et al.*, 1989). In the absence of metal catalysts, it is therefore readily removed from biological systems, as is superoxide anion, rendering it fairly harmless (Cheeseman & Slater, 1993).

2.1.3 Hydroxyl radicals

Hydroxyl radicals ($\cdot OH^-$) may be formed in the combined presence of superoxide anion, hydrogen peroxide and trace amounts of ferric iron which catalyses its formation by the Fenton reaction, thus:

$$Fe^{3+} + \cdot O_2^- \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^-$$

Ferric iron (Fe^{3+}) is stored within cells as ferritin, a form in which it is susceptible to attack from superoxide anion by the above reaction thus making it a potential source of available iron. Ferrous iron (Fe^{2+}) is therefore formed and, as shown, can react with hydrogen peroxide to generate hydroxyl radicals. Additionally, the formation of hydroxyl radical from hydrogen peroxide can be catalysed by cuprous copper (Cu^+), which may similarly be produced by reaction of superoxide anion with stored forms of cupric copper (Cu^{2+}), such as caeruloplasmin which is present in the circulation (Cheeseman & Slater, 1993).

Hydroxyl radicals are believed to be substantially more reactive than either superoxide anion or hydrogen peroxide and are able to attack biological molecules, usually by starting free-radical chain reactions (Halliwell & Gutteridge, 1989). In particular, hydroxyl radicals can initiate the process of lipid peroxidation (Halliwell & Chirico, 1993), a radical chain reaction

which results in the deterioration of polyunsaturated lipids, thereby impairing the integrity and function of cell membranes. Also, by attacking cellular DNA, causing both extensive strand breakage and degradation of deoxyribose, they may have a carcinogenic action (Halliwell & Gutteridge, 1984).

Being highly reactive, hydroxyl radicals have extremely short half-lives and therefore will not diffuse far within a cell from their site of production before reacting (Cheeseman & Slater, 1993). As such, they are capable of causing highly localised but extensive damage.

2.1.4 Hypochlorous acid

Hypochlorous acid (HOCl) is produced from hydrogen peroxide and chloride ions by the enzyme, myeloperoxidase, which is secreted by activated neutrophils (Harrison & Schultz, 1976). This reaction occurs as follows:

$$H_2O_2 + Cl^- \xrightarrow{\text{myeloperoxidase}} HOCl + OH^-$$

HOCl is believed to be the most toxic of the reactive oxygen species which neutrophils produce as part of the inflammatory response (Eley *et al.*, 1989) and has been shown *in vitro* to be able to kill both bacteria and fungi (Halliwell & Gutteridge, 1989). Also, it is able to oxidise many biological molecules and, in particular, will attack thiol groups. For instance, it has been shown to disrupt contractile function in ventricular myocytes by attacking thiol-dependent Ca^{2+} transport mechanisms (Eley *et al.*, 1991).

2.1.5 Peroxynitrite anion

Peroxynitrite anion ($ONOO^-$) may be formed by interaction of nitric oxide and superoxide anion by the following reaction as was proposed by Beckman *et al.* (1990):

$$NO^{\cdot} + {}^{\cdot}O_2^- \rightarrow ONOO^-$$

As well as being a strong oxidant in itself, with a half-life of round a second, Beckman *et al.* also showed that peroxynitrite can act as the source of a strong oxidising agent with properties identical to those of the hydroxyl radical. They proposed that this may be explained by its ability to undergo decomposition to generate hydroxyl radical as follows:

$$ONOO^- + H^+ \rightarrow ONOOH$$

$$ONOOH \rightarrow {}^{\cdot}OH + {}^{\cdot}NO_2$$

Hence, peroxynitrite may represent another pathway, in addition to the Fenton reaction, by which hydroxyl radical may be formed in biological systems and by a mechanism that does not require the presence of iron to act as a catalyst.

Furthermore, it has been suggested that peroxynitrite can be generated by cells such as macrophages, neutrophils, and possibly the vascular endothelium, by the simultaneous generation of both superoxide anion and nitric oxide (Hogg *et al.*, 1992).

2.2 Ischaemia-reperfusion injury

2.2.1 Ischaemia

The deprivation of oxygen i.e. ischaemia from a region of either the heart or the brain is a major cause of death in current Western society. The extent of the damage caused to the affected tissue is dependent on both the duration of the ischaemic period and on the extent of the level of oxygen deprivation (Braunwald & Kloner, 1986; Simpson & Lucchesi, 1987). If the ischaemic period is short then recovery of the tissue will occur, but with longer periods of ischaemia the damage becomes irreversible and cell death will result.

Ischaemia results from arterial blockage which prevents the supply to the affected area of oxygenated blood. The most common causes of such a blockage are that of atherosclerosis (see Section 2.3) and thrombosis.

2.2.2 Reperfusion

Reperfusion of an ischaemic tissue provides both beneficial and deleterious effects. Unless the period of ischaemia has been sufficiently long to render cell death in the affected tissue, reperfusion will ultimately prevent such cell death and facilitate tissue recovery (Halliwell & Gutteridge, 1989). Coupled with this restorative effect, however, reperfusion will also result in a fresh wave a cell injury including a hastening of the necrosis of irreversibly injured cells, cell swelling, an incomplete restoration of blood flow (the so-called "no-reflow" phenomenon), haemorrhagic infarction, post-ischaemic depression of ventricular function in the heart (myocardial "stunning") and the production of reactive oxygen species (Braunwald & Kloner, 1986).

2.2.3 The hypoxanthine-xanthine oxidase free radical generating system

The hypoxanthine-xanthine oxidase system has been implicated in playing a major role in the damage resulting from ischaemia and reperfusion. The first evidence for a role of this system in hypoxic injury was shown in the late 1960's. Jones et al. (1968) demonstrated that following hypoxia induced by haemorrhage in dogs, there was an accumulation within the blood of uric acid, a product of the hypoxanthine-xanthine oxidase system. Subsequent work showed that allopurinol, a xanthine oxidase inhibitor, exhibited a considerable protective effect on ischaemia-induced impairment of myocardial function in the hearts of both dogs and sheep (Crowell *et al.*, 1969; DeWall *et al.*, 1971). Similarly, allopurinol has been shown to inhibit reperfusion-induced injury in the kidney (Hansson *et al.*, 1983) and liver (Adkison *et al.*, 1986).

During ischaemia, the absence of oxygen forces the purine, adenosine triphosphate to follow an anaerobic catabolic pathway leading to an accumulation of hypoxanthine within cells (McCord, 1987; Werns & Lucchesi, 1990). This pathway is illustrated in Figure 2.1. At the same time, xanthine oxidase is formed within cells by conversion from its native state of xanthine dehydrogenase. This conversion was first demonstrated by Roy & McCord (1983) using segments of rat intestine and is believed to occur either by limited proteolysis (Phan *et al.*, 1989) or by an oxidation of essential thiol groups (Halliwell & Gutteridge, 1989).

Unlike xanthine dehydrogenase which uses NAD as an oxidant leading to the formation of NADH, xanthine oxidase utilises oxygen, thereby yielding superoxide anion on oxidation of either hypoxanthine or xanthine (Engerson *et al.*, 1987). Hence, as oxygen once again becomes available on reperfusion,

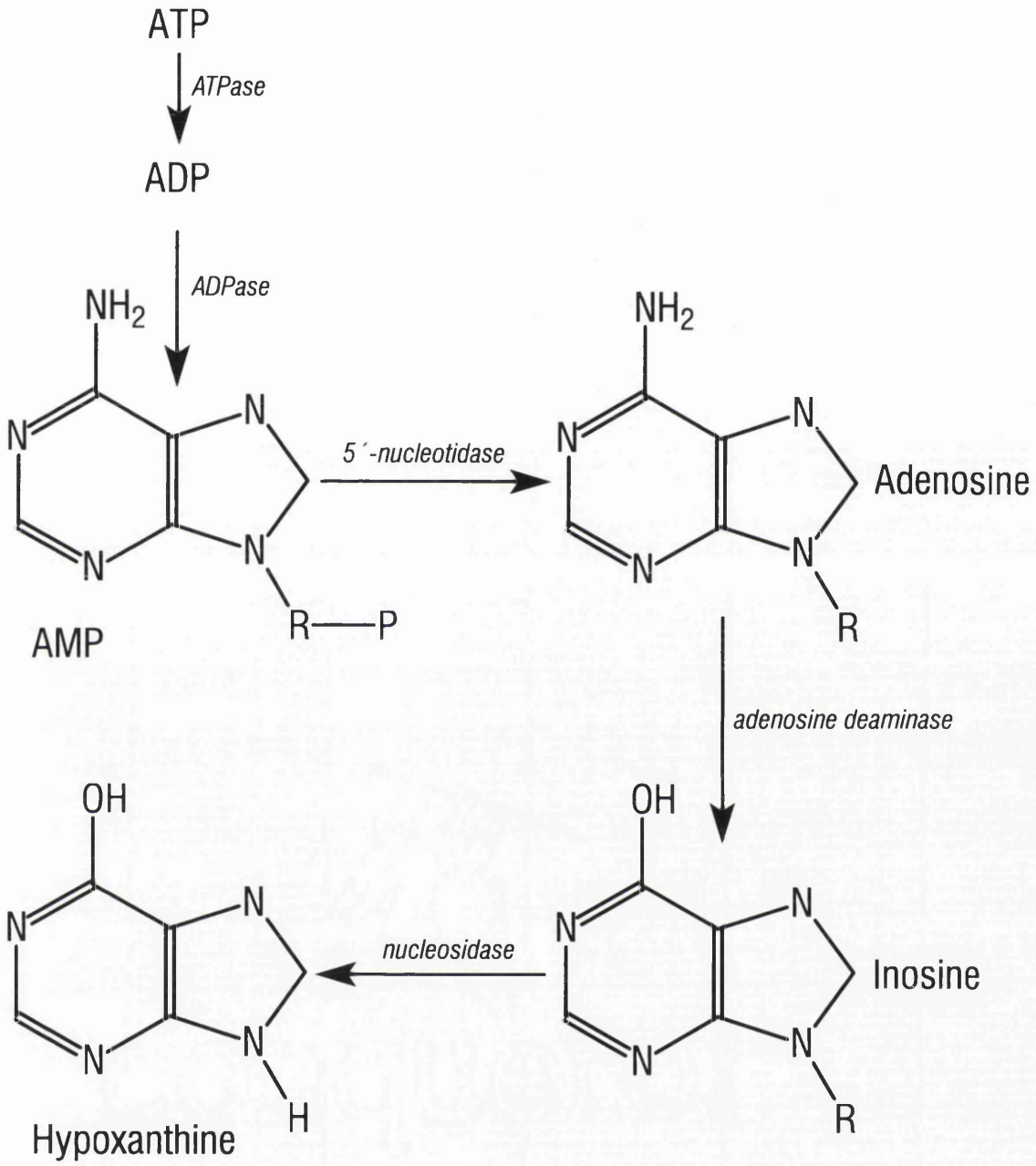


Figure 2.1 Pathway showing the formation of hypoxanthine under ischaemic conditions by the anaeorbic catabolism of adenosine triphosphate. ADP—adenosine diphosphate; AMP—adenosine monophophate; ATP—adenosine triphosphate.

xanthine oxidase, as illustrated in Figure 2.2, will convert hypoxanthine to xanthine and then to uric acid producing superoxide anion in the process of both these reactions. The production of superoxide anion during ischaemia-reperfusion injury has been confirmed in many tissues including intestine (Granger *et al.*, 1981; Parks *et al.*, 1982), liver (Adkison *et al.*, 1986) and skin (Im *et al.*, 1985) by the ability of superoxide dismutase, which rapidly converts superoxide anion to hydrogen peroxide, to inhibit the extent of the resultant tissue damage.

2.2.4 Role of neutrophils

Polymorphonuclear leucocytes, specifically neutrophils, have also been implicated in contributing to the injury induced by ischaemia-reperfusion (Romson *et al.*, 1983; Grisham *et al.*, 1986). Neutrophils play a key role in the development of the inflammatory response (Siflinger-Birnboim & Malik, 1993) and have been shown to contribute to endothelial injury, increased microvascular endothelial permeability and tissue damage at sites of inflammation (Harlan, 1985; Sibille & Reynolds, 1990). In particular, neutrophils have been identified as a major mediator of the tissue injury associated with inflammatory bowel disease (Grisham *et al.*, 1986).

Neutrophils secrete a wide variety of mediators including prostanoids and leukotrienes, as well as various digestive enzymes such as elastase which can induce endothelial cell detachment (Harlan *et al.*, 1981). They also produce reactive oxygen species, including superoxide anion and hydrogen peroxide (Shasby *et al.*, 1983; Siflinger-Birnboim *et al.*, 1992) through the activity of a membrane-bound enzyme, NADPH oxidase (Rangan & Bulkley, 1993). Additionally, neutrophils secrete the enzyme, myeloperoxidase, which catalyses the formation of the potent oxidant, hypochlorous acid, from hydrogen peroxide and chloride ions (Harrison & Schultz, 1976).

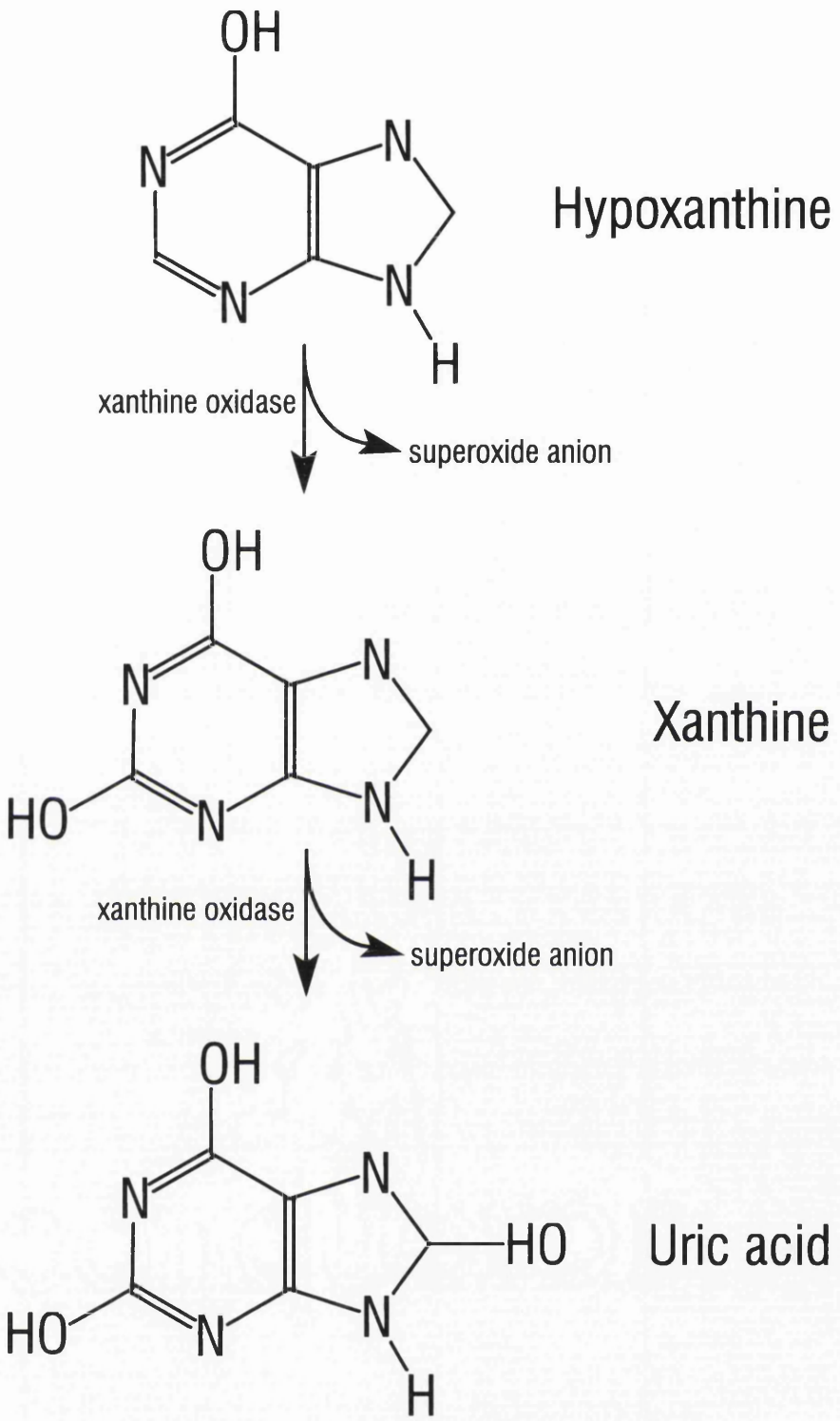


Figure 2.2 Pathway showing the formation of superoxide anion during the xanthine oxidase-catalysed conversions of hypoxathine to xanthine and xanthine to uric acid.

Various findings point to a role for neutrophils in the pathogenesis of ischaemia-reperfusion injury. In the heart, neutrophils have been observed to begin to infiltrate a site of injury at the onset of ischaemia with their numbers progressively increasing as the resultant myocardial infarct develops during the following 24 hours (Simpson & Lucchesi, 1987). Similarly, neutrophil infiltration has been documented in skeletal muscle following experimentally induced ischaemia and reperfusion (Smith *et al.,* 1989). Romson *et al.* (1983) demonstrated that by using neutrophil anti-sera to reduce the numbers of circulating neutrophils, myocardial infarcts following experimentally induced coronary artery occlusion and reperfusion in dogs were reduced by up to 50%. Moreover, neutrophil adhesion to endothelium and migration into sub-endothelial tissue has been observed following similarly induced coronary artery occlusion and subsequent reperfusion by Mullane *et al.* (1984). They also demonstrated that infarct size could be significantly reduced using hydroxyurea, which depletes circulating neutrophils, or BW755C, a combined cyclo-oxygenase and lipoxygenase inhibitor which inhibits neutrophil infiltration.

A link between neutrophils and the hypoxanthine-xanthine oxidase system has been proposed by various workers. Grisham *et al.* (1986) proposed that superoxide anion or other oxidants, produced during ischaemia-reperfusion by activation of the hypoxanthine-xanthine oxidase system, may act to attract neutrophils to a site of injury. This possibly occurs through the formation of some potent chemoattractant formed either by activation of the complement system or through reaction with lipoproteins. At the same time, certain evidence points to the ability of neutrophils, in turn, to activate the hypoxanthine-xanthine oxidase system. Phan *et al.* (1989) showed, for instance, that addition of neutrophils to cultured rat pulmonary artery endothelial cells stimulated the conversion of xanthine dehydrogenase to xanthine oxidase. Using an *in vitro* model of ischaemia-

reperfusion injury to cultured bovine coronary microvascular endothelial cells, Inauen *et al.* (1988) showed that neither a protective effect on endothelial cytotoxicity nor cell detachment was seen on addition of either superoxide dismutase or allopurinol unless neutrophils were present. Also, they showed that addition of neutrophils enhanced the degree of injury that was obtained.

Granger (1988) described an overall scheme for the interactions of ischaemia-reperfusion, lipid peroxidation and activation of neutrophils as shown in Figure 2.3.

2.3 Atherosclerosis

Atherosclerosis is the primary cause of heart attacks, stroke and gangrene, being responsible for about half of all deaths in Western civilisation (Ross, 1993). It is a progressive condition which is characterised by vascular lesions which are first observable as so-called "fatty streaks". These then develop over a long period of time, by a complex and not fully-understood process, to more substantial and potentially-occlusive lesions characterised by intimal proliferation of smooth muscle cells and the deposition of intracellular and extracellular lipid (Ross & Harker, 1976).

2.3.1 The "response to injury" hypothesis

The "response to injury" hypothesis of the atherogenic process has been advanced over the past twenty years or so and was recently outlined on current understanding by Ross (1993). The trapping of lipoproteins at various arterial sites, coupled with the appearance of adhesive glycoproteins on the surfaces of endothelial cells, is believed to attract both monocytes and T lymphocytes. These migrate across the endothelial

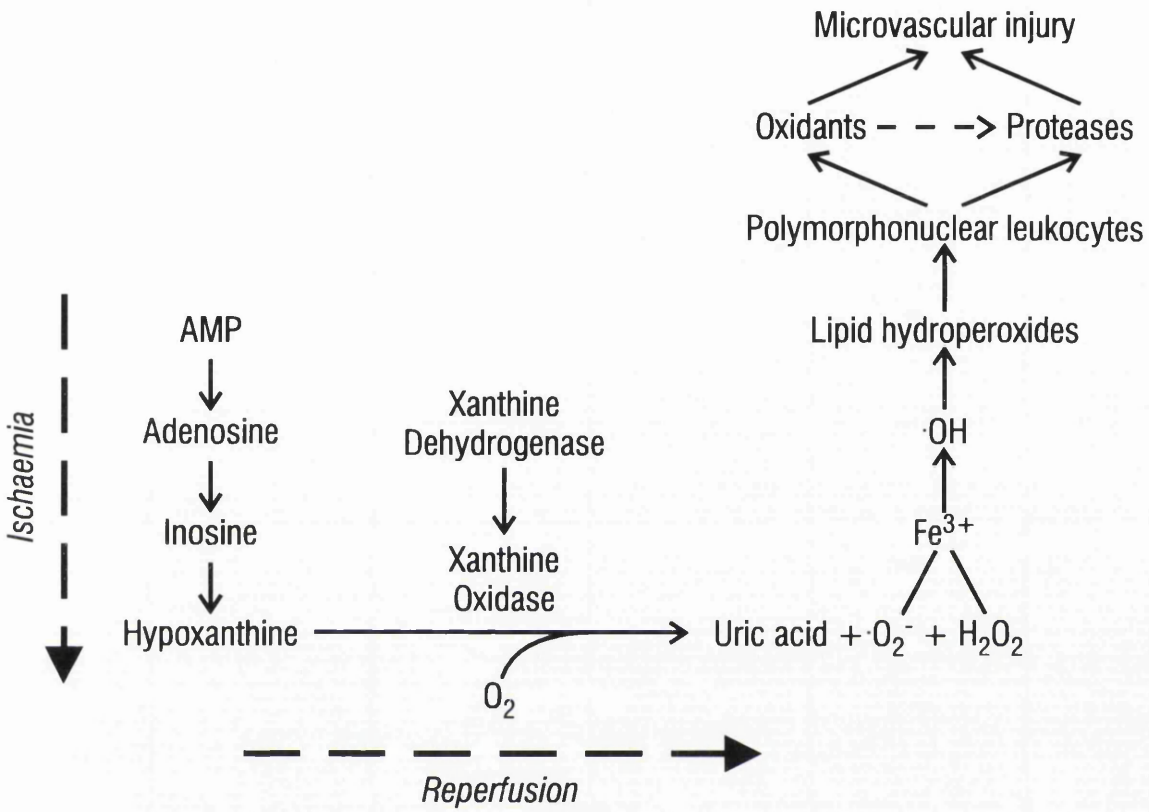


Figure 2.3 Schematic representation of the development of ischaemia-reperfusion-induced microvascular injury as proposed by Granger (1988). A suggested link between xanthine oxidase-derived reactive oxygen species, lipid peroxidation and activation of polymorphonuclear leukocytes is shown.

cell layer under the influence of both chemoattractants and growth factors which are released from the endothelium, adherent leucocytes and possibly underlying smooth muscle cells. The monocytes become macrophages, accumulate lipid and then become foam cells which in combination with lymphocytes form the basis of the fatty streak. Continued cell infiltration and proliferation leads to the development of more advanced lesions, and ultimately to the formation of fibrous plaques.

2.3.2 Role of endothelial dysfunction

By facilitating the initial migration across the endothelium of monocytes and lymphocytes, endothelial dysfunction, including barrier dysfunction, is clearly an important early step in the atherogenic process (Ross, 1986). It is reasonable therefore to hypothesise that factors which are injurious to the endothelium will act as risk factors for the development of atherosclerosis. Oxidised low-density lipoprotein, for example, is believed to play a significant role in promoting such migration through causing direct injury of the endothelium and also through inducing the formation of cell-surface adhesion molecules. Also, substances which initiate the generation of reactive oxygen species such as homocysteine (see below) could act as risk factors for atherosclerosis through causing injury to the endothelium.

2.4 Homocystinuria

The condition of homocystinuria is a recognised risk factor for the development of atherosclerosis (Malinow, 1990). It is so named as it can be characterised by an elevated level of homocystine secretion in urine. Homocystine is the disulphide form of the thiol-containing amino acid, homocysteine, an intermediate formed during the metabolism of both

methionine and cysteine (Refsum & Ueland, 1990). Defects in the metabolism of homocysteine, most commonly caused by a deficiency of cystathionine β-synthase (Clarke *et al.*, 1992), lead to an accumulation of both homocysteine and homocystine in the circulation, rising from a combined level of ~10 μM in normal subjects (Boers *et al.*, 1985) to as high as 250 μM in homozygotic homocystinura sufferers (Perry *et al.*, 1967).

Several studies have demonstrated a link between high circulating levels of homocyst(e)ine (hyperhomocyst(e)inemia) and the premature development of vascular disease including cerebrovascular (Brattstrom *et al.*, 1984), peripheral vascular (Boers *et al.*, 1985) and coronary vascular disease (Kang *et al.*, 1986; Clarke *et al.*, 1992). The mechanisms underlying the atherogenic actions of homocysteine are however unclear. Significant platelet accumulation has been observed at sites of vascular injury and at occlusive thrombi in both human sufferers and experimental models of homocystinuria (Harker *et al.*, 1974; Harker *et al.*, 1976) whilst, additionally, a direct stimulatory action of homocysteine on platelet aggregation has been reported (Graeber *et al.*, 1982; McCully & Carvalho, 1987).

In vitro studies have demonstrated the ability of homocysteine to exert a cytotoxic action on cultured endothelium (Wall *et al.*, 1980; Starkebaum & Harlan, 1986), confirming *in vivo* observations of homocysteine-induced endothelial injury (Harker *et al.*, 1976; Harker *et al.*, 1983). Starkebaum & Harlan (1986) proposed that the injury observed in their study resulted from the production of hydrogen peroxide during the copper-catalysed oxidation of homocysteine to homocystine (see Figure 2.4). This proposal was based on the findings that homocysteine-induced endothelial injury, as measured by a ^{51}Cr-release assay, was dependent on the presence of copper in the form of either copper sulphate or caeruloplasmin, and additionally was abolished in the presence of catalase.

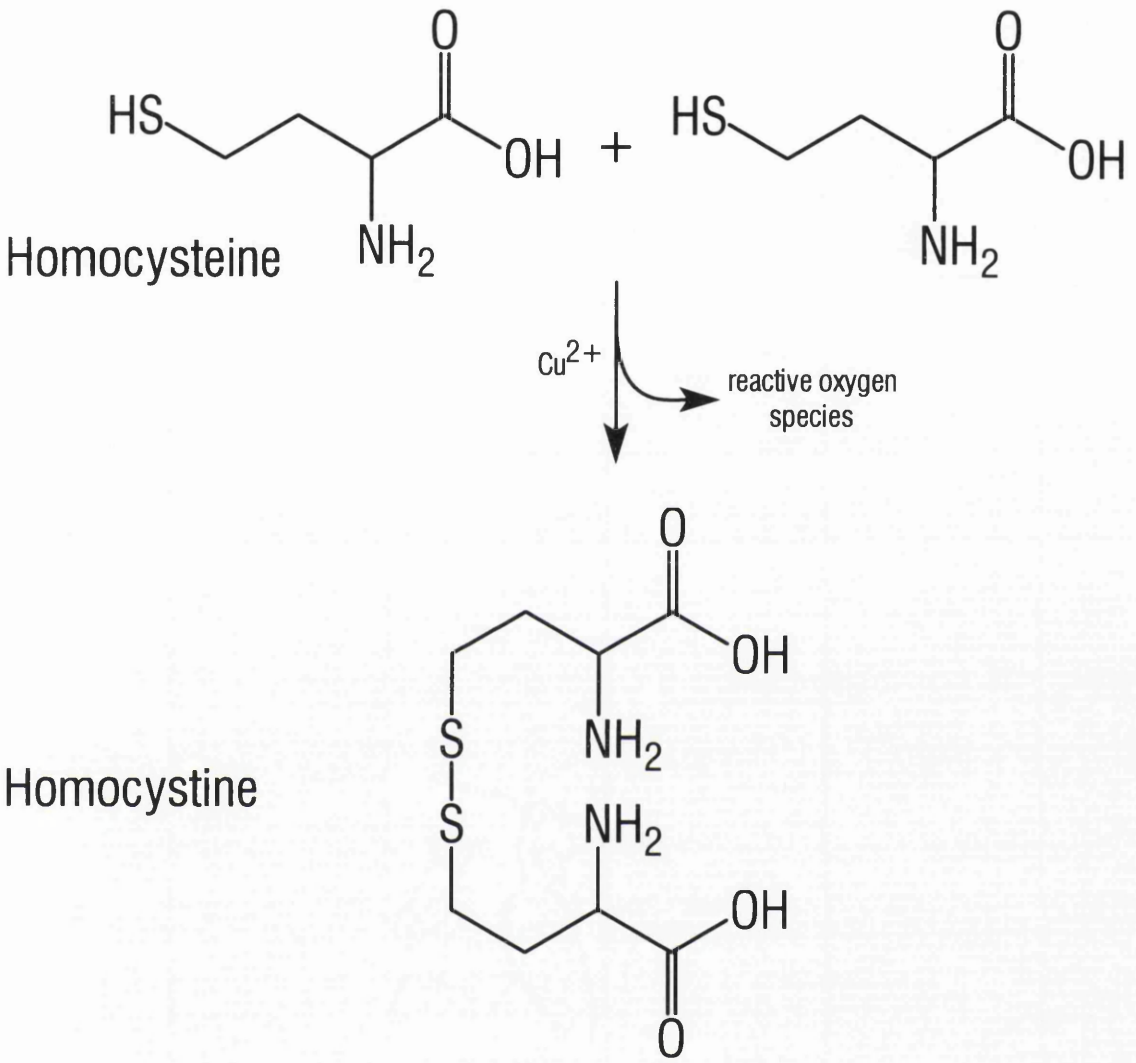


Figure 2.4 Pathway showing the production of reactive oxygen species during the copper-catalysed oxidation of homocysteine to its disulphide form, homocystine.

CHAPTER 3

3.1 Septic Shock

Septic shock, often caused by lipopolysaccharide (LPS) derived from gram-negative bacteria, is a condition which is growing in incidence. In the USA, for example, there are around 200,000 cases of septic shock currently diagnosed each year with around half of these cases resulting in death (Martin & Silverman, 1992). The main characteristics of septic shock are profound, persistent hypotension, hyporeactivity to vasoconstrictor agents, increased endothelial permeability, inadequate tissue perfusion, vascular damage and intravascular coagulation leading, in time, to multiple organ failure (Bone, 1991a; Wright *et al.*, 1992).

The majority of deaths from septic shock occur rapidly, within hours to days of onset, usually as a direct result of the persistent hypotension. About 25% of deaths, however, occur days to weeks after successful treatment for hypotension and are usually a consequence of the development of multiple organ failure (Martin & Silverman, 1992).

Septic shock is a severe shock state which follows on from the development firstly of sepsis and then, subsequently, the sepsis syndrome. Bone (1991b) attempted to define these different stages in the development of septic shock as follows:

Sepsis—clinical evidence of infection plus tachycardia, increased rate of respiration and either hyperthermia or hypothermia.

The sepsis syndrome—as sepsis *plus* evidence of altered organ perfusion.

Early septic shock—as sepsis syndrome *plus* hypotension (systolic blood pressure below 90 mm Hg or a 40 mm Hg decrease below baseline systolic blood pressure) which is responsive to conventional therapy within 1 hour of commencement of treatment.

Refractory septic shock—as sepsis syndrome *plus* hypotension (systolic blood pressure below 90 mm Hg or a 40 mm Hg decrease below baseline systolic blood pressure) which is not responsive to conventional therapy within 1 hour of commencement of treatment.

Septic shock is a serious condition, which develops in about 40% of patients with sepsis, and its diagnosis indicates a poor prognosis (Bone, 1991a). Different studies, however, have indicated different mortality rates in patients who have developed septic shock, ranging from 10% (Cunnion & Parillo, 1989), through 40–50% (The Veterans Administration Systemic Sepsis Cooperative Study Group, 1987; Tran *et al.*, 1990) to as high as 90% in one study (Parker & Parillo, 1983).

3.1.1 Causes of septic shock

Septic shock, as already stated, is often caused by LPS derived from gram-negative bacteria and is triggered following its release into the circulation (Bone, 1991a). LPS from gram-negative bacteria only accounts for around 30% of septic shock cases (Bone *et al.*, 1987), however, and several other substances have been observed to trigger its onset. These include enterotoxin, toxic shock syndrome toxin-1, cell wall products

released from both gram-positive bacteria and yeast, and also both viral and fungal antigens (Fong *et al.,* 1988; Tracey *et al.,* 1988).

3.1.2 Lipopolysaccharide (LPS)

LPS, which is also known as endotoxin, is a component of bacterial outer cell walls. Its structure varies from species to species but, in general, consists of three distinct elements (Westphal *et al.,* 1983):

Region I—an O-antigen-specific polysaccharide chain made up of repeating oligosaccharide units.

Region II—an R-antigen-specific core polysaccharide containing both an inner and an outer core.

Region III—a lipid domain, termed Lipid A.

Region I varies quite widely from species to species and carries the primary antigenicity of the associated bacterium (Liao & Florén, 1993). In contrast, both the core portion of the molecule, Region II, and the Lipid A portion, Region III, posses a morphology which is well conserved amongst different bacterial species and genera (Priest *et al.,* 1989), with Lipid A having been recognised as the region of the molecule which is mainly responsible for its ability to activate the host's immune system (Lüderitz *et al.,* 1973).

3.1.3 The pathogenesis of septic shock

The pathogenesis of septic shock and its preceding conditions is one which is complex and in which many factors are involved. Bone (1991a), however, proposed that one key factor is that of endothelial

dysfunction, in particular barrier dysfunction which results from repetitive, focused sites of inflammation thereby leading to a substantial increase in capillary permeability and contributing to a deterioration in organ perfusion. Such endothelial injury may become widespread and lead to the failure of various organs including heart, kidneys, brain, intestine and liver as well as leading, in some cases, to the development of the adult respiratory distress syndrome as a result of lung failure (Martin & Silverman, 1992).

On its release into the circulation, LPS is known to trigger a cascade of events leading to an increase in the circulation of many different mediators, all of which may contribute to the eventual development of sepsis and septic shock (shown schematically in Figure 3.1). LPS, for example, is believed to directly stimulate the release of tumour necrosis factor-α (TNF-α), interleukin-1, interleukin-6, interleukin-8 and platelet activating factor (PAF) from a variety of cells including both macrophages and endothelial cells themselves (Wolff, 1973; Michalek *et al.,* 1980; Johnston, Jr, 1988; Tracey *et al.,* 1989). The precise role played by each of these mediators is not clear, but many play a role in triggering the release of further mediators to generate the "sepsis cascade". For instance, both the complement system and the coagulation cascade are subsequently activated although it is not in fact known whether this is caused by LPS directly, or through LPS-stimulated release of TNF-α or some other mediator (Bone, 1991a). Following the release of TNF-α, interleukin-1 and PAF, the formation of arachidonic acid metabolites is stimulated. These include leukotrienes, thromboxane A_2, and prostaglandins (particularly prostaglandin E_2 and prostacyclin) (Petrak *et al.,* 1989). At the same time, interleukin-1 and interleukin-6 will activate T cells leading to the production on interferon-γ (IFN-γ), interleukin-2, interleukin-4 and granulocyte-monocyte colony-stimulating factor (Dinarello & Mier, 1987; Jacobs & Tabor, 1989; Kuhweide *et al.,* 1990; Thornhill *et al.,* 1990).

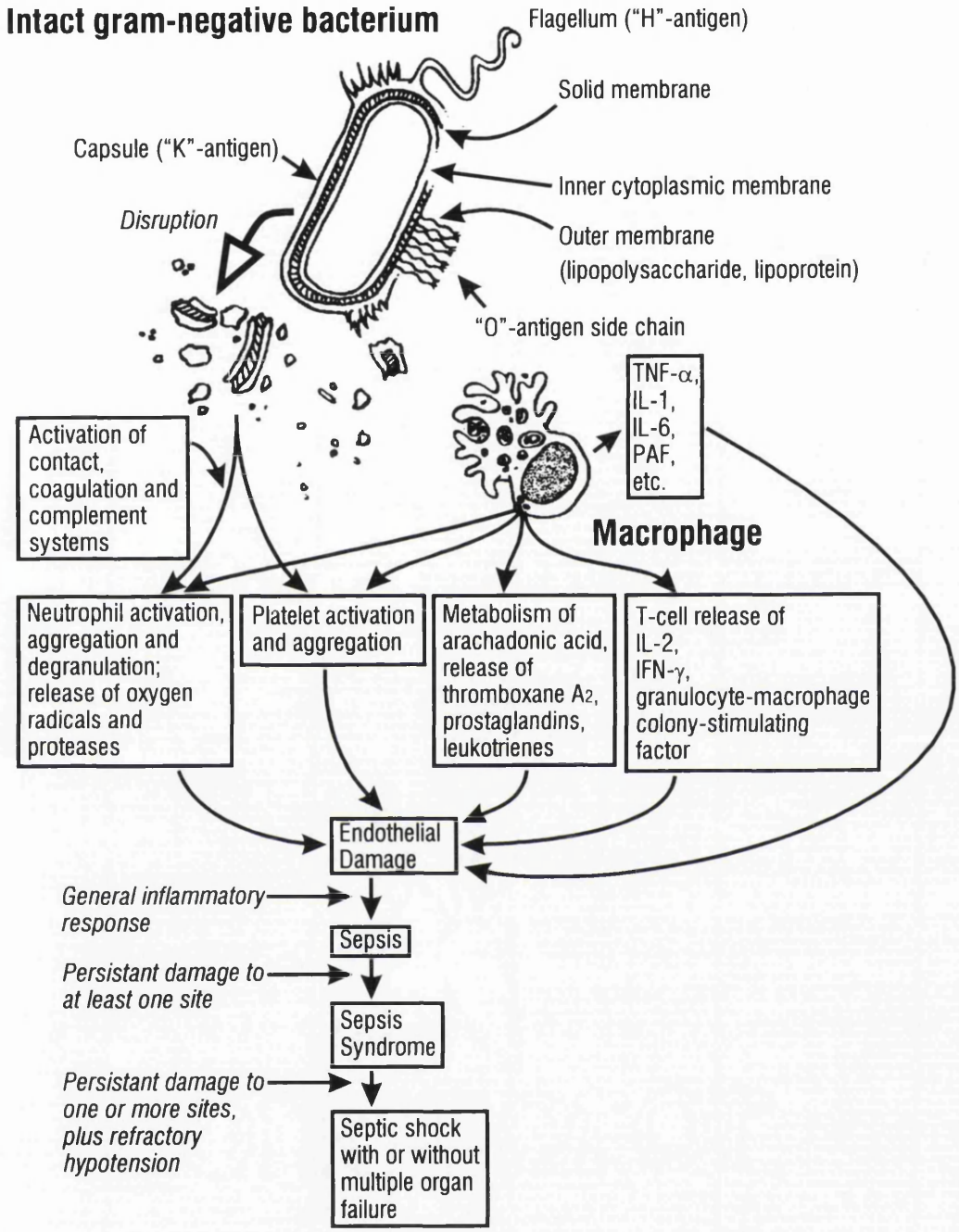


Figure 3.1 Schematic representation of the pathogenesis of septic shock. Modified from Bone (1991b). IFN-γ—interferon-γ; IL-1—interleukin-1; IL-2—interleukin-2; IL-6—interleukin-6; PAF—platelet activating factor; TNF-α—tumour necrosis factor-α.

Virtually all of these agents can exert a direct action on the vascular endothelium and many have been shown to have the ability to induce endothelial barrier dysfunction. For instance, *in vivo*, LPS has been observed to induce plasma leakage, shown by the leakage of Evan's Blue dye, in rat duodenum, stomach and lung (Ibbotson & Wallace, 1989), as well as to increase the permeability of the endothelium of rat aortas, shown by an increase in the accumulation in the intima and media of intravenously administered horseradish peroxidase (Penn & Chisolm, 1991). *In vitro*, LPS has been observed to induce an increase in the albumin permeability of bovine pulmonary artery endothelial cell monolayers (Meyrick *et al.*, 1986), whilst TNF-α has been shown to induce an increase in the albumin, inulin, sorbitol, cytochrome C and co-vitamin B_{12} permeability of monolayers of BAEC (Brett *et al.*, 1989; Royall *et al.*, 1989), as well as the albumin permeability of monolayers of human umbilical vein endothelial cells (Burke-Gaffney & Keenan, 1993a). Interleukin-1 has been shown to induce an increase in the albumin, cytochrome C and co-vitamin B_{12} permeability of bovine aortic endothelial cell monolayers (Royall *et al.*, 1989), whilst, additionally, both IFN-γ and interleukin-1 were found to induce an increase in the albumin permeability of human umbilical vein endothelial cell monolayers (Burke-Gaffney & Keenan, 1993b).

Activation of the complement cascade can also lead to vascular dysfunction (Lundberg *et al.*, 1987) and to neutrophil activation (Frank, 1987). Neutrophils may also be activated by many of the other already-mentioned mediators and can also, in themselves, bring about endothelial dysfunction through release of reactive oxygen species and lysosomal enzymes, through aggregation and through adherence to the endothelium causing vaso-dilatation (Bone, 1991a). Various studies have demonstrated the ability of neutrophils to induce endothelial barrier dysfunction. For instance, neutrophils have been observed, *in vitro*, to induce an increase in the

albumin permeability of bovine pulmonary microvessel endothelial cells (Siflinger-Birnboim *et al.,* 1993) which may result as a consequence of the generation of hydrogen peroxide (Siflinger-Birnboim & Malik, 1993).

Other mediators which have been implicated as being involved in the sepsis cascade include platelets (Heffner *et al.,* 1987), transforming growth factor β_1 (Wahl *et al.,* 1987), adhesion molecules (Cotran & Pober, 1990), kinins (Katori *et al.,* 1989), thrombin (Esmon, 1987), myocardial depressant substance (Reilly *et al.,* 1989), beta-endorphin (Hughes, Jr., 1985) and heat shock protein (Rinaldo *et al.,* 1990). The relative role that each of these mediators plays, however, has yet to be determined.

3.1.4 Role of TNF-α

Of all the mediators so far mentioned, TNF-α is believed to be the most important (Bone, 1991a) and, indeed, until a few years ago was regarded as the prime mediator of sepsis and septic shock (Tracey *et al.,* 1988). It was noted, for instance, that intravenous administration of TNF-α in animal models generated very similar cardiovascular, haematological and inflammatory effects to those observed in septic shock (Tracey *et al.,* 1986) and that circulating levels of TNF-α are often elevated in a number of septic states (Bone, 1991a). Furthermore, it has been observed that LPS can promote macrophage production of TNF-α (Tracey *et al.,* 1988) and, when injected, cause a substantial elevation of TNF-α levels in animals (Hesse *et al.,* 1988). Finally, administration of anti-TNF-α antibodies has been shown to prevent the induction of septic shock following subsequent challenge with LPS (Tracey *et al.,* 1987).

TNF-α levels, however, are also observed to rise in a variety of other conditions including cancer (Aderka *et al.,* 1985), the acquired immuno-

deficiency syndrome (AIDS) (Lähdevrita *et al.*, 1988), congestive heart failure (Levine *et al.*, 1990), leprosy and leishmaniasis (Pisa *et al.*, 1990). Also, TNF-α levels in septic shock patients are not always found to be raised (Debets *et al.*, 1989) and can often be found to be high in healthy individuals (Damas *et al.*, 1989). Furthermore, administration of other mediators, such as interleukin-1 and PAF, has also been shown to generate septic shock-like symptoms (Lefer *et al.*, 1984; Kurt-Jones *et al.*, 1987).

3.1.5 Circulatory failure in septic shock

As previously indicated, one of the primary features of septic shock is that of profound hypotension. Total blood flow may in fact be normal, however, but flow to metabolically active tissues is inadequate and this reduced level of perfusion can, in time, lead to organ failure (Dal Nogare, 1991). The hypotension observed in septic shock results from vasodilatation leading to a substantial decrease in peripheral vascular resistance (Brady & Poole-Wilson, 1993) and this is often accompanied, at least in the early stages of shock, by tachycardia (Dal Nogare, 1991). In early septic shock both stroke volume and cardiac output are generally maintained but as the condition develops further, left ventricular dilatation is observed leading to a decrease in ejection volume and, therefore, a fall in cardiac output (MacLean *et al.*, 1967; Ellrodt *et al.*, 1985). Additionally, a global deterioration in the contractility of cardiac myocytes has been observed (Brady & Poole-Wilson, 1993).

3.1.6 Role of the L-arginine nitric oxide system

Recently, much attention in elucidating the mechanisms by which septic shock is mediated has fallen on the L-arginine nitric oxide system. Nitric oxide (or a nitric oxide-like substance), synthesised in the vascular

endothelium from L-arginine by a *constitutive* nitric oxide synthase, plays a key physiological role in the regulation of both blood flow and blood pressure (see Chapter 1). This isoform of nitric oxide synthase is Ca^{2+}/calmodulin-dependent and generates nitric oxide in small (picomolar) quantities (Moncada, 1992). In contrast, many cell types can produce nanomolar amounts of nitric oxide when challenged with certain immunological stimuli, such as LPS and cytokines, through the induction of a Ca^{2+}-independent *inducible* form of nitric oxide synthase (Wright *et al.*, 1992; Moncada, 1992; Brady & Poole-Wilson, 1993). Cells types in which inducible nitric oxide synthases have been identified include macrophages (Marletta *et al.*, 1988), vascular smooth muscle cells (Rees *et al.*, 1990), hepatocytes (Curran *et al.*, 1989) and Kupffer cells (Billiar *et al.*, 1989). Endothelial cells themselves have also been shown to express this inducible isoform of nitric oxide synthase in response to appropriate stimulation such as with LPS and IFN-γ (Radomski *et al.*, 1990). In the case of macrophages, production of nitric oxide is believed to account for much of their cytotoxic activity against both tumour cells and invading micro-organisms (Pellat *et al.*, 1990; Nathan, 1992).

In contrast to the constitutive from of nitric oxide synthase, the inducible form has been shown not to require Ca^{2+} for its activity, although it does require calmodulin, which it binds with high affinity (Xie *et al.*, 1992), as well as NADPH, tetrahydrobiopterin, FAD and FMN (Marletta *et al.*, 1988; Tayeh & Marletta, 1989; Marletta, 1993). Also, it is only observed to be present in cells following appropriate stimulation which causes its induction, whereas in the cells in which the constitutive form is known to be found, it is present all the time (Moncada, 1992). The activity of the inducible form of nitric oxide synthase has been shown to be inhibited by L-NMMA, L-NAME, N^{ω}-iminoethyl-L-ornithine (L-NIO) and, unlike the constitutive form, by L-canavanine (Iyengar *et al.*, 1987; McCall *et al.*, 1991). Another important

way in which the inducible form of nitric oxide synthase differs from its constitutive counterpart is the fact that its induction (but not its activity) may be inhibited by glucocorticoids, including dexamethasone, hydrocortisone and cortisol (Moncada, 1992).

Over the last three years, growing evidence has been put forward suggesting the involvement of nitric oxide production septic shock. It has been shown, for example, that endothelium-denuded rings incubated *in vitro* with LPS (Fleming *et al.*, 1990), or obtained from rats that had been treated with LPS (Knowles *et al.*, 1990), exhibited production of nitric oxide by the vascular smooth muscle that was Ca^{2+}-independent. Rees *et al.* (1990) demonstrated using rat aortic rings with or without endothelium, and which were contracted with phenylephrine, that treatment with LPS resulted in a progressive loss of tone after a lag of 2 hours. This was accompanied by an increase in cyclic GMP levels, a decreased response to phenylephrine and an ability to relax in response to L-arginine. All these effects were prevented by treatment with inhibitors of nitric oxide synthase and were associated with production in the tissue of a Ca^{2+}-independent nitric oxide synthase.

Various studies have also demonstrated the induction by cytokines of nitric oxide synthase in cultured vascular smooth muscle cells. For instance, Busse & Mülsch (1990b) showed that TNF-α and interleukin-1 both induced a Ca^{2+}/calmodulin-independent increase in guanylate cyclase activity in rabbit aortic smooth muscle cells which was blocked in the presence of either L-NMMA or the protein synthesis inhibitor, cycloheximide. They also showed that the increase in guanylate cyclase activity induced by TNF-α was time-dependent and was enhanced in the presence of IFN-γ. Using rat aortic smooth muscle cells, Beasley *et al.* (1991) similarly showed that interleukin-1 induced an increase in guanylate cylcase activity and further

showed that this was dependent on the availability of L-arginine and was accompanied by the formation of nitrite, a breakdown product of nitric oxide. Also using rat aortic smooth muscle cells, Kilbourn *et al.* (1992) showed that incubation with interleukin-1 caused a time- and concentration-dependent increase in nitrite formation which was inhibited by L-NMMA. A Fe^{2+}-myoglobin assay confirmed that the nitrite was indeed being formed as a consequence of nitric oxide production.

The induction of nitric oxide synthase by cytokines in various cell types has been shown to be inhibited by transforming growth factor-β and by platelet-derived growth factors. For instance, Ding *et al.* (1990) showed that transforming growth factors-β_1, -β_2 and β_3 inhibited nitric oxide synthase induction in macrophages by IFN-γ, whilst Pfeilschifter & Vosbeck (1991) showed that transforming growth factor-β_2 inhibited nitric oxide synthase induction by interleukin-1 and TNF-α in rat mesangial cells. In rat aortic smooth muscle cells, transforming growth factor-β_1, platelet-derived growth factor-AB and platelet-derived growth factor-BB were all found to inhibit the induction of nitric oxide synthase by interleukin-1 (Schini *et al.*, 1992). Additionally, thrombin has also been shown to inhibit the induction of nitric oxide synthase by interleukin-1 in rat aortic smooth muscle cells (Schini *et al.*, 1993).

Using *in vivo* models of septic shock, inhibition of nitric oxide synthesis has been shown to reduce the hypotension induced by treatment with LPS in rat (Thiemermann & Vane, 1990) or rabbit (Wright *et al.*, 1992) or by treatment with either LPS or TNF-α in dog (Kilbourn *et al.*, 1990a; Kilbourn *et al.*, 1990b). In addition, the induction of nitric oxide synthase has been shown to be responsible for the hyporesponsiveness to vasoconstrictors induced by LPS in isolated rabbit hearts (Smith *et al.*, 1991). Further evidence supporting a role for nitric oxide are the findings that nitric oxide levels,

measured as nitrite and nitrate in plasma, are elevated in septic shock in both animals (Westenberger *et al.*, 1990) and humans (Ochoa *et al.*, 1991).

Time-course studies of the development of the hypotension, following administration of LPS, in *in vivo* animal models of septic shock, have revealed a biphasic time-course (Wright *et al.*, 1992). In addition to the sustained fall in blood pressure which develops after several hours of LPS challenge, and which is now attributed to induction of an inducible nitric oxide synthase, there has also been observed an immediate, transient fall in blood pressure which occurs within 1–5 minutes (Fleming *et al.*, 1992). Moreover, Salvemini *et al.* (1990) demonstrated the ability of LPS to cause the immediate release of a nitric oxide-like factor from bovine aortic endothelial cells *in vitro*. Using both human umbilical vein and porcine aortic endothelial cells, Fleming *et al.* (1992) showed that this immediate release of nitric oxide, following challenge with LPS, was prevented by a bradykinin B_2 receptor antagonist. This suggests that it may mediated by LPS-induced release of kinins which can activate the constitutive isoform of nitric oxide synthase within the endothelium.

Szabó *et al.* (1993) have also demonstrated that hyporeactivity to the vasoconstrictor effects of noradrenaline in anaesthetised rats, induced following intravenous injection with LPS, exhibits a biphasic development which parallels the time-course of the development of the hypotension. The immediate hyporeactivity to noradrenaline, preceded the induction of a Ca^{2+}-independent nitric oxide synthase but was reversed by the nitric oxide synthase inhibitor, L-NAME. From these findings, it was postulated that the early phase of the LPS response, in terms of the effects on both hyporeactivity to noradrenaline and on blood pressure, is mediated by nitric oxide production via a constitutive nitric oxide synthase. This is in

contrast to the later, sustained phase of the response which is mediated by nitric oxide production via an inducible nitric oxide synthase.

Thus, it is clear that production of nitric oxide, by activation of both constitutive and inducible nitric oxide synthases, is required for both the hypotension and the hyporeactivity to vasoconstrictors associated with septic shock. In contrast, little is known at present of the mechanism by which LPS induces endothelial barrier dysfunction.

METHODS

CHAPTER 4

4.1 Cytochrome C assay for the production of reactive oxygen species

Before assessing the effects of the hypoxanthine-xanthine oxidase free radical generating system on endothelial barrier function, it was necessary to validate the ability of the system to generate oxygen-derived free radicals. Therefore, the cytochrome C assay, a well-established assay for the production of superoxide anion (Fridovich, 1970), was employed.

4.1.1 Basis of the cytochrome C assay

As supplied commercially, cytochrome C exists predominantly in its oxidised form, ferricytochrome C. Superoxide anion has been demonstrated to cause the reduction of cytochrome C to its reduced form, known as ferrocytochrome C according to the equation:

$$\text{cytochrome } C^{+++} + {}^{\cdot}O_2^{-} \rightarrow \text{cytochrome } C^{++} + O_2$$

In this form, cytochrome C has an absorbance peak at 550 nm (Figure 4.1) and a linear relationship therefore exists between the absorbance of cytochrome C at this wavelength and the proportion of the sample which is in its reduced form. As such, by following the absorbance of a sample of cytochrome C at 550 nm with time, its oxidation state can also be followed. Since superoxide anion causes the reduction of cytochrome C, an increase in the absorbance at 550 nm can therefore be taken as an indicator of the production of the anion.

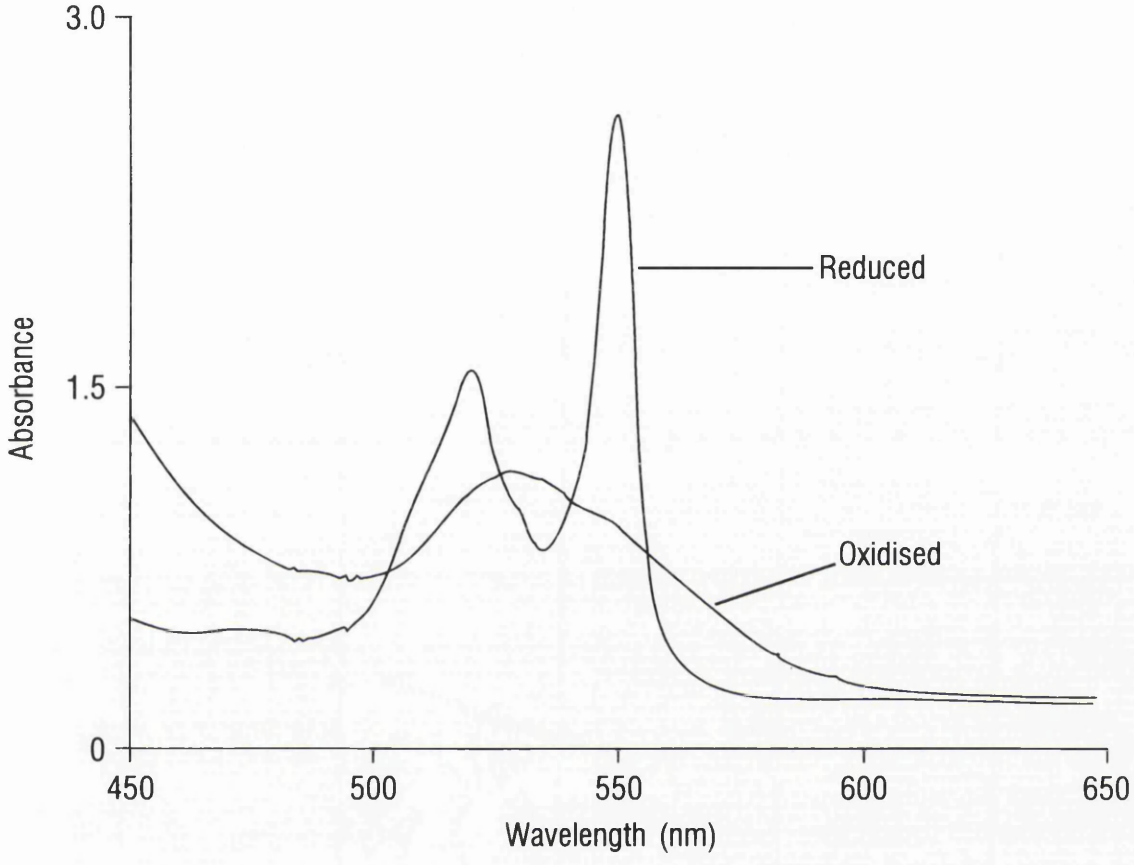


Figure 4.1 Absorption spectra (450–650 nm) generated from a 10 μM sample of cytochrome C both in its commercially supplied oxidised state and in its reduced state, induced by addition of the reducing agent, sodium dithionite (300 μM). The absorbance peak at 550 nm, by which the oxidation state of the sample can be determined, is clearly visible in the spectrum of the sample when in the reduced state.

Hydrogen peroxide may be formed by spontaneous dismutation of superoxide anion:

$$2 \cdot O_2^- + 2 H^+ \rightarrow O_2 + H_2O_2$$

In contrast to superoxide anion, hydrogen peroxide will oxidise cytochrome C, restoring it from its reduced form, ferrocytochrome C, to its oxidised form, ferricytochrome C:

$$\text{cytochrome } C^{++} + H_2O_2 \rightarrow \text{cytochrome } C^{+++} + OH^- + \cdot OH$$

Therefore, as hydrogen peroxide is produced, the absorbance of the cytochrome C sample at 550 nm will decrease as it re-oxidises.

4.1.2 Measurement of superoxide anion and hydrogen peroxide production by the hypoxanthine-xanthine oxidase system

In order to measure the production of superoxide anion and hydrogen peroxide by the hypoxanthine-xanthine oxidase free radical generating system, a 1 ml sample of 10 μM cytochrome C was placed in a glass cuvette. A reference blank sample was also prepared using 1 ml of distilled water. Using a Shimadzu dual-beam spectrophotometer (Model No. UV-240), the absorbance of the cytochrome C at 550 nm was then recorded for the duration of the experiment. Where appropriate according to the protocol being followed, superoxide dismutase (6000 U ml^{-1}) and/or catalase (100 U ml^{-1}) was first added to the cytochrome C sample, following which both hypoxanthine (0.2 mM) and then xanthine oxidase (20 mU ml^{-1}) were added. All drugs were added in volumes of 20 μl.

4.2 Isolation of bovine aortic endothelial cells (BAEC)

Bovine thoracic aortae were obtained from a local abattoir shortly after death and were initially flushed with sterile saline supplemented with 200 U ml^{-1} benzyl penicillin and 200 μg ml^{-1} streptomycin. The end of each aorta which had been proximal to the heart was then ligated with strong string whilst the distal end was canulated with an adapter and a 60 ml syringe which also contained this supplemented sterile saline. The saline was infused into each aorta to keep it moist during transportation to the laboratory where the remainder of the isolation procedure was conducted.

At the laboratory, all subsequent work was carried out under aseptic conditions in a laminar flow cabinet (Flow Laboratories—Model No. Gelaire TC48). Using fresh, sterile saline, the aorta was again flushed through to remove any remaining blood and other debris. The aortic intercostal arteries were cleared of adhering fat to allow ligation with surgical thread, thus sealing off the aorta. Using a syringe, the aorta was drained of saline which was then replaced with 10 ml of sterile collagenase solution (Type II, Sigma; 0.1% in Dulbecco's modification of Eagle's medium (DMEM)). The aorta was incubated at 37°C for 20 minutes after which it was gently massaged and the resulting cell suspension harvested using a syringe. 20 ml of sterile saline was then infused into the vessel lumen to remove any remaining cells. The collected cell suspensions were then centrifuged (200g; 4 minutes; 10°C) and the supernatants discarded. The resulting cell pellets were resuspended in 10 ml of growth medium (DMEM supplemented with 10% foetal calf serum, 10% newborn calf serum, 4 mM glutamine, 200 U ml^{-1} penicillin and 200 μg ml^{-1} streptomycin) and centrifuged again (200g; 4 minutes, 10°C). After again discarding the supernatants, the pellets from each aorta were resuspended in 20 ml of growth medium and seeded

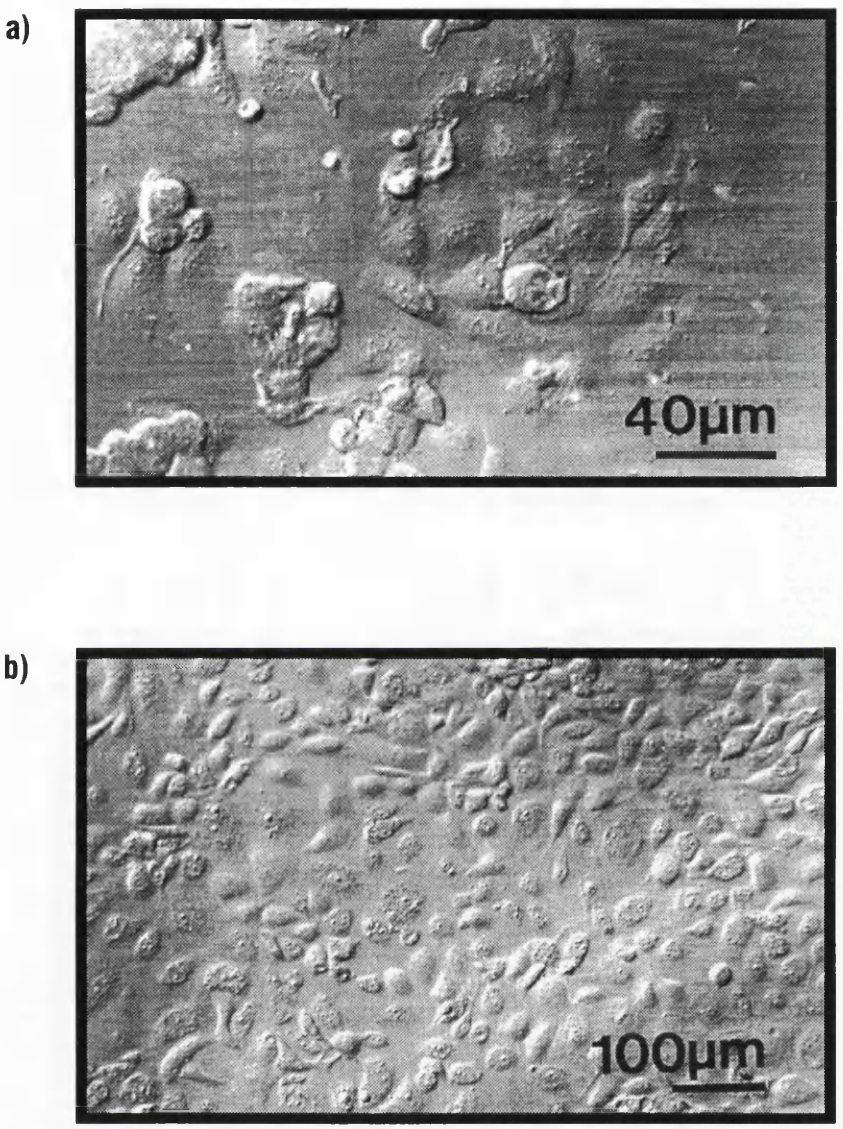


Figure 4.2 Differential interference contrast micrographs of primary cultures of bovine aortic endothelial cells are shown **a)** 1 day and **b)** 3 days after isolation by collagenase treatment. The cells, which were grown on glass coverslips to facilitate micrographic visualisation, are initially isolated as clumps which adhere to the underlying surface within 24 hours. After subsequent division, the cells multiply to form a confluent monolayer which exhibits the characteristic "cobblestone" appearance of the vascular endothelium.

into 80 cm^2 culture flasks (Nunc). The flasks were placed in an incubator (Flow Laboratories—Model No. 220) at 37°C in an atmosphere of 5% CO_2 and 95% air and grown until confluent (3-7 days). During this period the growth medium in each flask was renewed, initially one day following the isolation and, subsequently, every 2-3 days.

The cells were validated as endothelial cells by the fact that they formed a single layer with a characteristic "cobblestone" morphology. Previous work in this laboratory has further confirmed the identification of pig aortic endothelial cells, which had been isolated in a similar manner, by their ability to produce both prostacyclin (Martin *et al.*, 1989) and EDRF (Martin *et al.*, 1988), and by using a fluorescent LDL assay, the cell population has been shown to have >98% purity (Martin *et al.*, 1988).

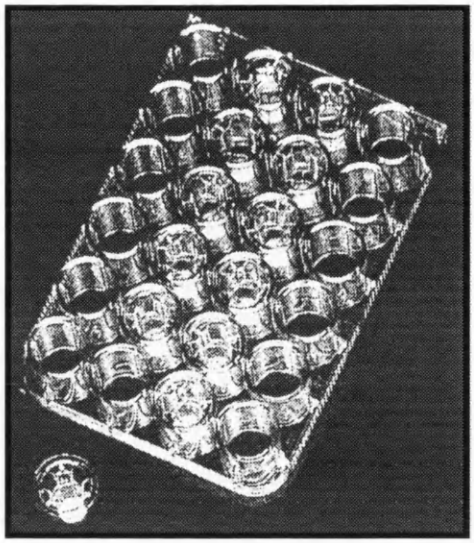
4.3 Assessment of endothelial barrier function

4.3.1 *In vitro* model of the vascular endothelial barrier

Endothelial barrier function was investigated in this study using an *in vitro* model of the arterial endothelial barrier (Gudgeon & Martin, 1989). Such a model is a useful tool since, unlike *in vivo* models, it facilitates the elimination of both the indirect influences on permeability of other physiological actions of the agents being studied, and any interactions which occur with other cell types (see Chapter 1). Therefore, it enables the direct actions of agents solely on endothelial barrier function to be observed.

The model used in this study consisted of a two-chamber system in which monolayers of BAEC were grown on Costar Transwell membranes (Figure 4.3). Albumin was chosen as a marker of endothelial barrier function since it is an endogenous substance and, being a major component of blood, is

a)



b)

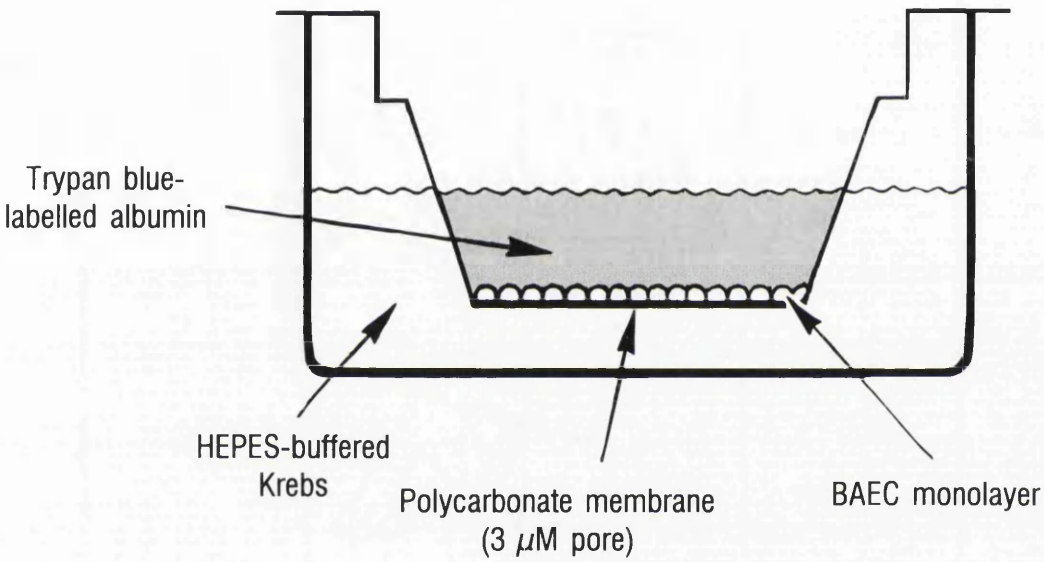


Figure 4.3 a) Photographic and **b)** diagrammatic representation of Transwell membrane filter units. First passage BAEC's were cultured on polycarbonate membranes for 3–4 days after which, where appropriate, drugs were added to both upper and lower chambers as timed pre-treatments. The membranes were then transferred to wells, the lower chamber of which was filled with HEPES-buffered Krebs, whilst trypan blue-labelled albumin (4% in HEPES-buffered Krebs) was placed in the upper chamber. Again, where appropriate, drugs were added to both chambers and the wells placed on an orbital shaker for 90 minutes. After this time, samples were taken from each lower chamber and the transfer of trypan blue-labelled albumin assessed by colourometric assay.

believed to be an important determinant of the hydraulic conductivity of the endothelium *in vivo* (McCandless *et al.,* 1991). Endothelial cell monolayers were grown on polycarbonate membranes (diameter 6.5 mm; pore size 3 μm) and the passage of trypan blue-labelled albumin across these monolayers was measured by a spectrophotometric assay.

4.3.2 Preparation of endothelial monolayers

Upon reaching confluence, an 80 cm^2 flask of BAEC was taken, the growth medium removed and the cells washed twice using 20 ml of sterile saline. 10 ml of a solution of trypsin (0.05%) and EDTA (0.02%) was then added, and the flask was incubated at 37°C for approximately 2–4 minutes to facilitate cell detachment. The resultant cell suspension was added to 2 ml of newborn calf serum, which acted to inhibit the trypsin, and then twice centrifuged (200g; 4 minutes; 10°C) followed by resuspension in 5 ml of growth medium. Transwell membranes were prepared by adding 1 ml of growth medium to the lower chamber of each of 48 wells, with a polycarbonate filter being placed on top. 100 μl of the BAEC suspension was then added to the upper chamber of each well on top of the polycarbonate filter. The cells were incubated again at 37°C in an atmosphere of 95% air/5% CO_2 for 3–4 days to allow these secondary cultures to attain confluence after which drug treatments and albumin transfer studies were carried out to assess endothelial barrier function.

4.3.3 Preparation of trypan blue-labelled albumin

Bovine serum albumin (Fraction V; 4g) was dissolved in 100 ml of Krebs containing (mM): NaCl 118, KCl 4.8, $MgSO_4$ 1, KH_2PO_4 1.2, $NaHCO_3$ 2.4, glucose 11 and HEPES (N-2-hydroxyethylpiperazine-N´-2-ethanesuplhonic acid) 5. Trypan blue (180 mg) was then added to form

a stable complex with an absorption peak at 590 nm. Precipitation with 6% trichloroacetic acid showed that the trypan blue was >99.8% bound to albumin. The stable trypan blue-labelled albumin complex appeared therefore to constitute a suitable high molecular weight marker for use in assessing the barrier function of endothelial monolayers.

4.3.4 Addition of drugs as pretreatments

In all experiments in which drug pretreatments were performed, the drugs were initially added to confluent monolayers in culture at appropriate time intervals prior to the assessment of barrier function. These drugs, which included deferoxamine when added as an overnight pretreatment, lipopolysaccharide (LPS) and all drugs added concomitantly with LPS, were added to both upper and lower chambers of each Transwell in volumes of 11 μl. The monolayers were then returned to the incubator at 37°C in 5% CO_2/95% air for the duration of the pretreatments. Full details of timings and concentrations used are listed in the appropriate sections in the Results.

4.3.5 Measurement of albumin transfer across endothelial monolayers

Transwell membranes with BAEC monolayers attached were initially washed twice by gentle immersion in HEPES-buffered Krebs (as described in section 4.3.3) at 37°C and pH 7.4. The membranes were transferred to a 24-well cluster plate with 600 μl of HEPES-buffered Krebs being placed in the lower chamber and 100 μl of trypan blue-labelled albumin (4%) being placed in the upper chamber of each well above the endothelial monolayer. The volumes were chosen so as to avoid generating a hydrostatic gradient across the monolayer. With the exception of LPS and

dexamethasone, which were added as pretreatments, drugs were then added in volumes of 7 μl to both upper and lower chambers and the plates were transferred to an orbital shaker (Luckham) which ensured gentle, continuous agitation, and placed in air in an incubator (ICN Flow—Model No. 160) at 37°C for 90 minutes. At the end of this period, 100 μl samples were removed from the lower chamber of each well and transferred to plastic cuvettes for quantification of albumin transfer.

In some experiments involving LPS pretreatments, a shorter incubation time than 90 minutes with trypan-blue labelled albumin was employed. This was because the monolayers in these cases were found to be more permeable than normal. Thus, the albumin transfer rate was found to be high enough to have enabled sufficient albumin transfer to have occurred by either 30 or 60 minutes to facilitate quantification of transfer. Such modifications of the procedure could only be performed in experiments, such as those with LPS, in which the drugs were added as pretreatments and whose action was therefore not dependent on the time period of this incubation. Since each experiment was performed with its own internal controls, valid qualitative analysis could still be performed.

4.3.6 Quantification of albumin transfer

The 100 μl samples collected from the lower chamber of each well were diluted 1 in 10 by addition of 900 μl HEPES-buffered Krebs to each cuvette. The content of trypan blue-labelled albumin in each sample was subsequently determined using a dual-beam spectrophotometer (Shimadzu) to measure the absorbance of each at 590 nm. These results were then expressed as a percentage of that which would have been obtained had full equilibrium occurred. 1 ml of HEPES-buffered Krebs was used as the 0% reference sample whilst the 100% reference sample was

prepared by adding 14.3 μl of trypan blue-labelled albumin to 985.7 μl of HEPES-buffered Krebs.

4.4 Materials

4.4.1 Tissue culture reagents and materials

DMEM, foetal calf serum, newborn calf serum, glutamine and penicillin/ streptomycin were all obtained from Gibco, Paisley, UK. Trypsin/ EDTA was obtained from Flow Laboratories, Irvine, UK whilst bovine serum albumin and collagenase (Type II) were obtained from Sigma, Poole, UK. Collagenase was dissolved in DMEM to yield a 0.1% solution which was then sterilised by filtration through a millipore filter (0.22 μm; Flow Laboratories).

Tissue culture flasks (80 cm^2) were obtained from Life Technologies, Uxbridge, UK. Transwell membranes (6.5 mm diameter; 3 μM pore size) were obtained from Costar (UK) Ltd., High Wycombe, UK. Sterile centrifuge tubes (15 ml and 50 ml; Falcon) were obtained from R.& J. Wood, Paisley, UK. Sterile saline (0.9% w/v) was obtained from Baxter Health Care, Thetford, UK. Cylinders of CO_2 were obtained from B.O.C., Ltd., UK.

4.4.2 Drugs

Allopurinol, catalase (bovine liver), cytochrome C (horse heart), deferoxamine mesylate, dexamethasone, DL-dithiothreitol, DL-homocysteine, hydrogen peroxide (30%), hypoxanthine, lipopolysaccharide from *Salmonella typhosa* (prepared by phenol extraction), D-mannitol, N-(2-mercaptopropionyl)-glycine, N^{ω}-nitro L-arginine, N^{ω}-nitro-L-arginine methyl ester, superoxide dismutase (bovine erythrocyte), polymixin B

sulphate, xanthine and xanthine oxidase (buttermilk) were obtained from Sigma, Poole, UK. 1,3-dimethyl-2-thiourea was obtained from Aldrich, Gillingham, UK. Human recombinant interferon-γ was obtained from the National Institute for Biological Standards and Control, Potters Barr, Hertfordshire. Sodium flurbiprofen dihydrate was a generous gift from Dr. R.V. Holland, Boots Pure Drug Co., UK, whilst N^{ω}-monomethyl-L-arginine acetate was a generous gift from Dr. D.D. Rees, Wellcome Laboratories, Beckenham, Kent, UK. All drugs were dissolved in distilled water except for hypoxanthine and xanthine, which were dissolved in sodium hydroxide (10 mM), and dexamethasone which was dissolved in absolute ethanol. The final concentration of sodium hydroxide in experiments did not exceed 0.2 mM whilst that of ethanol did not exceed 0.2%. These concentrations did not, in themselves, affect albumin transfer across monolayers of BAEC. None of the drugs used displaced trypan blue from the trypan blue-labelled albumin complex.

4.5 Statistical analysis of results

Results are expressed as means±s.e. mean and were compared by one-way analysis of variance to determine if any significant differences existed between treatment groups. This was followed by Fisher's test to determine where differences lay. Analysis was conducted using Minitab Release 8, (Minitab Inc., State College, USA). Due to the variability encountered in resting transfer of albumin amongst different batches of cells, each experiment was performed with its own internal controls. A probability of 0.05 or less was taken to be significant.

RESULTS

CHAPTER 5

5.1 Production of reactive oxygen species by the hypoxanthine-xanthine oxidase system

The cytochrome C assay enables the time-course of the production of free radical species by the hypoxanthine-xanthine oxidase system to be observed (Fridovich, 1970). As purchased, cytochrome C is found predominantly in its oxidised form. At 550 nm, the absorbance of a sample of cytochrome C increases as it becomes reduced, but in the absence of any added agents it will remain at a stable level.

Addition of either hypoxanthine or xanthine oxidase alone to oxidised cytochrome C has no effect on its oxidation state and hence does not alter its absorbance at 550 nm. However, as shown in Figure 5.1a, when the combination of 0.2 mM hypoxanthine and 20 mU ml^{-1} xanthine oxidase was added to a 10 μM sample of oxidised cytochrome C, a rapid increase in its absorbance at 550 nm occurred, indicating that the sample was being rapidly reduced. The absorbance reached a maximum within a time period of 1–2 minutes, levelled off and then, after around 15 minutes, began to slowly decrease as the cytochrome C sample became re-oxidised.

When the experiment was repeated in the presence of 6000 U ml^{-1} superoxide dismutase which rapidly converts superoxide anion to hydrogen peroxide, the initial rapid increase in absorbance at 550 nm was significantly inhibited (Figure 5.1b). This would indicate that the initial rapid reduction of cytochrome C induced by addition of the hypoxanthine-xanthine oxidase system, results from the production of superoxide anion.

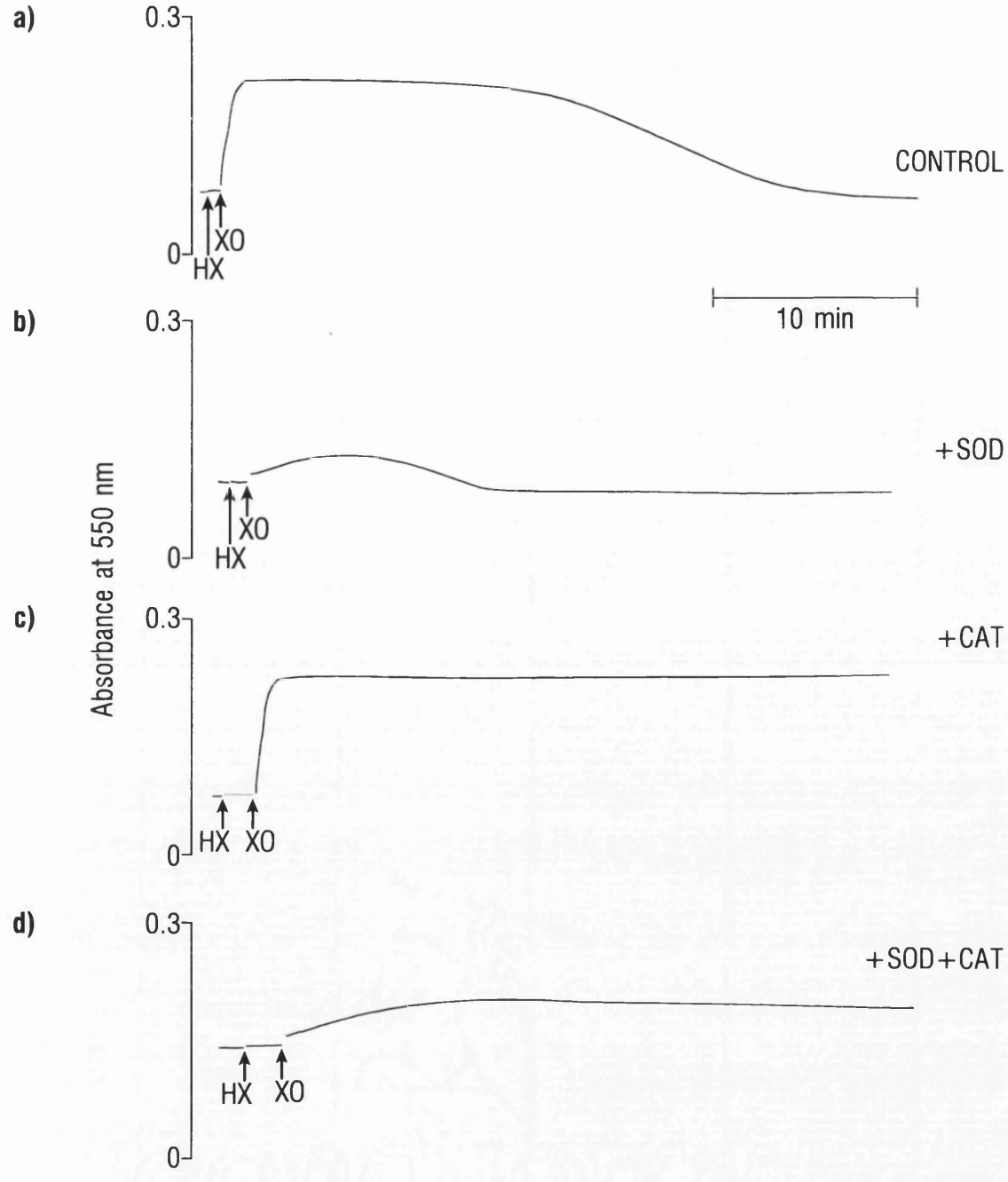


Figure 5.1 Traces showing absorption changes at 550 nm with time of 10 μM samples of cytochrome C on addition of 0.2 mM hypoxanthine (HX) followed by 20 mU ml^{-1} xanthine oxidase (XO). **a)** shows the control response. **b)** shows the response obtained in the presence of 6000 U ml^{-1} superoxide dismuase (SOD). **c)** shows the response obtained in the presence of 100 U ml^{-1} catalase (CAT). **d)** shows the response obtained in the combined presence of 6000 U ml^{-1} superoxide dismuase and 100 U ml^{-1} catalase.

In contrast, in the presence of 100 U ml^{-1} catalase which catalyses the breakdown of hydrogen peroxide to both water and oxygen, the initial rise in absorption at 550 nm induced by hypoxanthine (0.2 mM) and xanthine oxidase (20 mU ml^{-1}) was unaffected, but the subsequent decrease in absorption was completely blocked (Figure 5.1c). This would indicate that the re-oxidation phase of the response is induced by the formation of hydrogen peroxide which can be formed through spontaneous dismutation of superoxide anion.

As would be expected, in the presence of a combination of both superoxide dismutase and catalase (Figure 5.1d), both the initial rapid reduction and the subsequent slower re-oxidation of the cytochrome C sample were inhibited since the actions of both superoxide anion and hydrogen peroxide are being blocked.

These results confirm the ability of the hypoxanthine-xanthine oxidase system to produce both superoxide anion and, subsequently, hydrogen peroxide, thereby validating its use as a free radical generating system for use in studying the effects of free radicals on endothelial barrier function. Additionally, since the system produces both superoxide anion and hydrogen peroxide, it is also possible that further reactive oxygen species may be generated such as hydroxyl radical, which given a source of iron, can be formed in the presence of both these species by the Fenton reaction (Repine *et al.*, 1981), and also hypochlorous acid which may be produced by an interaction between hydrogen peroxide and chloride ions in the presence of the enzyme, myeoloperoxidase (Halliwell & Gutteridge, 1989)—see Introduction.

5.2 Validation of the *in vitro* model of endothelial permeability

Endothelial cells grown on membranes have been shown to maintain a profile of barrier function similar to that obtained *in vivo* (Del-Vecchio *et al.,* 1987). The validity of the *in vitro* model employed in this study to assess endothelial permeability has been demonstrated by previous work in the same laboratory. A time-course study was undertaken to determine the passage of trypan blue-labelled albumin (4%) across Transwell membranes both alone and with monolayers of first passage pig aortic endothelial cells attached (Figure 5.2). In the absence of cells, equilibration of albumin across the membranes was rapid reaching maximum (100% equilibration) within 20–40 minutes. In the presence of endothelial cells, however, albumin transfer was substantially restricted, thereby demonstrating the ability of the model to recreate *in vitro* the barrier properties of the vascular endothelium.

Similarly, in this study, monolayers of BAEC were found to be able to restrict the passage of albumin across Transwell membranes. Typically, after a 90 minutes incubation period, control levels of albumin transfer ranged from 2–25% (Figures 5.3–5.7).

5.3 Study of the effects of the hypoxanthine-xanthine oxidase system on barrier function

5.3.1 Effects of hypoxanthine and xanthine oxidase on albumin transfer

Using the *in vitro* model of bovine aortic endothelial permeability employed in this study, the effects of the hypoxanthine-xanthine

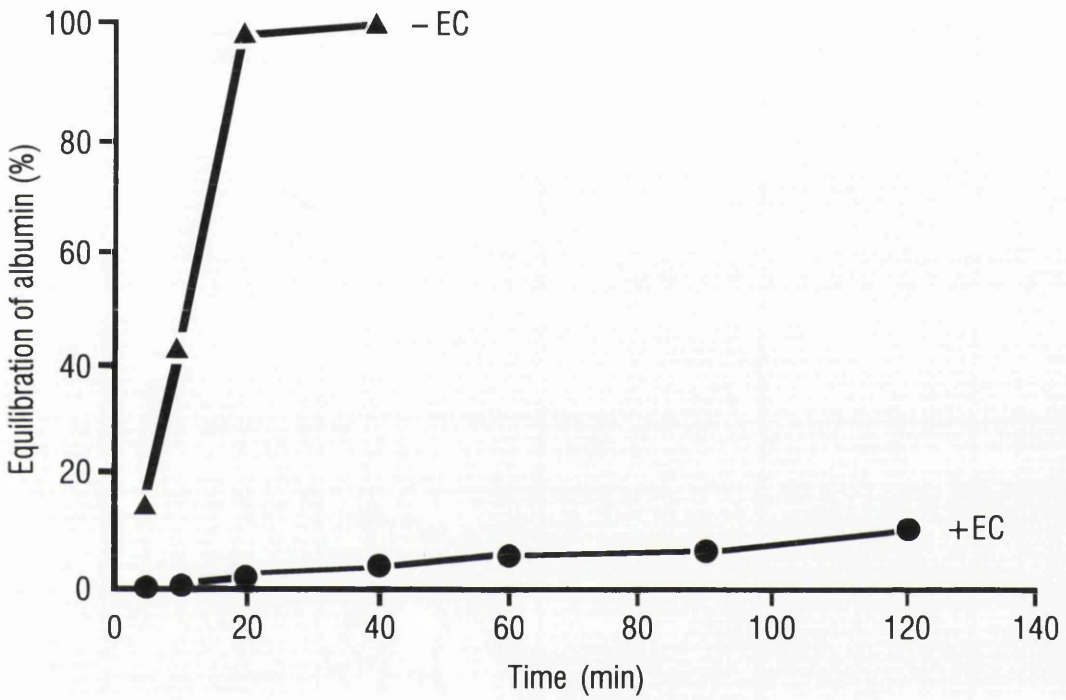


Figure 5.2 Time-course showing the passage of trypan blue-labelled albumin across Transwell membranes in the presence (+EC) and absence (–EC) of pig aortic endothelial cells. HEPES-buffererd Krebs containing 4% trypan blue-labelled albumin was placed in the upper chamber and its passage into the lower chamber, containing HEPES-buffered Krebs only, was measured at various time points. Each point is the mean of 2 observations with full equilibration between both chambers being 100%. Modified from Gudgeon and Martin (1989).

oxidase free radical generating system were investigated. When either hypoxanthine (0.2 mM) or xanthine oxidase (20 mU ml^{-1}) was added alone to monolayers of BAEC during a 90 minute incubation, there was no significant alteration in the equilibration of albumin across the monolayers relative to the control level (4.6±0.9%) in the absence of any added drug (Figure 5.3). However when hypoxanthine and xanthine oxidase were added in combination, a significant increase in albumin transfer (18.2±1.8%) was observed demonstrating the ability of the hypoxanthine-xanthine oxidase system to induce an increase in endothelial permeability.

In the presence of a fixed concentration of hypoxanthine (0.2 mM), xanthine oxidase (3–300 U ml^{-1}) was shown to induce a concentration-dependent increase in albumin transfer across monolayers of BAEC (Figure 5.4). The maximum increase obtained was 47.6±1.3% at a xanthine oxidase concentration of 300 U ml^{-1}. Thus, the xanthine oxidase concentration of 20 U ml^{-1} was not a maximal concentration in terms of its effects on endothelial permeability. This concentration of xanthine oxidase, together with a fixed hypoxanthine concentration of 0.2 mM, was chosen as a standard concentration in all subsequent experiments involving the hypoxanthine-xanthine oxidase system.

The effects of concomitant addition of the xanthine oxidase inhibitor, allopurinol (4 mM) on the increase in albumin transfer across monolayers of BAEC induced by hypoxanthine and xanthine oxidase (added at the chosen standard concentrations of 0.2 mM and 20 U ml^{-1}, respectively) are shown in Figure 5.5. As would be expected, allopurinol significantly reduced the increase in albumin transfer induced by the hypoxanthine-xanthine oxidase system, confirming the need for the complete enzyme-substrate system to induce endothelial barrier dysfunction.

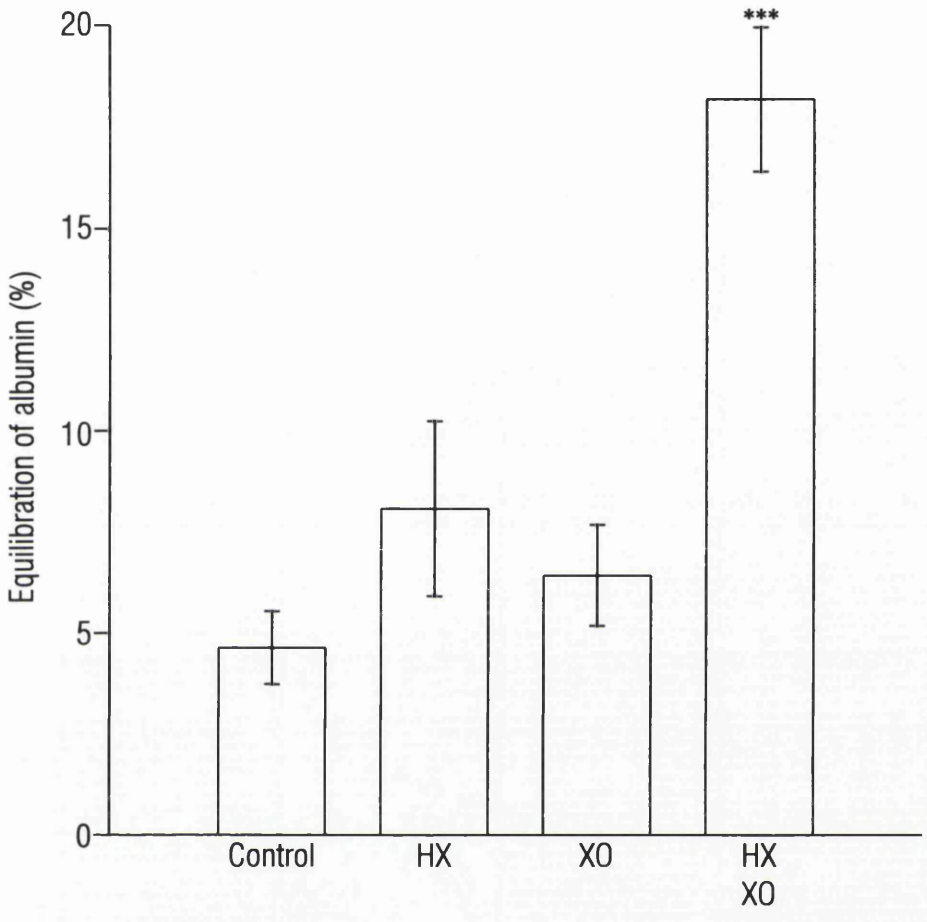


Figure 5.3 Graph showing the effects of 90 minutes' exposure to 0.2 mM hypoxanthine (HX) and 20 mU ml^{-1} xanthine oxidase (XO), added alone and in combination, on albumin transfer across monolayers of bovine aortic endothelial cells. Each column is the mean of 6 observations with vertical bars representing s.e. mean. ***$p<0.001$, indicates a significant difference from Control.

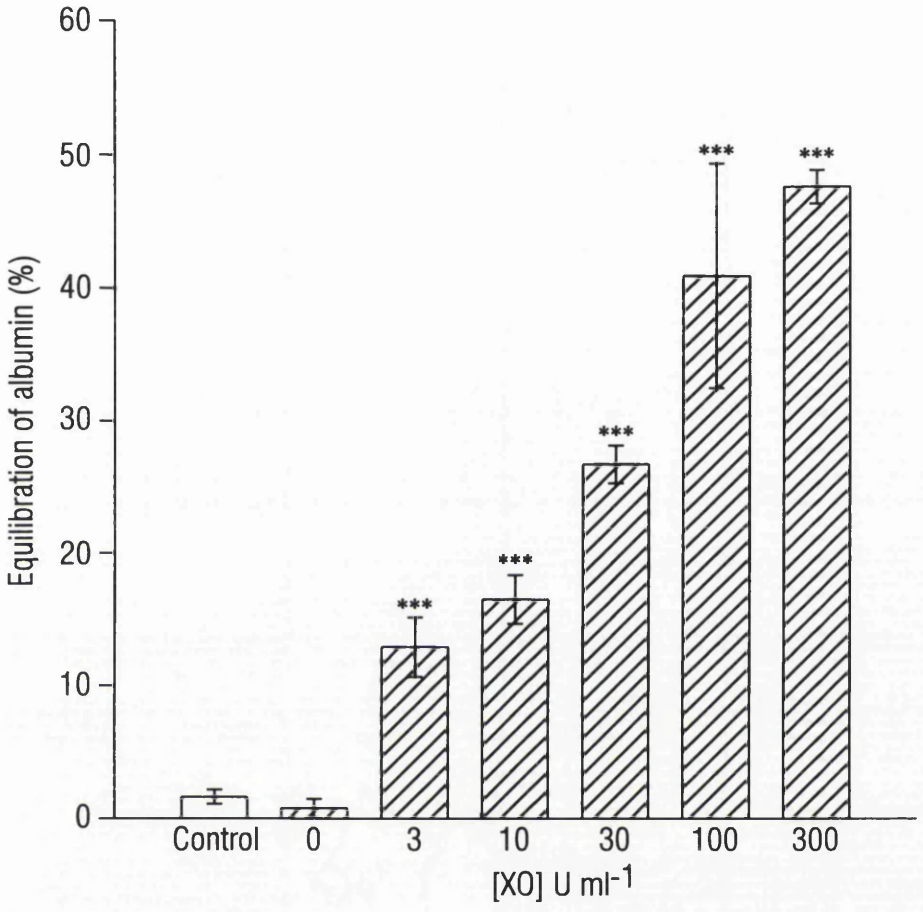


Figure 5.4 Graph showing the effects of 90 minutes' exposure to 0–300 mM xanthine oxidase (XO) on albumin transfer across monolayers of bovine aortic endothelial cells in presence of 0.2 mM hypoxanthine (▨). Control responses in the absence of both xanthine oxidase and hypoxanthine are also shown (□). Each column is the mean of 6 observations with vertical bars representing s.e. mean. ***$p<0.001$, indicates a significant difference from Control.

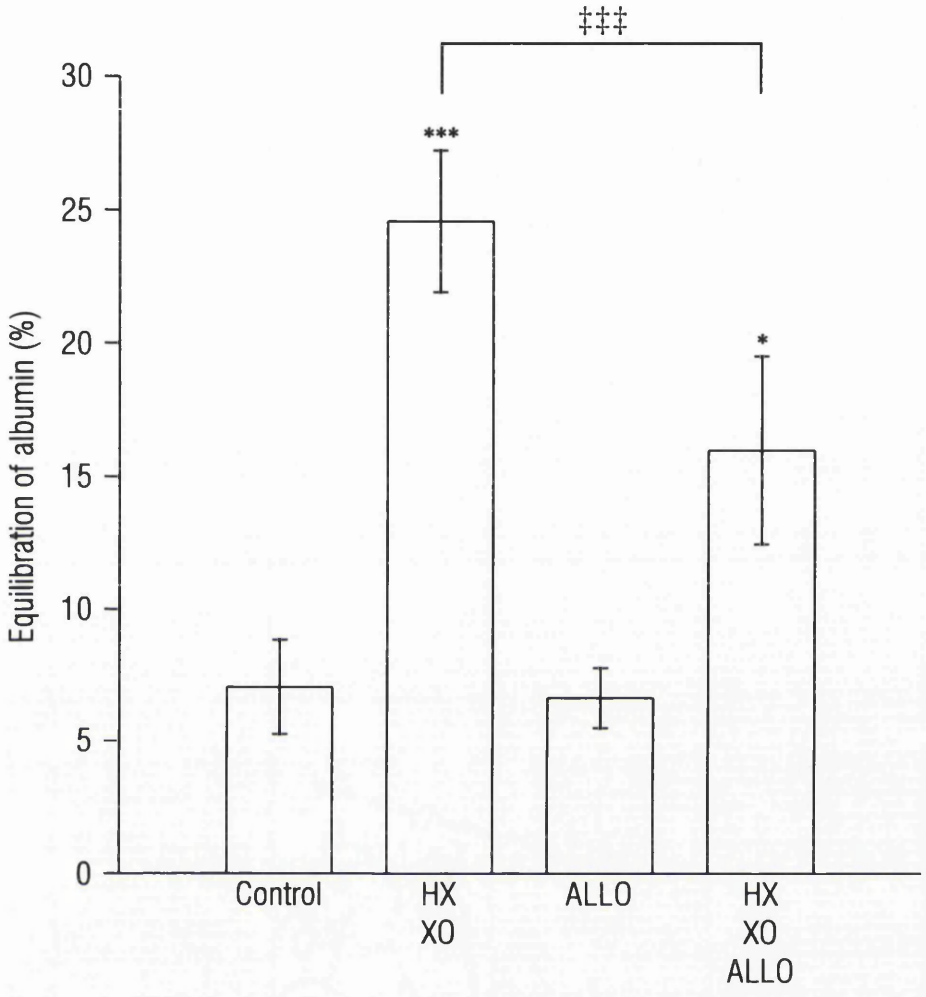


Figure 5.5 Graph showing the effects of 90 minutes' exposure to 0.2 mM hypoxanthine (HX) and 20 mU ml^{-1} xanthine oxidase (XO) on albumin transfer across monolayers of bovine aortic endothelial cells. The effects of concomitant addition of 4 mM allopurinol (ALLO) are also shown. Each column is the mean of 12 observations with vertical bars representing s.e. mean. *$p<0.05$; ***$p<0.001$, indicates a significant difference from Control, whilst ‡‡‡$p<0.001$, indicates a significant difference between groups joined by a bracket.

The inhibition obtained on addition of allopurinol was incomplete, only producing approximately a 51% reduction of the hypoxanthine-xanthine oxidase-induced permeability increase. Higher concentrations of allopurinol, however, were not tested due to its relative insolubility in aqueous solutions.

In order to investigate any involvement of superoxide anion in mediating the permeability increase induced by the hypoxanthine-xanthine oxidase system, the effects of superoxide dismutase (30 U ml^{-1}) were investigated (Figure 5.6). When added alone, superoxide dismutase had no effect on the level of albumin transfer compared to control and when it was added in combination with hypoxanthine (0.2 mM) and xanthine oxidase (20 mU ml^{-1}), it showed no inhibitory effect. This result would indicate that superoxide anion does not mediate the permeability increase.

Since it is possible that the reason for its failure to show an inhibitory effect was because the superoxide dismutase was not added at a sufficiently high concentration, this experiment was repeated at a much increased superoxide dismutase concentration of 6000 U ml^{-1} to ensure maximum effectiveness of the drug. However, when 6000 U ml^{-1} superoxide dismutase, which itself had no effect of albumin transfer across monolayers of BAEC (Figure 5.7), was added in combination with hypoxanthine (0.2 mM) and xanthine oxidase (20 mU ml^{-1}), it was also found to have no inhibitory effect on the increase in albumin transfer stimulated by the free radical generating system. This would therefore confirm the observation that superoxide anion is not involved in mediating the hypoxanthine-xanthine oxidase-induced increase in endothelial permeability.

To investigate any involvement of hydrogen peroxide in the increase in albumin transfer across monolayers of BAEC, catalase (0.3–30 U ml^{-1}) was co-incubated with hypoxanthine (0.2 mM) and xanthine oxidase

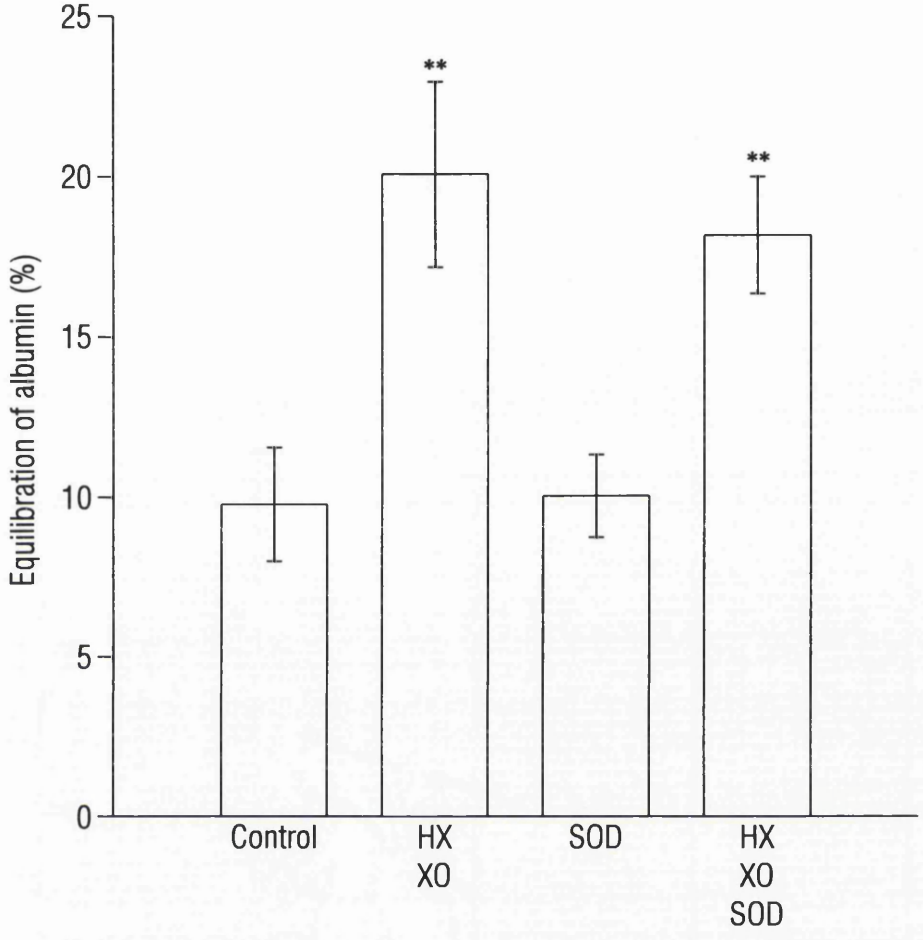


Figure 5.6 Graph showing the effects of 90 minutes' exposure to 0.2 mM hypoxanthine (HX) and 20 mU ml^{-1} xanthine oxidase (XO) on albumin transfer across monolayers of bovine aortic endothelial cells. The effects of concomitant addition of 30 U ml^{-1} superoxide dismutase (SOD) are also shown. Each column is the mean of 6 observations with vertical bars representing s.e. mean. **$p<0.01$, indicates a significant difference from control.

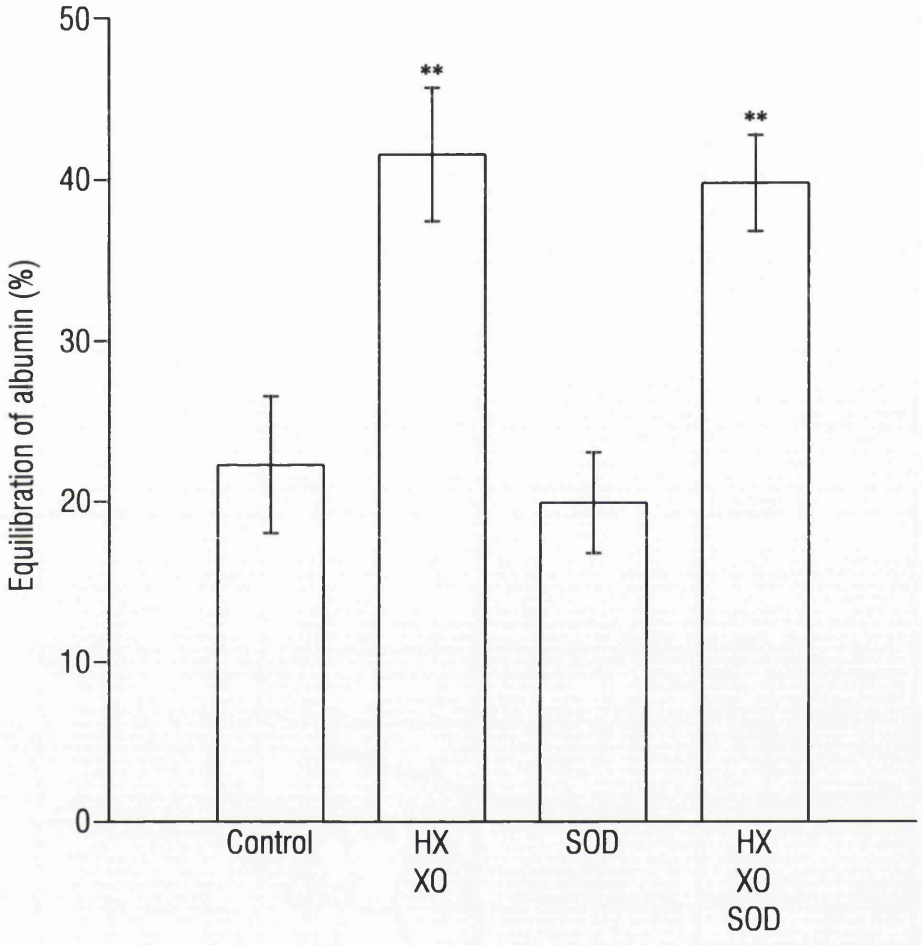


Figure 5.7 Graph showing the effects of 90 minutes' exposure to 0.2 mM hypoxanthine (HX) and 20 mU ml^{-1} xanthine oxidase (XO) on albumin transfer across monolayers of bovine aortic endothelial cells. The effects of concomitant addition of 6000 U ml^{-1} superoxide dismutase (SOD) are also shown. Each column is the mean of 6 observations with vertical bars representing s.e. mean. **$p<0.01$, indicates a significant difference from control.

(20 mU ml^{-1}) (Figure 5.8). At concentrations of 0.3–1 U ml^{-1}, catalase had no effect but at 3 U ml^{-1} and above, it was found to abolish the increase in albumin transfer stimulated by the hypoxanthine-xanthine oxidase system. This therefore indicates that hydrogen peroxide must play a key role in mediating the barrier dysfunction induced by the hypoxanthine-xanthine oxidase system.

5.3.2 Effects of xanthine and xanthine oxidase on albumin transfer

In order to establish if xanthine can equally well be used as a substrate for xanthine oxidase in terms of its effects on barrier function, certain experiments were repeated substituting xanthine in place of hypoxanthine at the same concentration.

Figure 5.9 shows that like hypoxanthine, xanthine (0.2 mM) had no effect on albumin transfer across monolayers of BAEC compared to control levels (6.4±.05%) when added alone. However, when xanthine was added in combination with xanthine oxidase (20 mU ml^{-1}), which again was shown to have no significant effect by itself, a significant increase in albumin transfer was observed (30.8±1.6%).

When catalase (0.3–30 U ml^{-1}) was added concomitantly with xanthine (0.2 mM) and xanthine oxidase (20 mU ml^{-1}), it was observed to be without effect at concentrations of 0.3–1 U ml^{-1}, but at concentrations of 3 U ml^{-1} and greater, it was found to abolish the induced increase in albumin transfer (Figure 5.10).

These two experiments revealed that there was little difference observed in this system in either the increase in albumin transfer obtained, or the susceptibility of this increase to be inhibited by catalase, when xanthine

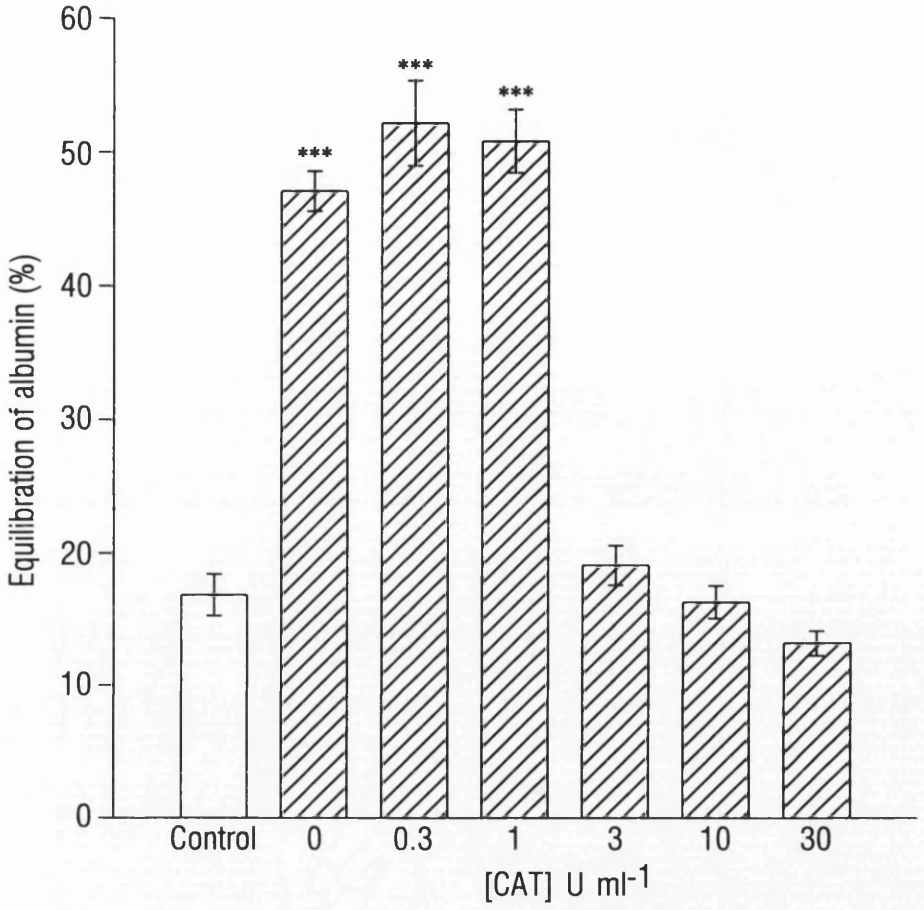


Figure 5.8 Graph showing albumin transfer across monolayers of bovine aortic endothelial cells after 90 minutes' incubation both in the presence (▨) and absence (□) of 0.2 mM hypoxanthine and 20 mU ml^{-1} xanthine oxidase. The effects of concomitant addition of 0.3–30 U ml^{-1} catalase (CAT) are also shown. Each column is the mean of 6–9 observations with vertical bars representing s.e. mean. ***$p<0.001$, indicates a significant difference from Control.

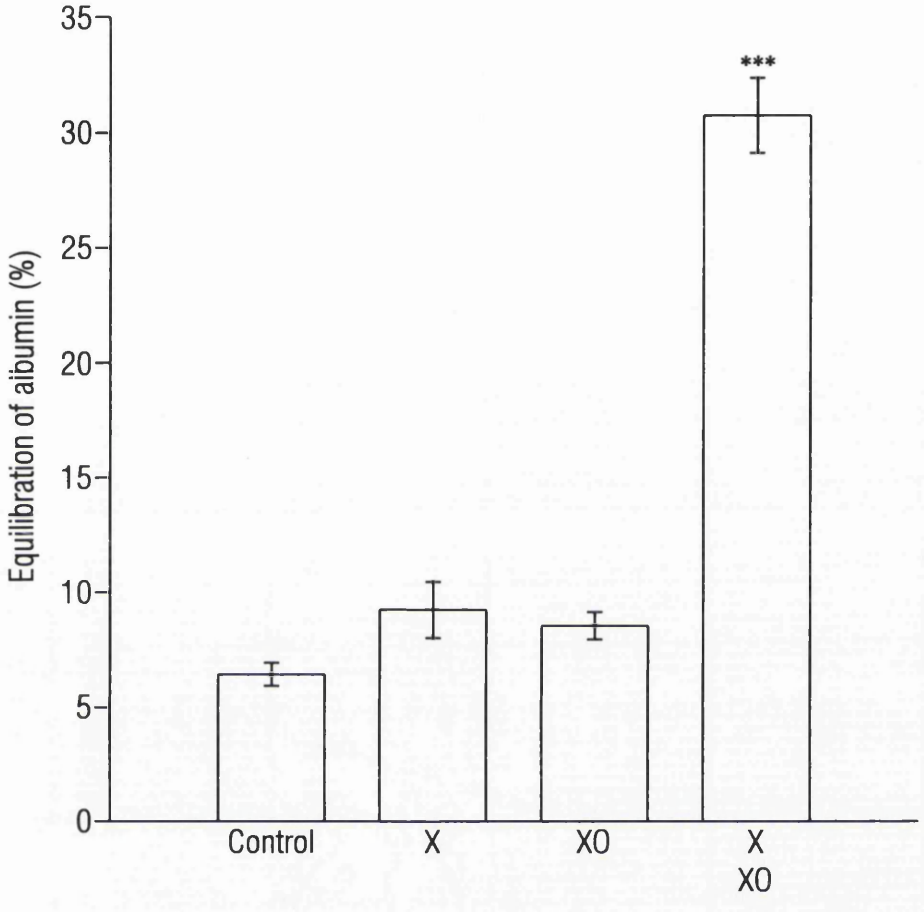


Figure 5.9 Graph showing the effects of 90 minutes' exposure to 0.2 mM xanthine (X) and 20 mU ml^{-1} xanthine oxidase (XO), added alone and in combination, on albumin transfer across monolayers of bovine aortic endothelial cells. Each column is the mean of 6 observations with vertical bars representing s.e. mean. ***$p<0.001$, indicates a significant difference from Control.

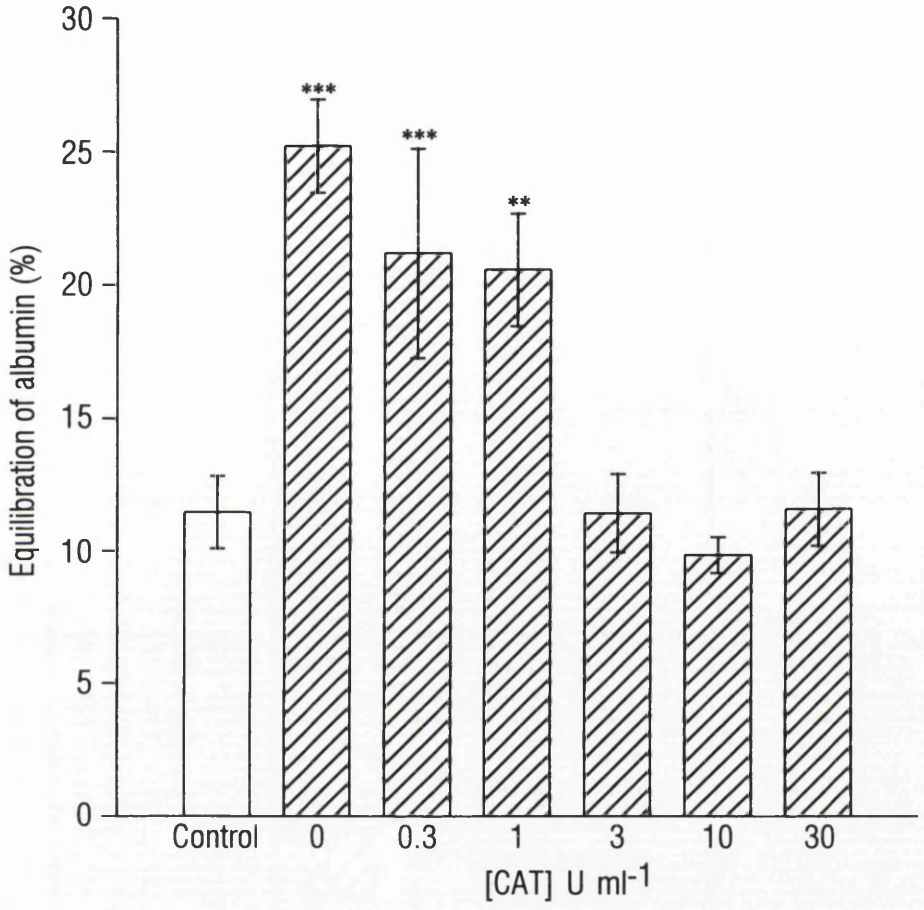


Figure 5.10 Graph showing albumin transfer across monolayers of bovine aortic endothelial cells after 90 minutes' incubation both in the presence (▨) and absence (□) of 0.2 mM xanthine and 20 mU ml^{-1} xanthine oxidase. The effects of concomitant addition of 0.3–30 U ml^{-1} catalase (CAT) are also shown. Each column is the mean of 6-9 observations with vertical bars representing s.e. mean. ***$p<0.001$; **$p<0.01$, indicates a significant difference from Control.

(0.2 mM) was substituted in place of hypoxanthine and used in combination with xanthine oxidase at the chosen standard concentration of 20 mU ml^{-1}.

5.3.3 Effects of hydrogen peroxide on albumin transfer

Having shown through the effectiveness of catalase that hydrogen peroxide is implicated in mediating the increase in albumin transfer produced by the hypoxanthine-xanthine oxidase system, the effects of addition to monolayers of BAEC of native hydrogen peroxide itself were then examined (Figure 5.11). Hydrogen peroxide was found to induce a biphasic concentration-response curve in terms of its effects on albumin transfer across monolayers of BAEC. A lower peak was observed at 0.1–0.3 mM (with a maximum of 12.2±1.0% at 0.1 mM) which then declined towards basal levels as the concentration was increased, whilst a higher peak was observed at around 10–30 mM (with a maximum of 25.6±1.8% at 10 mM). This therefore clearly shows that hydrogen peroxide can itself induce endothelial barrier dysfunction.

Figure 5.12 shows the effects of catalase (0.3 and 1 U ml^{-1}) on the increase in albumin transfer across BAEC induced by 0.1 mM hydrogen peroxide. As expected, at both concentrations tested, catalase was found to abolish the increase stimulated by hydrogen peroxide.

Similarly in Figure 5.13, the effects of catalase (30–3000 U ml^{-1}) on the increase in albumin transfer induced by 10 mM hydrogen peroxide are shown. Catalase showed no inhibitory effect at concentrations of 30–300 U ml^{-1}, but at concentrations of 1000 U ml^{-1} and above, it was observed to abolish the hydrogen peroxide-induced permeability increase.

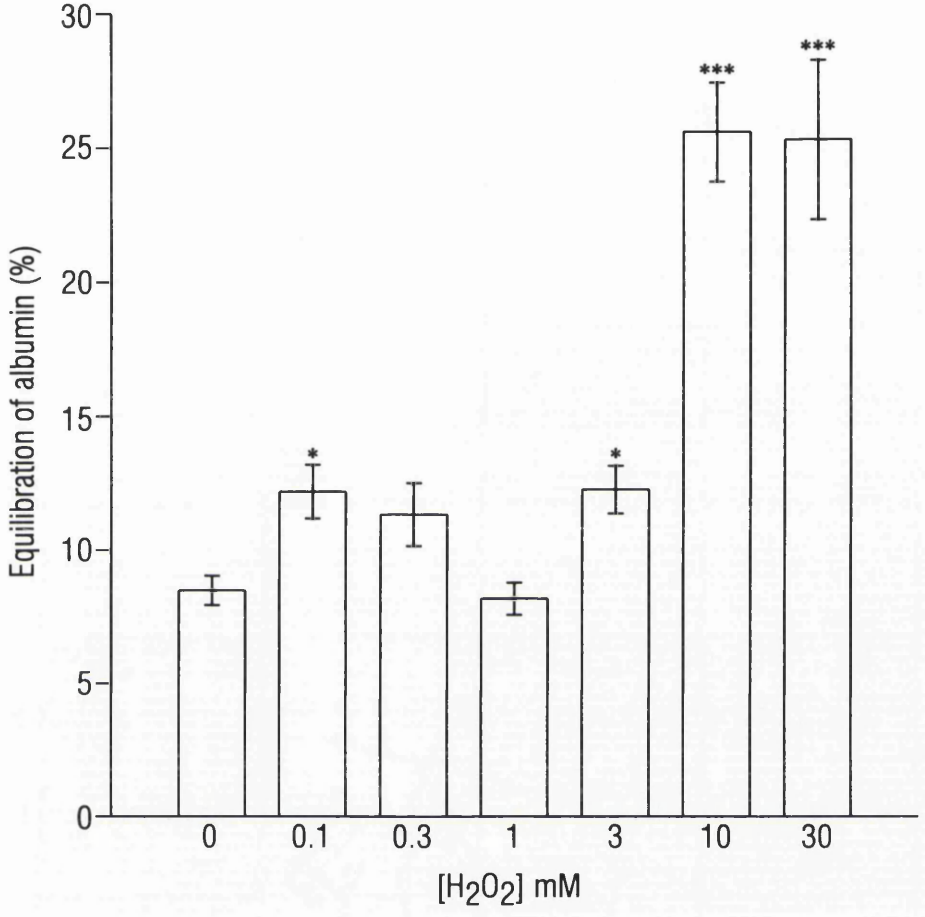


Figure 5.11 Graph showing the effects of 90 minutes' exposure to 0–30 mM hydrogen peroxide [H_2O_2] on albumin transfer across monolayers of bovine aortic endothelial cells. Each column is the mean of 6 observations with vertical bars representing s.e. mean. *$p<0.05$; ***$p<0.001$, indicates a significant difference from control (no H_2O_2).

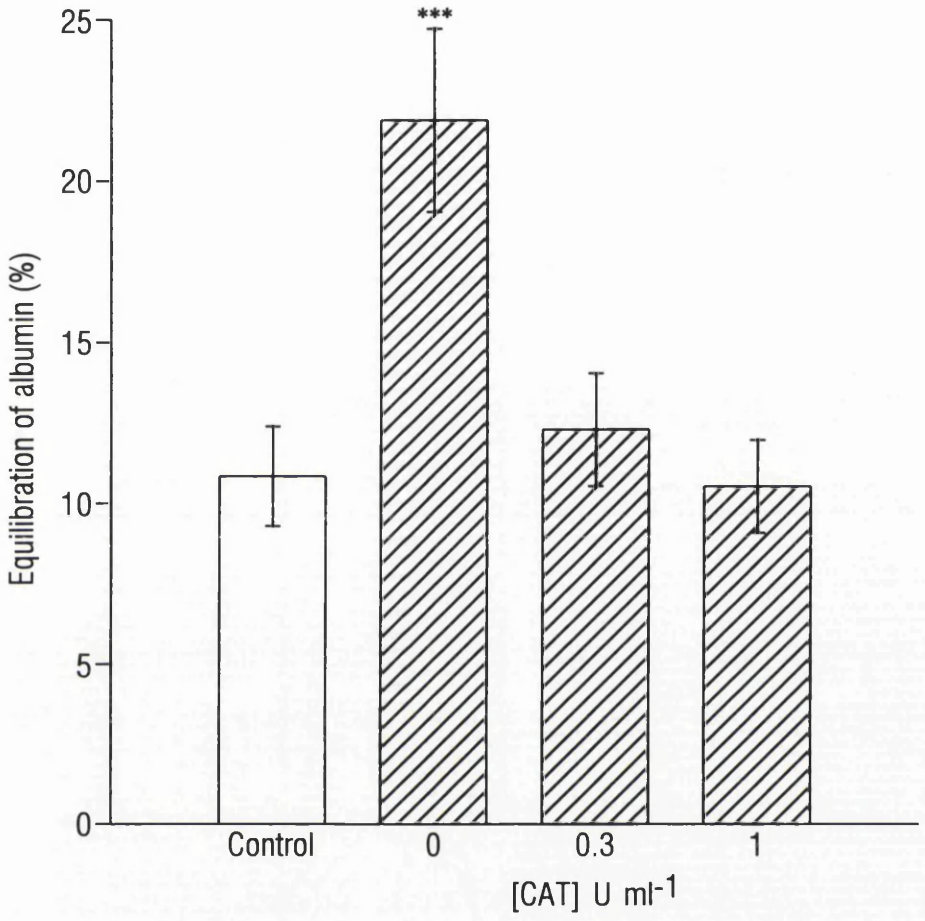


Figure 5.12 Graph showing albumin transfer across monolayers of bovine aortic endothelial cells after 90 minutes' incubation both in the presence (▨) and absence (□) of 0.1 mM hydrogen peroxide. The effects of concomitant addition of 0.3 and 1 U ml^{-1} catalase (CAT) are also shown. Each column is the mean of 6 observations with vertical bars representing s.e. mean. ***$p<0.001$, indicates a significant difference from Control.

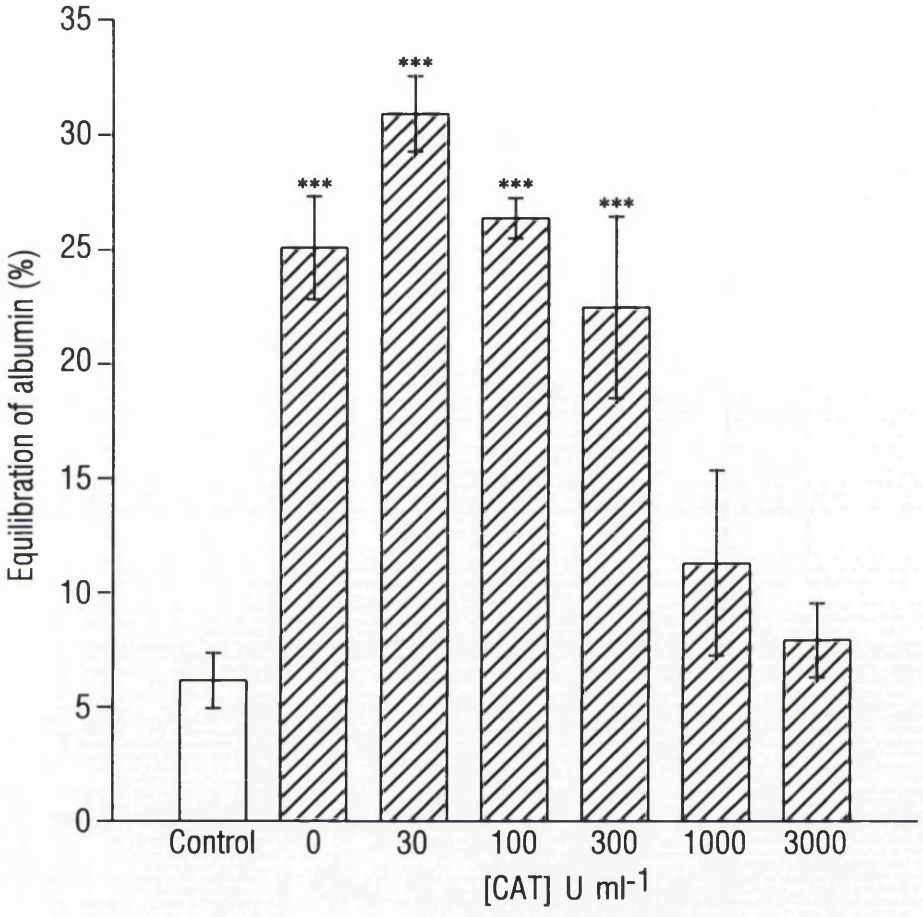


Figure 5.13 Graph showing albumin transfer across monolayers of bovine aortic endothelial cells after 90 minutes' incubation both in the presence (▨) and absence (□) of 10 mM hydrogen peroxide. The effects of concomitant addition of 30–3000 U ml^{-1} catalase (CAT) are also shown. Each column is the mean of 6–9 observations with vertical bars representing s.e. mean. ***$p<0.001$, indicates a significant difference from Control.

5.3.4 Involvement of the hydroxyl radical in the hypoxanthine-xanthine oxidase-induced increase in albumin transfer

Since it is possible that hydrogen peroxide may not be the sole mediator of the permeability increase induced by the hypoxanthine-xanthine oxidase system, investigation of the involvement of other reactive oxygen species was undertaken. Hydroxyl radical may be formed in the combined presence of superoxide anion, hydrogen peroxide and iron (Repine *et al.*, 1981; Starke & Farber, 1985). Both hydrogen peroxide and superoxide anion have been shown to be formed by the hypoxanthine-xanthine oxidase system, whilst iron is present within endothelial cells in the form of ferritin (Dognin & Crichton, 1975). Thus, the effects of hydroxyl radical scavengers were studied on the increase in albumin transfer across BAEC induced by hypoxanthine and xanthine oxidase.

Figure 5.14 shows the effects of mannitol, a membrane-impermeant hydroxyl radical scavenger, on the actions of the hypoxanthine-xanthine oxidase system. Mannitol itself (15 mM) did not affect the level of albumin transfer across monolayers of BAEC relative to the control value and, additionally, when it was co-incubated with hypoxanthine (0.2 mM) and xanthine oxidase (20 mU ml^{-1}) it failed to show any inhibitory effect on the increase in albumin transfer observed. This result would indicate that there is no involvement of hydroxyl radical in mediating the barrier dysfunction induced by hypoxanthine and xanthine oxidase.

However, being a membrane-impermeant scavenger, mannitol can only scavenge extracellularly produced hydroxyl radical and so, in order to investigate any involvement of intracellularly produced hydroxyl radical, two membrane-permeant hydroxyl radical scavengers, dimethylthiourea and N-(2-mercaptopropionyl) glycine, were employed.

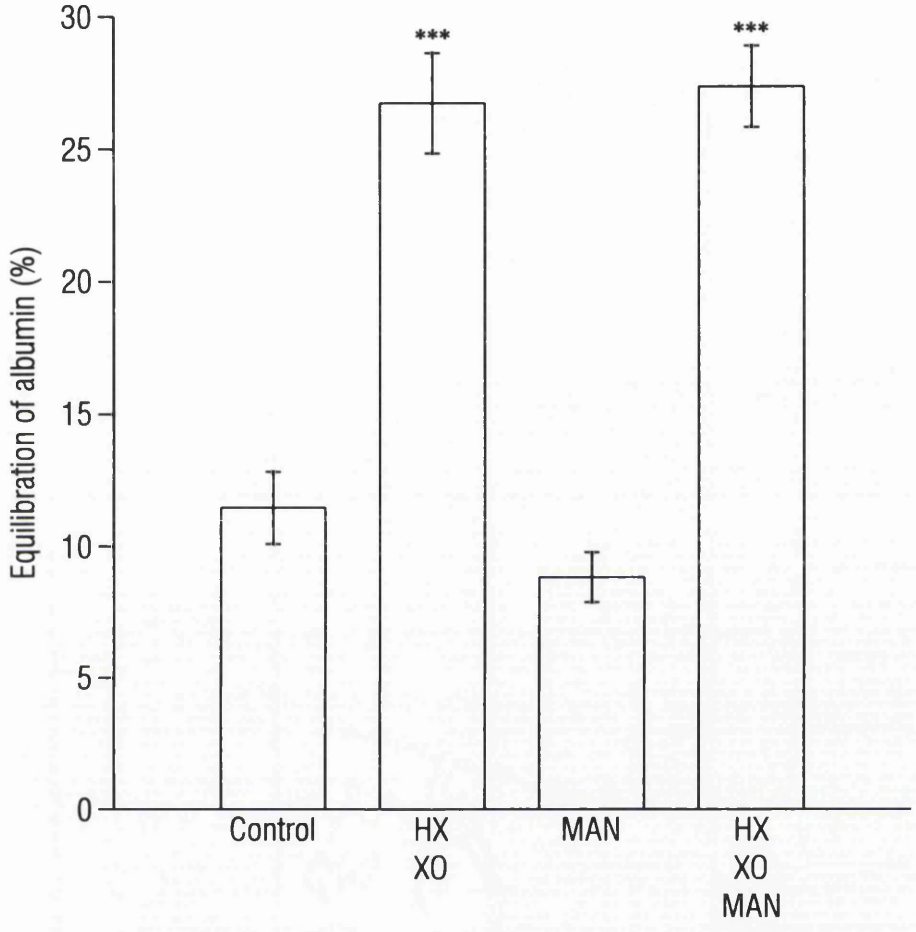


Figure 5.14 Graph showing the effects of 90 minutes' exposure to 0.2 mM hypoxanthine (HX) and 20 mU ml^{-1} xanthine oxidase (XO) on albumin transfer across monolayers of bovine aortic endothelial cells. The effects of concomitant addition of 15 mM mannitol (MAN) are also shown. Each column is the mean of 6 observations with vertical bars representing s.e. mean. ***$p<0.001$, indicates a significant difference from Control.

The effects of dimethylthiourea (10 mM) are shown in Figure 5.15. When added alone, dimethylthiourea had no effect on albumin transfer across monolayers of BAEC and when it was co-incubated with hypoxanthine (0.2 mM) and xanthine oxidase (20 mU ml^{-1}), it showed no inhibitory effect on the increase in albumin transfer observed.

Further investigation employing higher concentrations of dimethylthiourea, in order to show that the reason for its failure to cause an inhibition was not merely because it was not added at a high enough concentration, was not possible. This was because at higher concentrations than 10 mM, dimethylthiourea itself stimulated an increase in albumin transfer across monolayers of BAEC.

As shown in Figure 5.16, N-(2-mercaptopropionyl)-glycine (1 mM) similarly had neither an effect by itself on albumin transfer across monolayers of BAEC relative to control levels, nor did it inhibit the increase in albumin transfer induced when it was added in combination with hypoxanthine (0.2 mM) and xanthine oxidase (20 mU ml^{-1}).

As with dimethylthiourea, the use of higher concentrations of N-(2-mercaptopropionyl)-glycine was not possible since above 1 mM, it was found itself to induce an irreversible block in albumin transfer across endothelial cell monolayers. This may have been due to some interaction occurring between N-(2-mercaptopropionyl)-glycine and the endothelial cells themselves, the polycarbonate membranes on which they were grown, or some component of the trypan blue-labelled albumin complex.

These results (Figures 5.14–5.16) therefore indicate that it is unlikely that hydroxyl radical plays a role in mediating the endothelial barrier dysfunction

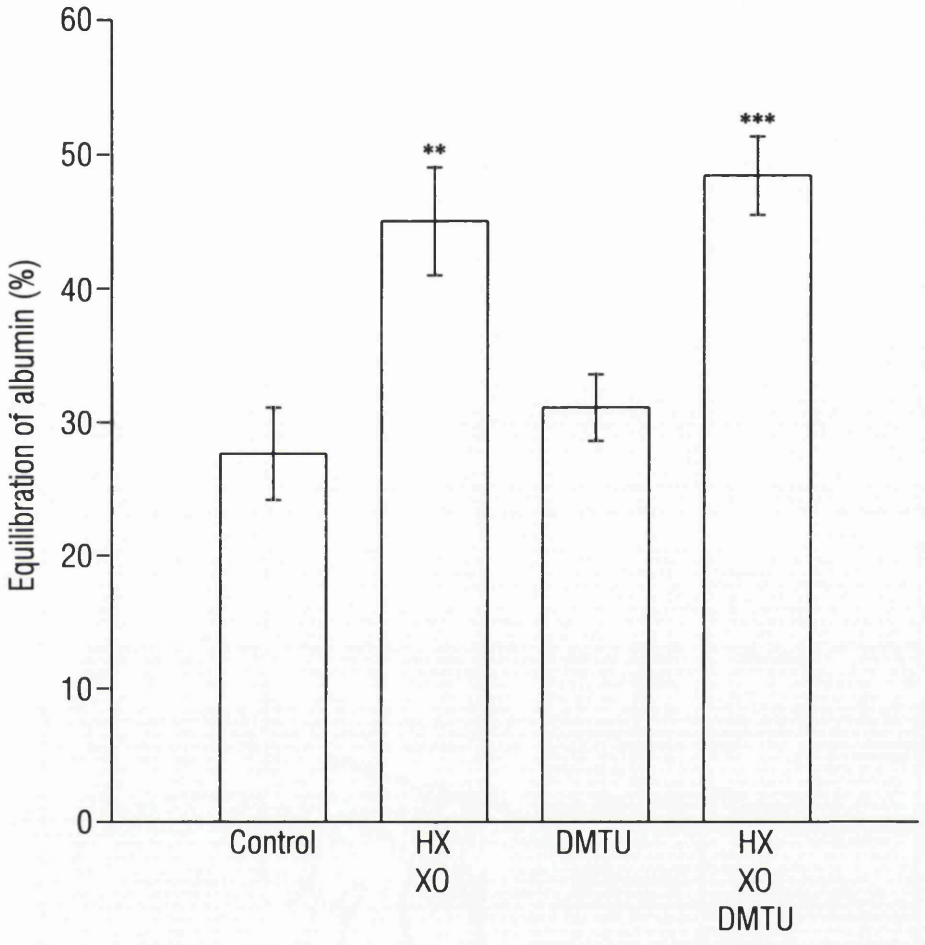


Figure 5.15 Graph showing the effects of 90 minutes' exposure to 0.2 mM hypoxanthine (HX) and 20 mU ml^{-1} xanthine oxidase (XO) on albumin transfer across monolayers of bovine aortic endothelial cells. The effects of concomitant addition of 10 mM dimethylthiourea (DMTU) are also shown. Each column is the mean of 6 observations with vertical bars representing s.e. mean. **$p<0.01$; ***$p<0.001$, indicates a significant difference from Control.

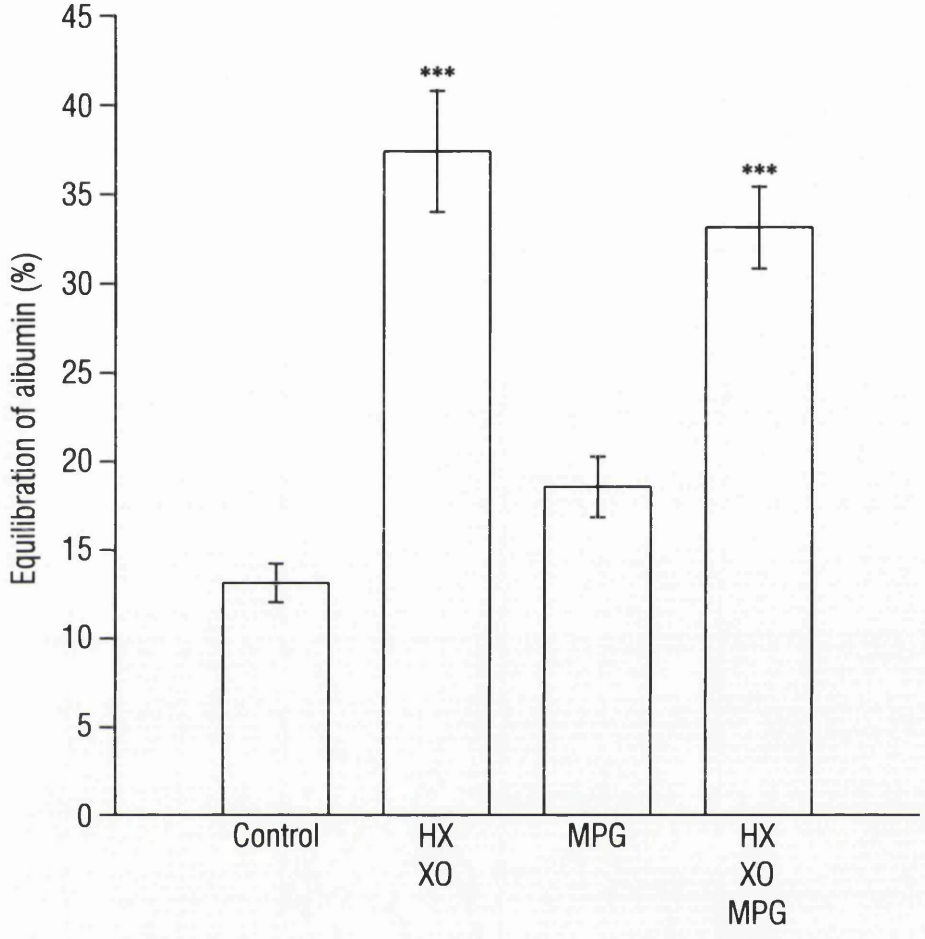


Figure 5.16 Graph showing the effects of 90 minutes' exposure to 0.2 mM hypoxanthine (HX) and 20 mU ml^{-1} xanthine oxidase (XO) on albumin transfer across monolayers of bovine aortic endothelial cells. The effects of concomitant addition of 1 mM N-(2-mercaptopropionyl)-glycine (MPG) are also shown. Each column is the mean of 6 observations with vertical bars representing s.e. mean. ***$p<0.001$, indicates a significant difference from Control.

induced by the hypoxanthine-xanthine oxidase free radical generating system.

If hydroxyl radical is being produced by the hypoxanthine-xanthine oxidase system, however, it is most probably being formed through the Fenton reaction which relies on a source of intracellular iron. Hence, in order to independently assess if production of hydroxyl radical via the Fenton reaction is involved in mediating the hypoxanthine-xanthine oxidase-induced permeability increase, the effects of both removing and augmenting available iron were then investigated.

The effects of deferoxamine, an iron chelator (Starke & Farber, 1985), on the permeability increase induced by the hypoxanthine-xanthine oxidase system are shown in Figure 5.17. Deferoxamine (500 μM) was found to have no effect compared to control levels when added alone to monolayers of BAEC and was observed to have no inhibitory effect on the increase in albumin transfer obtained when added concomitantly with hypoxanthine (0.2 mM) and xanthine oxidase (20 mU ml^{-1}).

Since, when added concomitantly in this way, deferoxamine is unable to enter the endothelial cells, it would therefore only be able to chelate extracellular iron. Thus, as shown in Figure 5.18, the experiment was repeated with deferoxamine (500 μM) being added to monolayers of BAEC as an overnight pretreatment prior to the addition of hypoxanthine (0.2 mM) and xanthine oxidase (20 mU ml^{-1}). This enabled the drug sufficient time to enter the endothelial cells and so chelate both intracellular and extracellular iron (Starke & Farber, 1985). Following this pretreatment, for the purposes of measuring albumin transfer, deferoxamine was re-added to both chambers in each monolayer preparation to ensure maximum effectiveness. The overnight deferoxamine pretreatment itself was found to

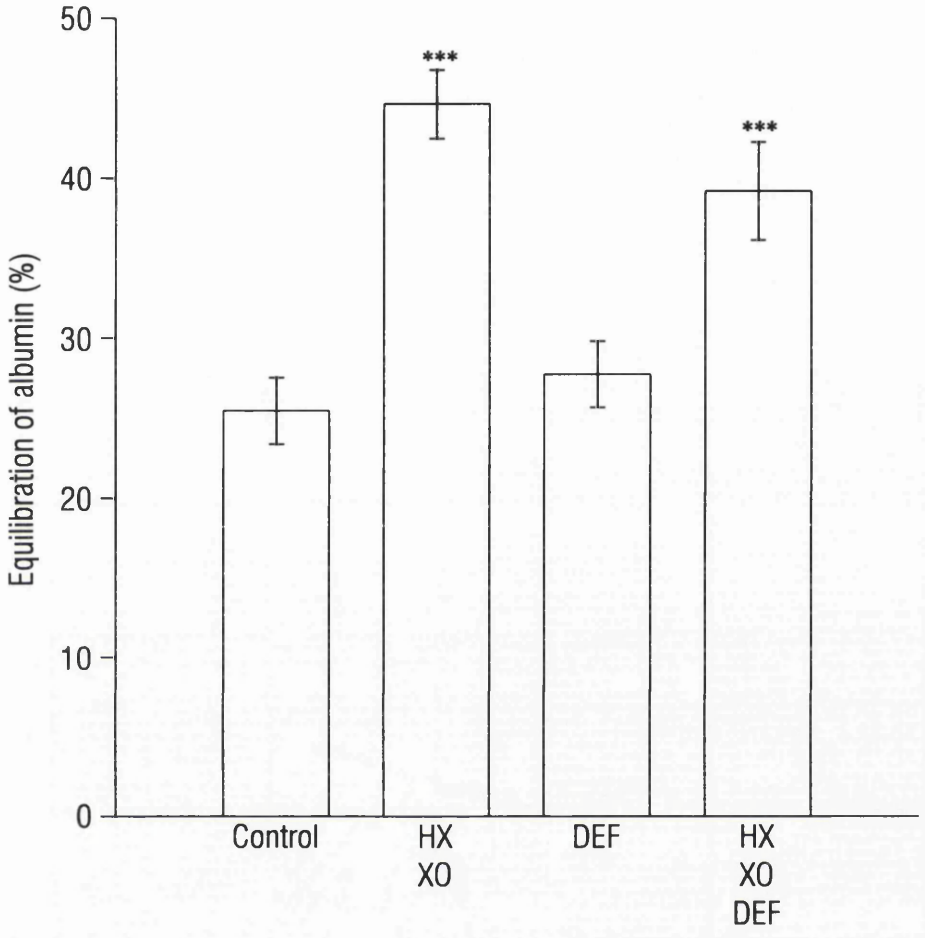


Figure 5.17 Graph showing the effects of 90 minutes' exposure to 0.2 mM hypoxanthine (HX) and 20 mU ml^{-1} xanthine oxidase (XO) on albumin transfer across monolayers of bovine aortic endothelial cells. The effects of concomitant addition of 500 μM deferoxamine (DEF) are also shown. Each column is the mean of 6 observations with vertical bars representing s.e. mean. ***$p<0.001$, indicates a significant difference from Control.

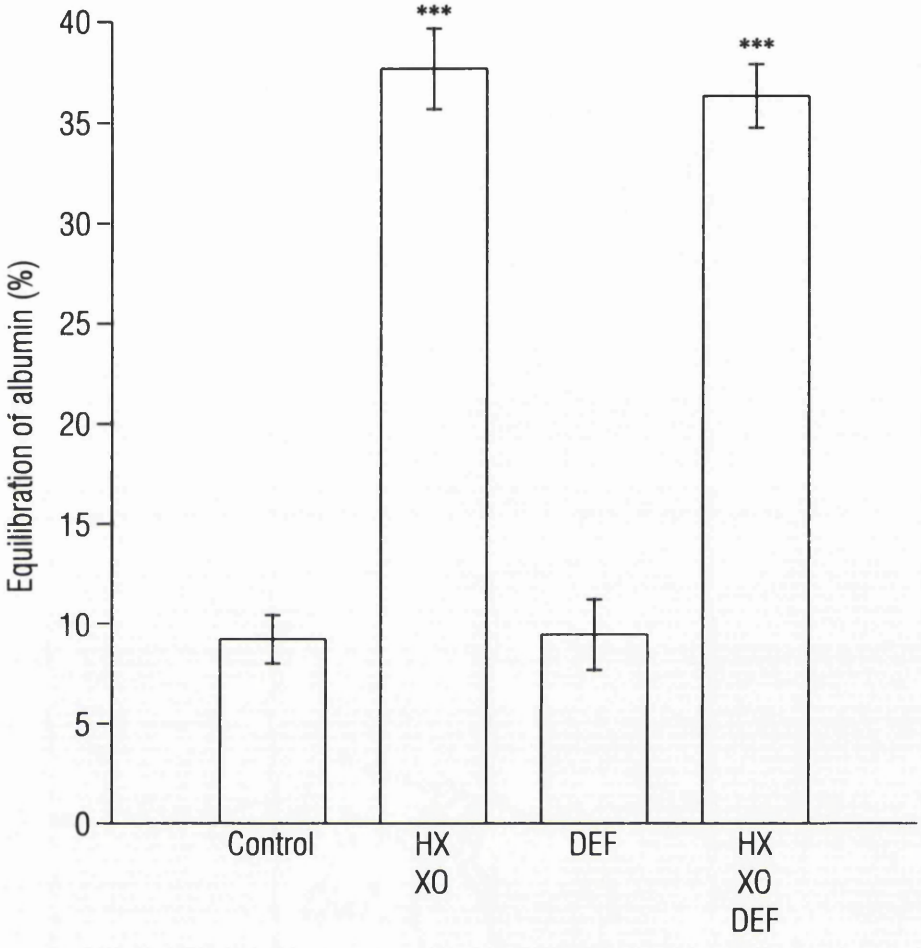


Figure 5.18 Graph showing the effects of 90 minutes' exposure to 0.2 mM hypoxanthine (HX) and 20 mU ml^{-1} xanthine oxidase (XO) on albumin transfer across monolayers of bovine aortic endothelial cells. The effects of overnight pretreatment with 500 μM deferoxamine (DEF) are also shown. Each column is the mean of 6 observations with vertical bars representing s.e. mean. ***$p<0.001$, indicates a significant difference from Control.

have no effect on albumin transfer relative to control levels. Similarly, the pretreatment was found to have no effect on the increase in albumin transfer across monolayers of BAEC resulting from subsequent addition of hypoxanthine (0.2 mM) and xanthine oxidase (20 mU ml^{-1}).

The effects of augmenting available iron were investigated through addition of ferric chloride in combination with hypoxanthine and xanthine oxidase (Figure 5.19). Ferric chloride (50 μM) itself had no effect on albumin transfer across monolayers of BAEC relative to control levels after a 90 minute incubation. Also, when ferric chloride was added in combination with hypoxanthine (0.2 mM) and xanthine oxidase (20 mU ml^{-1}), it failed to enhance the observed increase in albumin transfer.

Taken together, the results of this series of experiments (Figures 5.14–5.19) would indicate that hydroxyl radical plays no role in mediating the endothelial barrier dysfunction induced by the hypoxanthine-xanthine oxidase free radical generating system.

5.3.5 Involvement of nitric oxide and peroxynitrite anion in the hypoxanthine-xanthine oxidase-induced increase in albumin transfer

Beckman *et al.* (1990) proposed that an interaction can take place between superoxide anion produced by the hypoxanthine-xanthine oxidase system and nitric oxide released from endothelial cells, leading to the formation of the peroxynitrite anion. This anion is itself a powerful oxidant and, additionally, undergoes decomposition leading to the formation of nitrogen dioxide radical and hydroxyl radical, by a mechanism which does not involve iron catalysation. The inability of superoxide dismutase (30 and 6000 U ml^{-1}) to inhibit the permeability increase induced by the

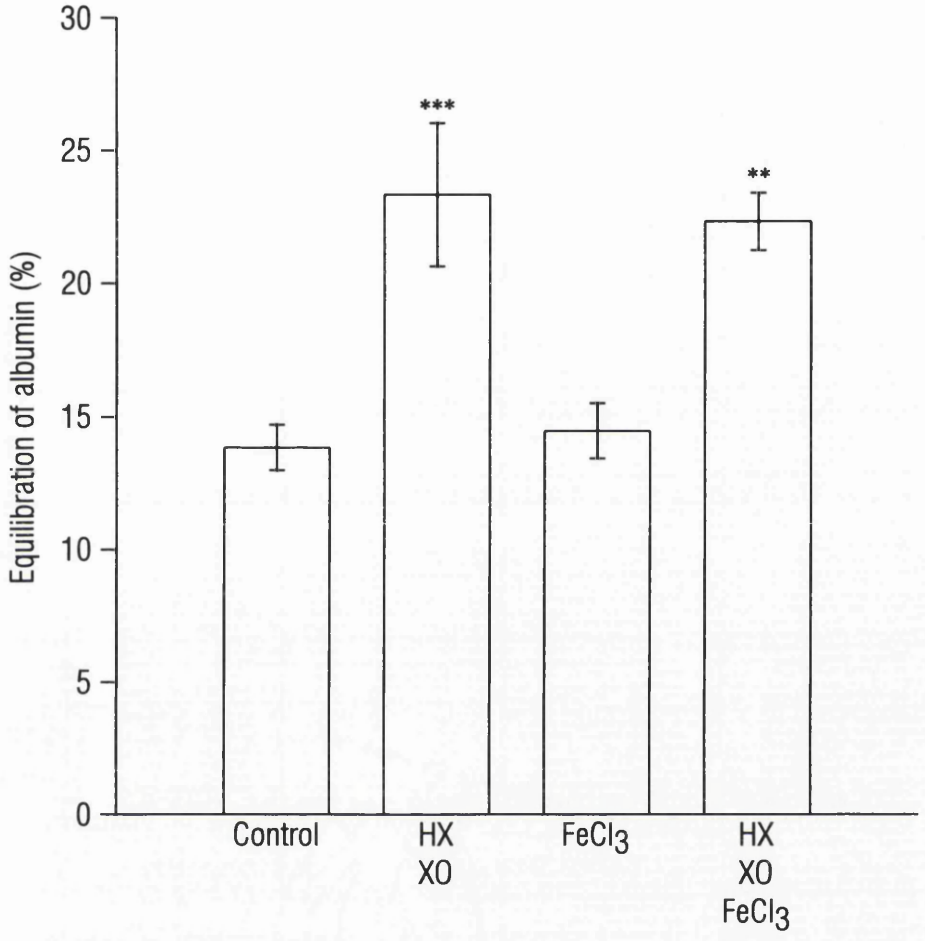


Figure 5.19 Graph showing the effects of 90 minutes' exposure to 0.2 mM hypoxanthine (HX) and 20 mU ml^{-1} xanthine oxidase (XO) on albumin transfer across monolayers of bovine aortic endothelial cells. The effects of concomitant addition of 50 μM ferric chloride ($FeCl_3$) are also shown. Each column is the mean of 6 observations with vertical bars representing s.e. mean. **p<0.01; ***p<0.001, indicates a significant difference from Control.

hypoxanthine-xanthine oxidase system has already been demonstrated (Figures 5.6–5.7). However, in order to independently investigate the involvement of nitric oxide and/or peroxynitrite anion in mediating this permeability increase, the effects of the nitric oxide synthase inhibitor, L-NOARG were studied.

Figure 5.20 shows that L-NOARG (30 μM) itself had no effect on albumin transfer across monolayers of BAEC relative to control levels during a 90 minute incubation. Additionally, when added in combination with hypoxanthine (0.2 mM) and xanthine oxidase (20 mU ml^{-1}), it exhibited no inhibitory effect on the resultant increase in albumin transfer indicating that nitric oxide does not play a role in mediating this increase. This would suggest that it is unlikely that peroxynitrite, formed through the interaction of superoxide anion and nitric oxide, contributes to the endothelial barrier dysfunction induced by the hypoxanthine-xanthine oxidase system.

5.3.6 Involvement of hypochlorous acid in the hypoxanthine-xanthine oxidase-induced increase in albumin transfer

Hypochlorous acid is a known product of the activated neutrophil (Lewis & Granger, 1986) which may be formed by the enzyme myeloperoxidase in the presence of both hydrogen peroxide and chloride ions (Halliwell & Gutteridge, 1989). Dithiothreitol has been shown to reverse the damaging effects of hypochlorous acid on both calcium homeostasis in rabbit isolated ventricular myocytes (Eley *et al.*, 1991) and cardiac muscle contractile function during ischaemia-reperfusion injury (Eley *et al.*, 1989). Thus, in order to determine if hypochlorous acid also plays a role in the permeability increase induced by the hypoxanthine-xanthine oxidase free radical generating system, the effects of dithiothreitol were studied.

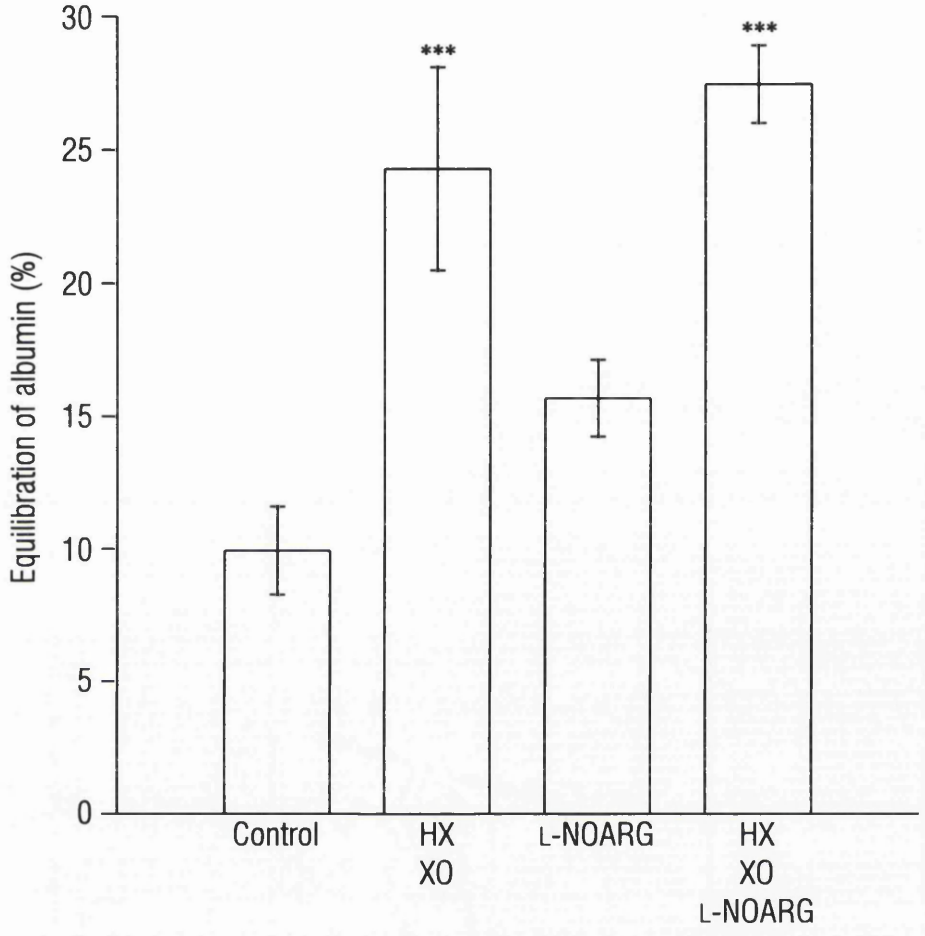


Figure 5.20 Graph showing the effects of 90 minutes' exposure to 0.2 mM hypoxanthine (HX) and 20 mU ml^{-1} xanthine oxidase (XO) on albumin transfer across monolayers of bovine aortic endothelial cells. The effects of concomitant addition of 30 μM N^{ω}-nitro-L-arginine (L-NOARG) are also shown. Each column is the mean of 6 observations with vertical bars representing s.e. mean. ***$p<0.001$, indicates a significant difference from Control.

When added alone, dithiothreitol (3 mM) had no effect on albumin transfer across BAEC relative to the control value during a 90 minute incubation, and when it was added concomitantly with hypoxanthine (0.2 mM) and xanthine oxidase (20 mU ml^{-1}), it failed to show any inhibitory effect on the observed increase in albumin transfer (Figure 5.21). This would indicate that hypochlorous acid plays no role in mediating the permeability increase induced by hypoxanthine and xanthine oxidase.

5.4 Study of the effects of homocysteine on barrier function

5.4.1 Effects of homocysteine and copper sulphate on albumin transfer

Homocysteine is a recognised risk factor for atherosclerosis, a condition in which a key role of its development is played by dysfunction of the endothelial barrier. Therefore, having established more about the way in which the hypoxanthine-xanthine free radical generating oxidase system modulates barrier function, the ability of homocysteine to affect endothelial monolayer permeability was then investigated.

When added alone to monolayers of BAEC at either 0.5 mM or 1.5 mM, homocysteine was found to exhibit no significant alteration in albumin transfer relative to control levels during a 90 minute incubation (Figure 5.22). Starkebaum & Harlan (1986) demonstrated, however, that homocysteine can undergo copper-catalysed oxidation. This process may occur *in vivo* through the actions of copper present as circulating caeruloplasmin but may be simulated *in vitro* using copper in the form of copper sulphate for sake of both convenience and economy. Thus, the effects of addition to endothelial monolayers of homocysteine in the presence of copper

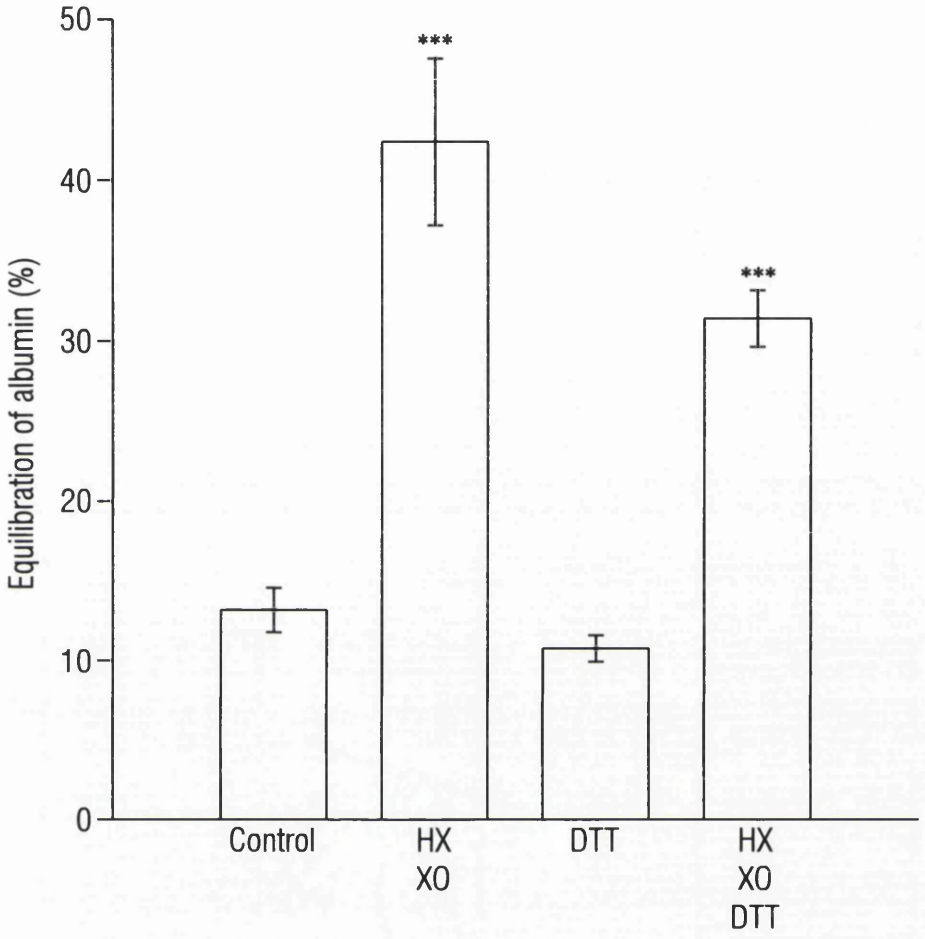


Figure 5.21 Graph showing the effects of 90 minutes' exposure to 0.2 mM hypoxanthine (HX) and 20 mU ml^{-1} xanthine oxidase (XO) on albumin transfer across monolayers of bovine aortic endothelial cells. The effects of concomitant addition of 3 mM dithiothreitol (DTT) are also shown. Each column is the mean of 6 observations with vertical bars representing s.e. mean. ***$p<0.001$, indicates a significant difference from Control.

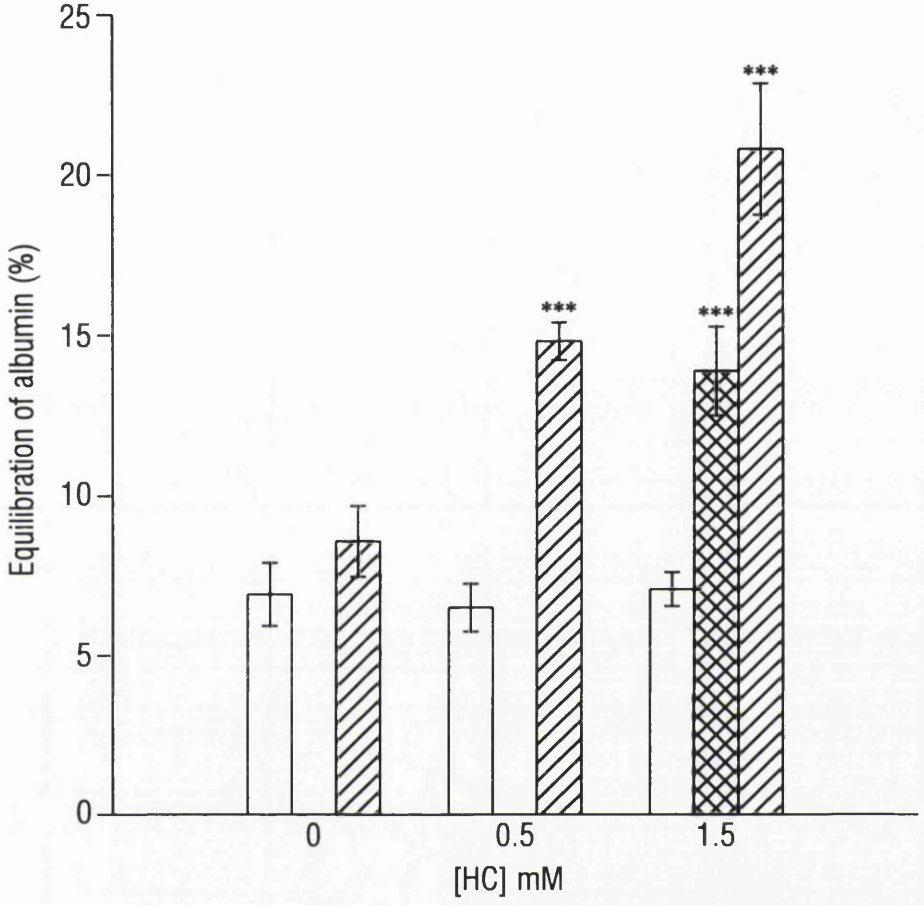


Figure 5.22 Graph showing the effects of 90 minutes' exposure to 0, 0.5 and 1.5 mM homocysteine (HC) on albumin transfer across monolayers of bovine aortic endothelial cells (□). The effects of concomitant addition of copper sulphate at 5 μM (▩) and 50 μM (▨) are also shown. Each column is the mean of 6 observations with vertical bars representing s.e. mean. ***$p<0.001$, indicates a significant difference from control (no HC, no copper sulphate).

sulphate were studied. When homocysteine at either 0.5 mM or 1.5 mM was added in combination with copper sulphate at a concentration of 50 μM, significant increases in albumin transfer were observed. Within the limited concentration range tested, this increase would appear to be concentration-dependent on the concentration of homocysteine, giving an increase in albumin equilibration from the control level of 6.9±1.0 to 14.8±0.6% at 0.5 mM homocysteine and 20.8±2.0% at 1.5 mM homocysteine. Similarly, the increase was found to be concentration-dependent on the copper sulphate concentration. When 1.5 mM homocysteine was added in combination with copper sulphate at 5 μM, it produced a significant increase in monolayer permeability reaching 13.9±1.4% (compared to 20.8±2.0% for the combination of 1.5 mM homocysteine and 50 μM copper sulphate). It can be clearly seen, therefore, that copper-catalysed oxidation of homocysteine, rather than addition of homocysteine itself, can cause an increase in endothelial permeability.

In all further experiments involving homocysteine and copper sulphate, standard concentrations of 1.5 mM homocysteine and 50 μM copper sulphate were chosen to induce an increase in albumin transfer across endothelial monolayers. These concentrations gave a sufficient stimulation of albumin transfer against which the effects of potential inhibitors could be guaged.

To establish whether superoxide anion plays a contributory role in mediating the endothelial barrier dysfunction induced by the copper-catalysed oxidation of homocysteine, superoxide dismutase (6000 U ml^{-1}) was co-incubated with homocysteine and copper sulphate (added to endothelial monolayers at the chosen standard concentrations of 1.5 mM and 50 μM, respectively) (Figure 5.23). Superoxide dismutase itself had no effect on albumin transfer across monolayers of BAEC and was also found to be

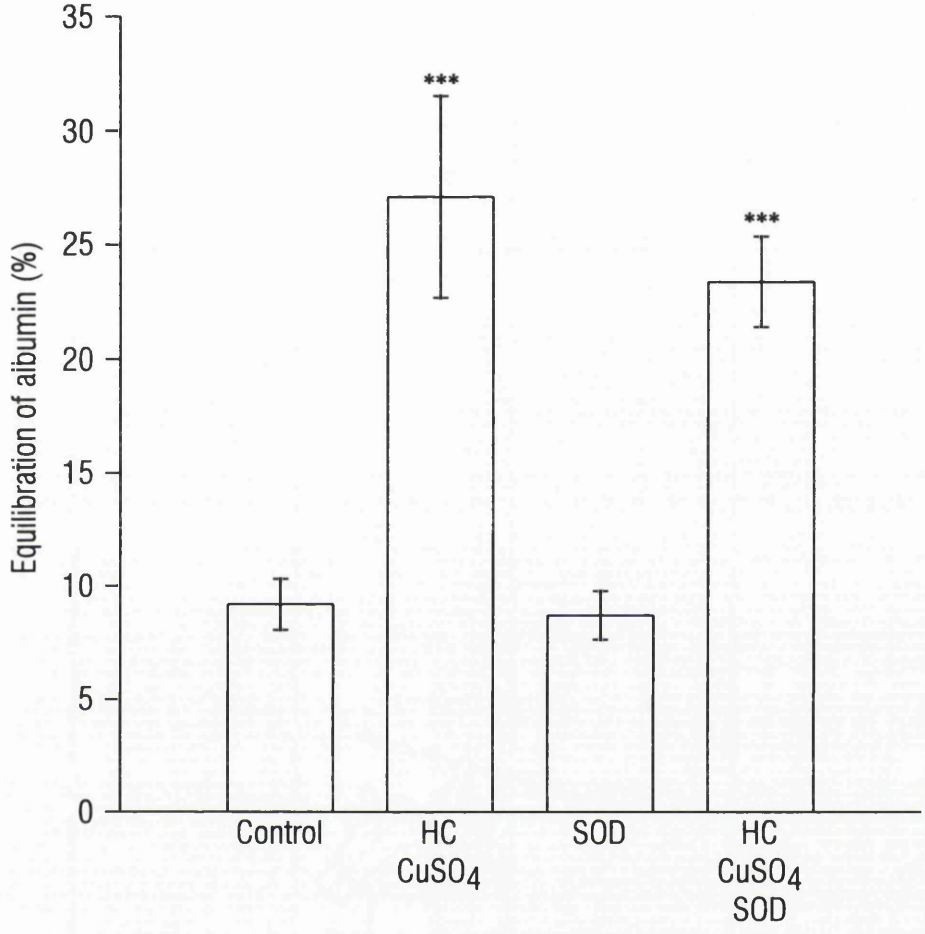


Figure 5.23 Graph showing the effects of 90 minutes' exposure to 1.5 mM homocysteine (HC) and 50 μM copper sulphate ($CuSO_4$) on albumin transfer across monolayers of bovine aortic endothelial cells. The effects of concomitant addition of 6000 U ml^{-1} superoxide dismutase (SOD) are also shown. Each column is the mean of 6 observations with vertical bars representing s.e. mean. ***$p<0.001$, indicates a significant difference from Control.

without effect when added in combination with homocysteine and copper sulphate. This would indicate that superoxide anion is not involved in mediating the endothelial barrier dysfunction induced by homocysteine and copper sulphate.

To investigate any involvement of hydrogen peroxide in the homocysteine-copper sulphate-induced permeability increase, the effects of catalase (1–100 U ml^{-1}) were studied (Figure 5.24). At all the concentrations of catalase investigated, it was found to abolish the increase in albumin transfer across monolayers of BAEC stimulated by homocysteine (1.5 mM) and copper sulphate (50 μM). Thus, as was the case with the hypoxanthine-xanthine oxidase system, hydrogen peroxide appears to play a key role in mediating the endothelial barrier dysfunction.

5.4.2 Involvement of hydroxyl radical in the homocysteine-copper sulphate-induced increase in albumin transfer

As was considered with the hypoxanthine-xanthine oxidase system, hydrogen peroxide may not be the sole mediator of the homocysteine-copper sulphate-induced increase in endothelial monolayer permeability and may instead, for example, be a necessary precursor for some other damaging species. Therefore, the involvement of hydroxyl radical was investigated.

Figure 5.25 shows the effects of the membrane-impermeant hydroxyl radical scavenger, mannitol (15 mM), on the increase in albumin transfer across monolayers of BAEC induced by homocysteine (1.5 mM) and copper sulphate (50 μM) during a 90 minute incubation. Addition of mannitol itself did not effect the level of albumin transfer relative to the control level. Similarly, when it was co-incubated with homocysteine and copper

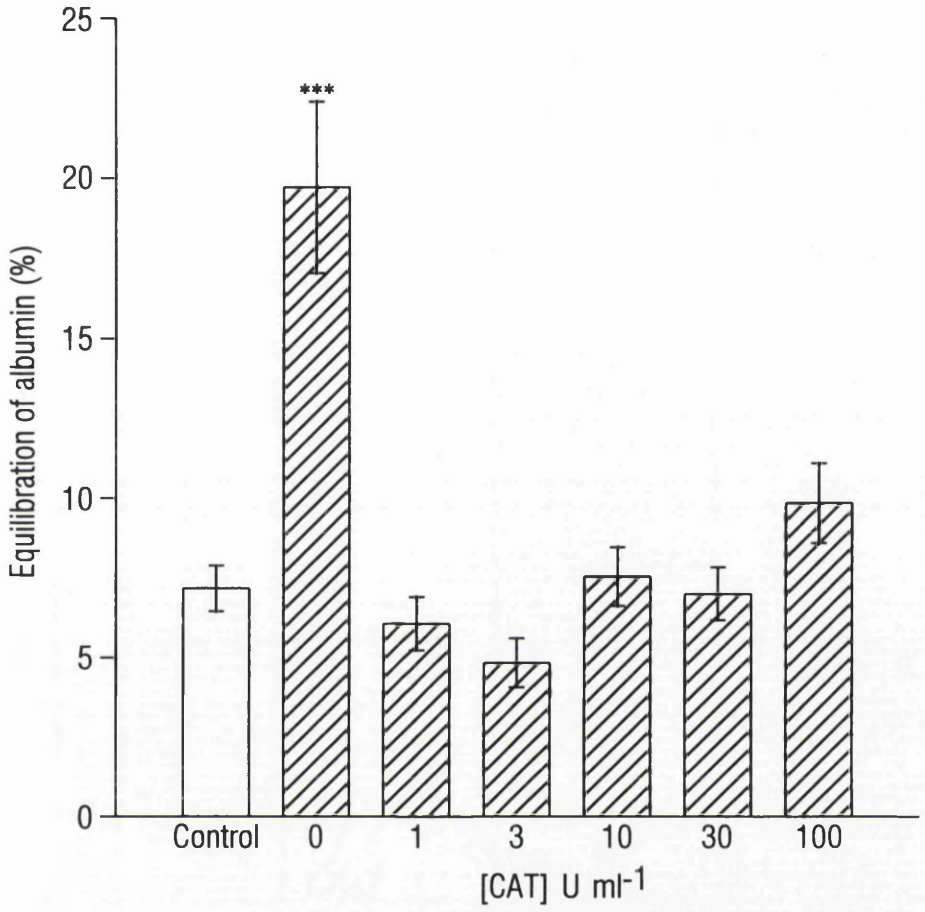


Figure 5.24 Graph showing albumin transfer across monolayers of bovine aortic endothelial cells after 90 minutes' incubation both in the presence (▨) and absence (□) of 1.5 mM homocysteine and 50 μM copper sulphate. The effects of concomitant addition of 1–100 U ml^{-1} catalase (CAT) are also shown. Each column is the mean of 6–9 observations with vertical bars representing s.e. mean. ***$p<0.001$, indicates a significant difference from Control.

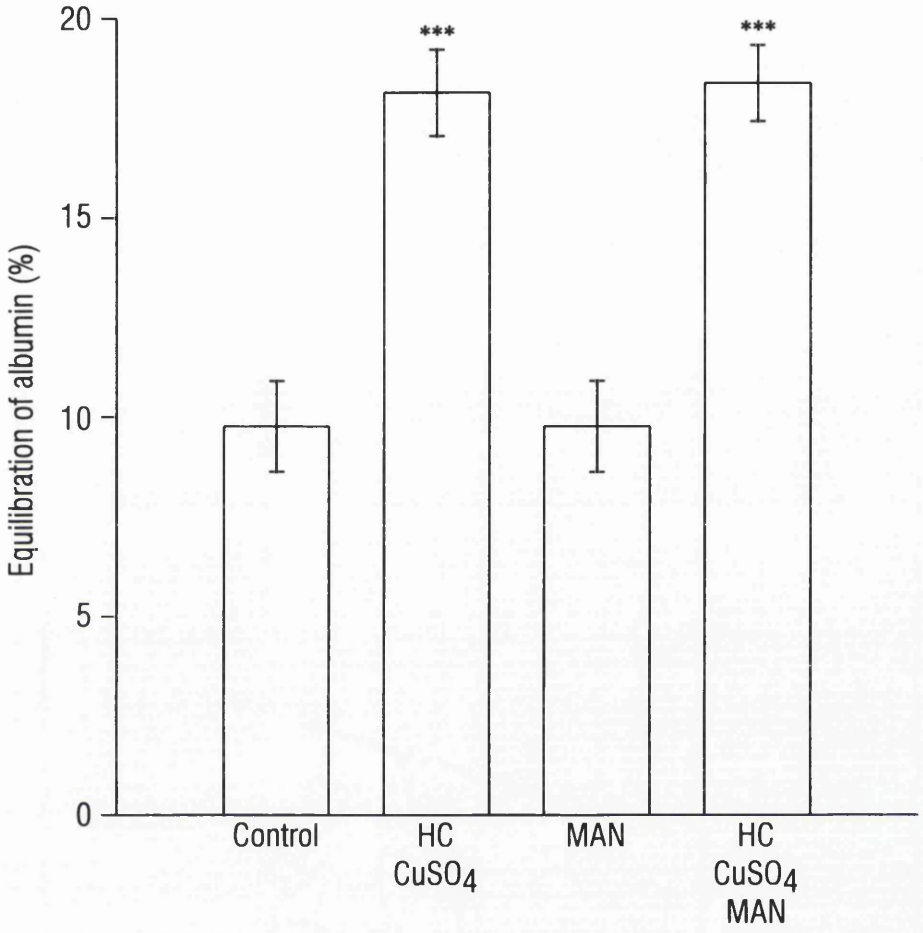


Figure 5.25 Graph showing the effects of 90 minutes' exposure to 1.5 mM homocysteine (HC) and 50 μM copper sulphate ($CuSO_4$) on albumin transfer across monolayers of bovine aortic endothelial cells. The effects of concomitant addition of 15 mM mannitol (MAN) are also shown. Each column is the mean of 6 observations with vertical bars representing s.e. mean. ***$p<0.001$, indicates a significant difference from Control.

sulphate, mannitol had no effect on the observed increase in albumin transfer.

The effects of the membrane-permeant hydroxyl radical scavenger, dimethylthiourea (1 mM), are shown in Figure 5.26. When added alone to monolayers of BAEC, dimethylthiourea was found to have no effect on albumin transfer. Furthermore, when it was co-incubated with homocysteine (1.5 mM) and copper sulphate (50 μM), it was found to have no inhibitory effect on the increase in albumin transfer observed.

In order to investigate any involvement of the Fenton reaction which could lead to the generation of hydroxyl radical (Repine *et al.,* 1981), the effects of the iron chelator, deferoxamine (500 μM), were then investigated (Figure 5.27). As before, deferoxamine was added to monolayers of BAEC as an overnight pretreatment prior to the addition of homocysteine (1.5 mM) and copper sulphate (50 μM). For the purposes of measuring albumin transfer, deferoxamine was re-added on commencing the experiment. The overnight pretreatment itself was found to have no effect on albumin transfer relative to control levels during a 90 minute incubation and also was without effect on the increase in albumin transfer stimulated by the subsequent addition of homocysteine and copper sulphate.

Taking these results together (Figures 5.25–5.27), it would therefore seem unlikely that the generation of hydroxyl radical plays a role in mediating the barrier dysfunction induced by the homocysteine-copper sulphate system.

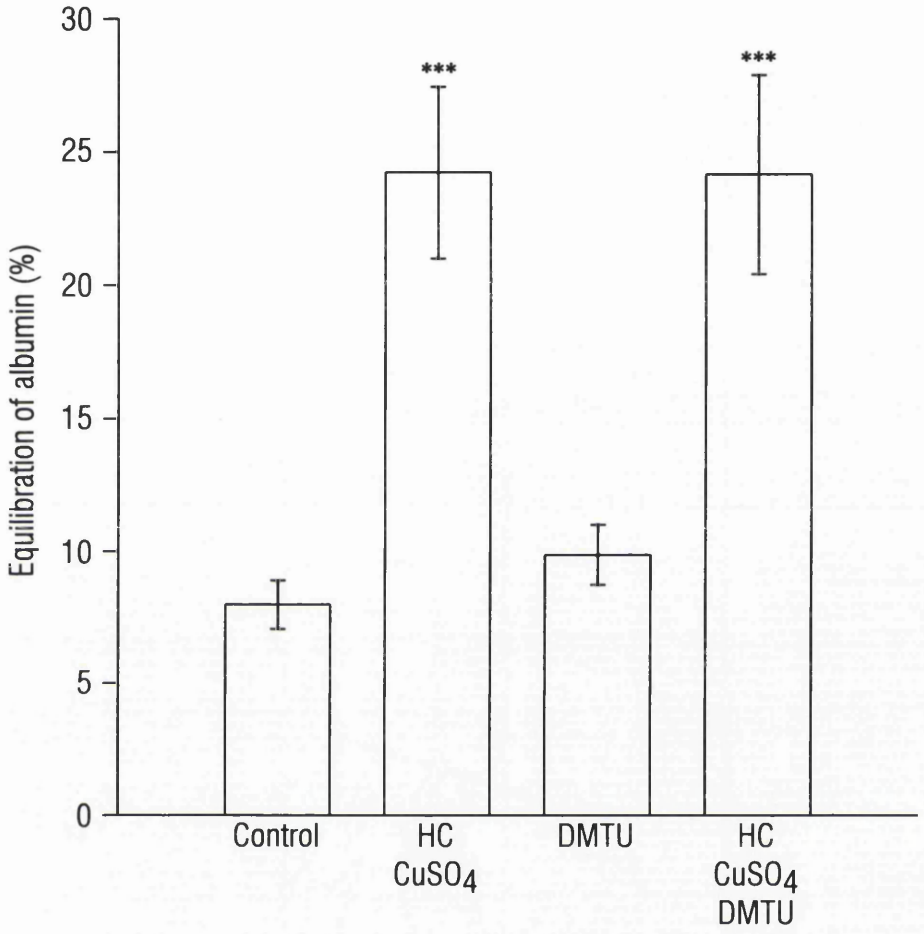


Figure 5.26 Graph showing the effects of 90 minutes' exposure to 1.5 mM homocysteine (HC) and 50 μM copper sulphate ($CuSO_4$) on albumin transfer across monolayers of bovine aortic endothelial cells. The effects of concomitant addition of 1 mM dimethylthiourea (DMTU) are also shown. Each column is the mean of 6 observations with vertical bars representing s.e. mean. ***$p<0.001$, indicates a significant difference from Control.

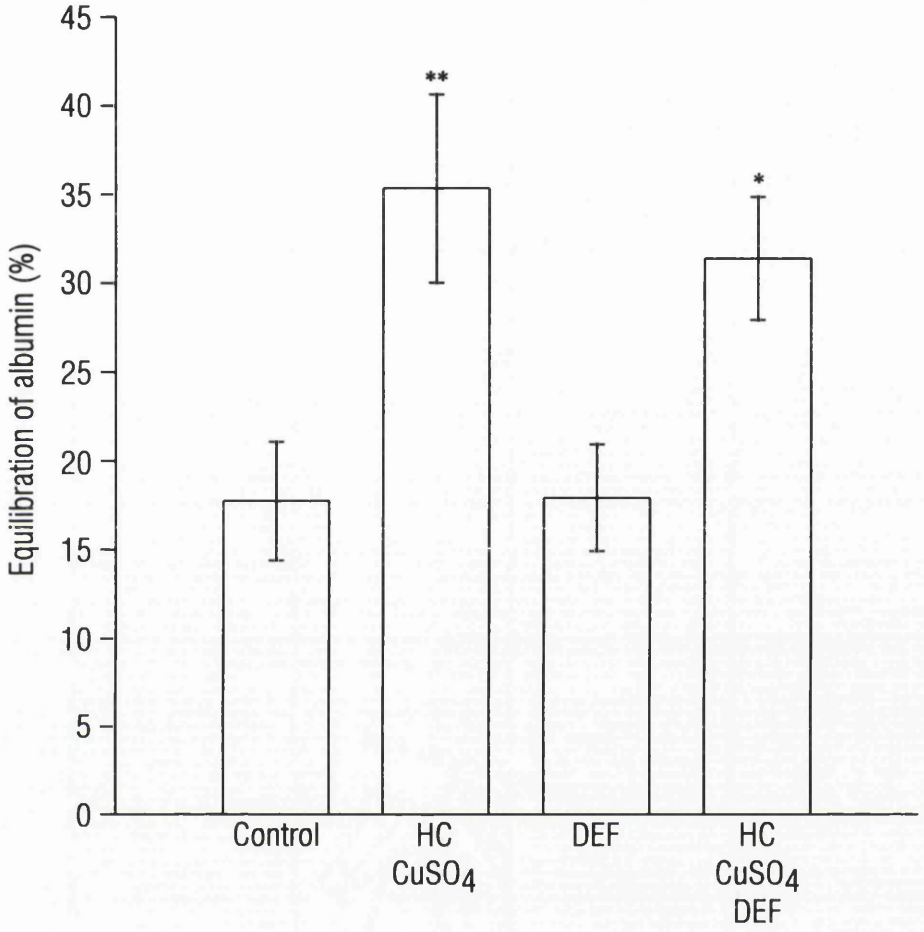


Figure 5.27 Graph showing the effects of 90 minutes' exposure to 1.5 mM homocysteine (HC) and 50 μM copper sulphate ($CuSO_4$) on albumin transfer across monolayers of bovine aortic endothelial cells. The effects of overnight pretreatment with 500 μM deferoxamine (DEF) are also shown. Each column is the mean of 6 observations with vertical bars representing s.e. mean. *$p<0.05$; **$p<0.01$, indicates a significant difference from Control.

CHAPTER 6

6.1 Study of the effects of bacterial endotoxin on barrier function

6.1.1 Effects of lipopolysaccharide (LPS) on albumin transfer

Endotoxin, also known as lipopolysaccharide (LPS), derived from gram-negative bacteria is a common cause of septic shock, a condition characterised by severe hypotension, hyporeactivity to vasoconstrictors and profound vascular leakage leading to multiple organ failure and ultimately death (Pinsky *et al.*, 1993). Whilst it is now accepted that the severe hypotension of septic shock results from the production of nitric oxide (Parker & Adams, 1993), little is known of the mediator(s) responsible for the associated vascular leakage. Hence, by using the *in vitro* model of endothelial permeability employed in this study, the ability of LPS to induce barrier dysfunction was investigated.

Figure 6.1 shows the effects of LPS (0–1000 ng ml^{-1}) from *Salmonella typhosa* on albumin transfer, measured during a 90 minute incubation period, following the exposure of BAEC monolayers in culture to LPS for 24 hours. LPS was found to exhibit a concentration-dependent increase in albumin transfer, demonstrating statistically significant increases at concentrations of 3 ng ml^{-1} and greater. The maximum level of albumin transfer achieved was 39.9±2.6% at a concentration of 1000 ng ml^{-1} of LPS. This therefore demonstrates the ability of LPS to induce endothelial barrier dysfunction through a direct action on the endothelium.

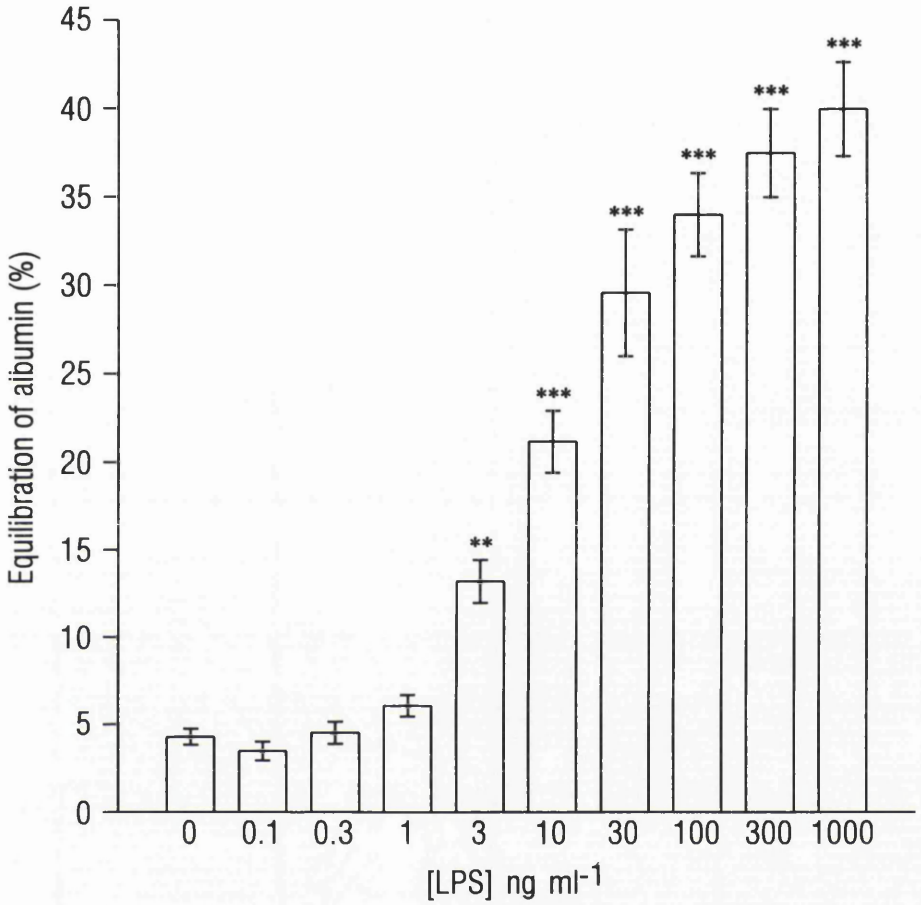


Figure 6.1 Graph showing the effects of 24 hours' prior exposure to 0–1000 ng ml^{-1} lipopolysaccharide (LPS) on albumin transfer across monolayers of bovine aortic endothelial cells during a 90 minute incubation period. Each column is the mean of 6–23 observations with vertical bars representing s.e. mean. **$p<0.01$; ***$p<0.001$, indicates a significant difference from control (0 ng ml^{-1} LPS).

From the concentration-response curve, a standard LPS concentration of 30 ng ml^{-1} was chosen for use in all subsequent experiments. This concentration was sufficiently high to yield a consistently reproducible response in terms of its effects on albumin transfer whilst yet being sufficiently sub-maximal to enable the effects of possible inhibitors of its action to be investigated.

The time-course of the development of the increase in albumin transfer induced by 30 ng ml^{-1} LPS was examined as shown in Figure 6.2. This time-course was found to be biphasic in nature with two distinct peaks. An early, transient peak (45.6±3.7%) was observed after around 2 hours' exposure to LPS which was followed by a decline in the level of albumin transfer to near basal levels. The level of transfer was then observed to rise once again, approaching a maximum (44.4±4.7%) by 24 hours. In order to ensure that this biphasic time-course was not merely the result of experimental error, this experiment was repeated on three separate occasions with three different batches of cells (data not shown). A similar biphasic profile was, however, observed on these occasions demonstrating that the original observation was clearly not just one aberrant finding.

The increases in albumin transfer stimulated following both 2 hours' and 24 hours' exposure to LPS are conceivably mediated by different mechanisms. Thus, in subsequent experiments in which the mechanisms of action of LPS were investigated, these were in the main conducted on both 2 hours' and 24 hours' exposures to LPS.

Polymixin B is an inhibitor of the actions of LPS which acts by binding the LPS molecule with high affinity (Lasfargues *et al.*, 1989). Figure 6.3 shows the effects of concomitant addition of Polymixin B (10 μg ml^{-1}) on a 2 hour exposure to LPS (added at the chosen standard concentration of

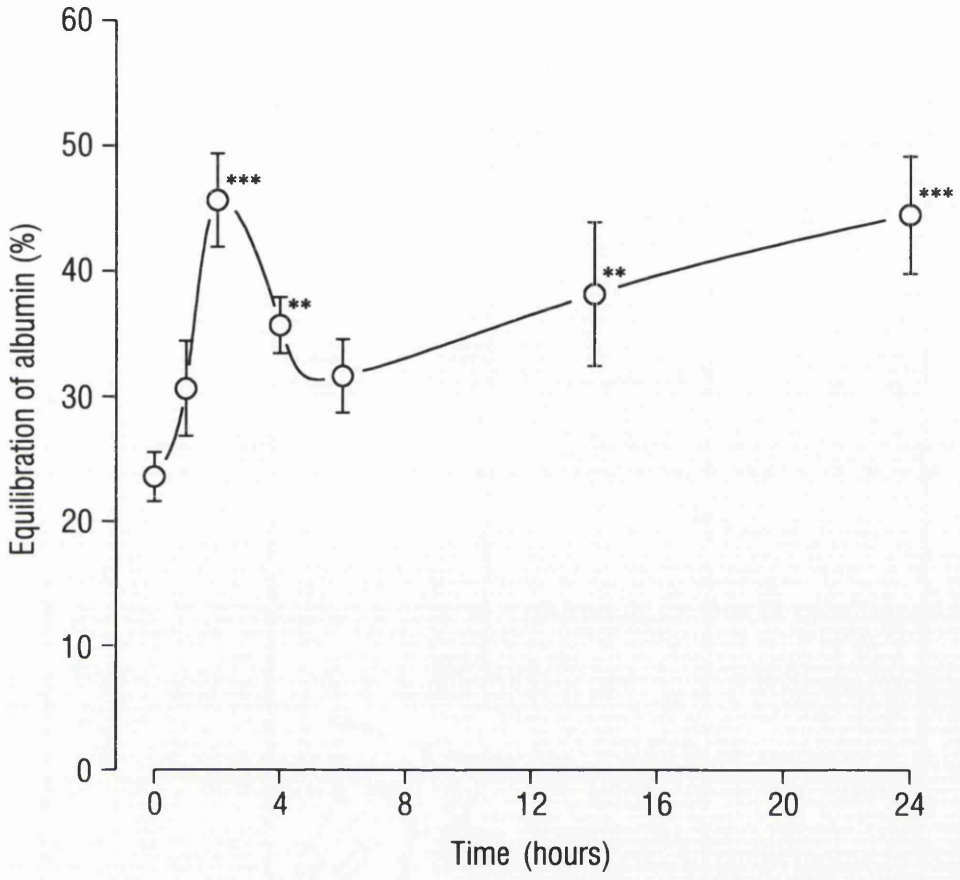


Figure 6.2 Graph showing the time-course of the development of the increase in albumin transfer across monolayers of bovine aortic endothelial cells induced by 30 ng ml^{-1} lipopolysaccharide. The time points indicate the duration of prior exposure to LPS but in each case albumin transfer was measured over a subsequent 90 minute period. Each point is the mean of 12–24 observations with vertical bars representing s.e. mean. **$p<0.01$; ***$p<0.001$, indicates a significant difference from control (0 hours).

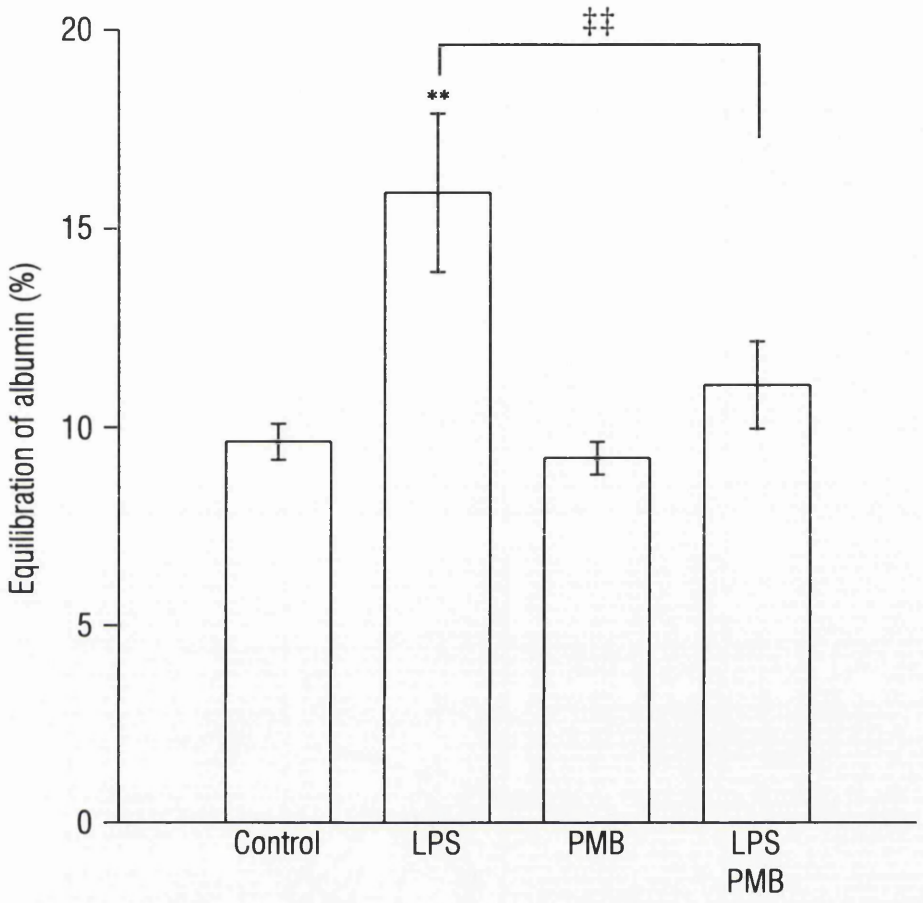


Figure 6.3 Graph showing the effects of 2 hours' exposure to 30 ng ml^{-1} lipopolysaccharide (LPS) on albumin transfer across monolayers of bovine aortic endothelial cells during a 90 minute incubation period. The effects of concomitant addition of 10 μg ml^{-1} polymixin B (PMB) are also shown. Each column is the mean of 6 observations with vertical bars representing s.e. mean. **$p<0.01$, indicates a significant difference from Control. ‡‡$p<0.01$, indicates a significant difference between groups joined by a bracket.

30 ng ml^{-1}). Polymixin B itself did not affect the level of albumin transfer, measured during a 90 minute incubation period, relative to the control value. However, when it was added in combination with LPS, it was found to abolish the resultant increase.

Similarly, in Figure 6.4 the effects of polymixin B (10 μg ml^{-1}) on a 24 hour exposure to LPS (30 ng ml^{-1}) are shown. Again, by itself, polymixin B had no effect compared to control and, again, when it was added in combination with LPS, it abolished the induced increase in albumin transfer observed during a 90 minute incubation period.

These results (Figures 6.3–6.4) show that is possible, using this *in vitro* model of the endothelial barrier, to effectively demonstrate an inhibition of the increases in albumin transfer induced by LPS.

6.1.2 Involvement of the L-arginine-nitric oxide system in the LPS-induced increase in albumin transfer

In order to establish if the L-arginine-nitric oxide system plays a role in mediating the endothelial barrier dysfunction induced by incubation with LPS, various experiments were subsequently conducted. The first set of these experiments involved looking at the effects of various nitric oxide synthase inhibitors on the increases in albumin transfer across BAEC induced by LPS.

Figure 6.5 shows the effects of 100 μM N^{ω}-nitro-L-arginine (L-NOARG) on the increase in albumin transfer stimulated by 24 hours' exposure to LPS (30 ng ml^{-1}) and measured during a subsequent 60 minute incubation period. When added alone to BAEC monolayers, L-NOARG did not itself significantly affect the level of albumin transfer compared to control levels

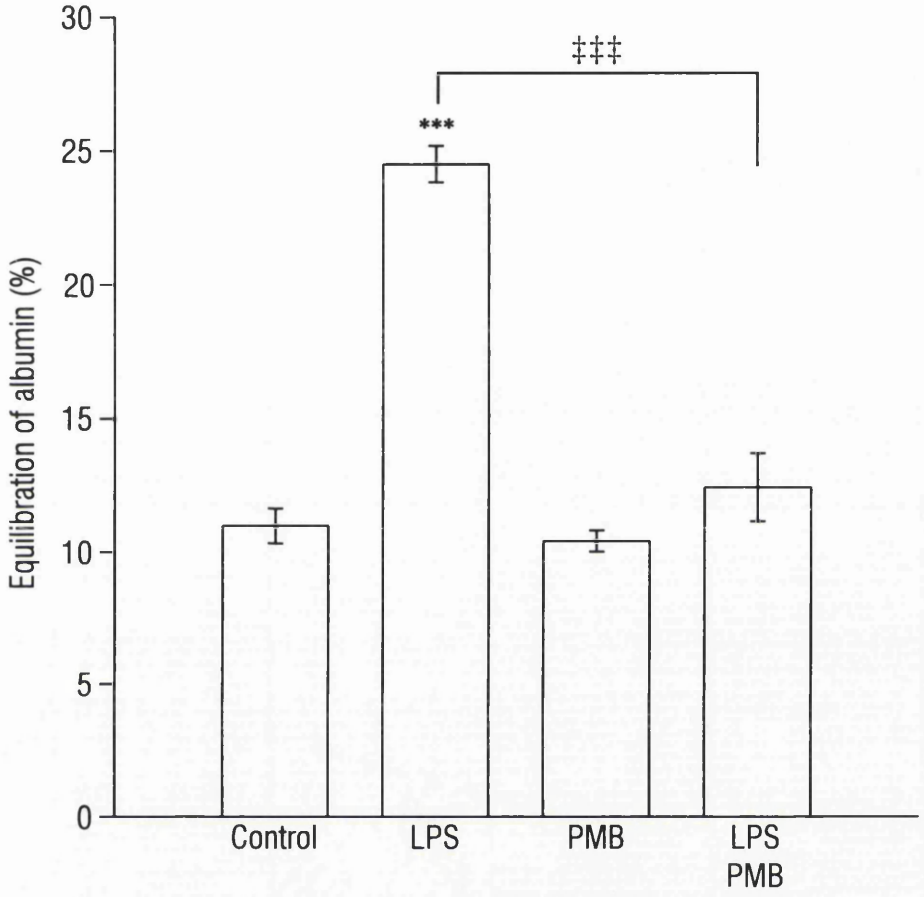


Figure 6.4 Graph showing the effects of 24 hours' exposure to 30 ng ml^{-1} lipopolysaccharide (LPS) on albumin transfer across monolayers of bovine aortic endothelial cells during a 90 minute incubation period. The effects of concomitant addition of 10 μg ml^{-1} polymixin B (PMB) are also shown. Each column is the mean of 6 observations with vertical bars representing s.e. mean. ***$p<0.001$, indicates a significant difference from Control. ‡‡‡$p<0.001$, indicates a significant difference between groups joined by a bracket.

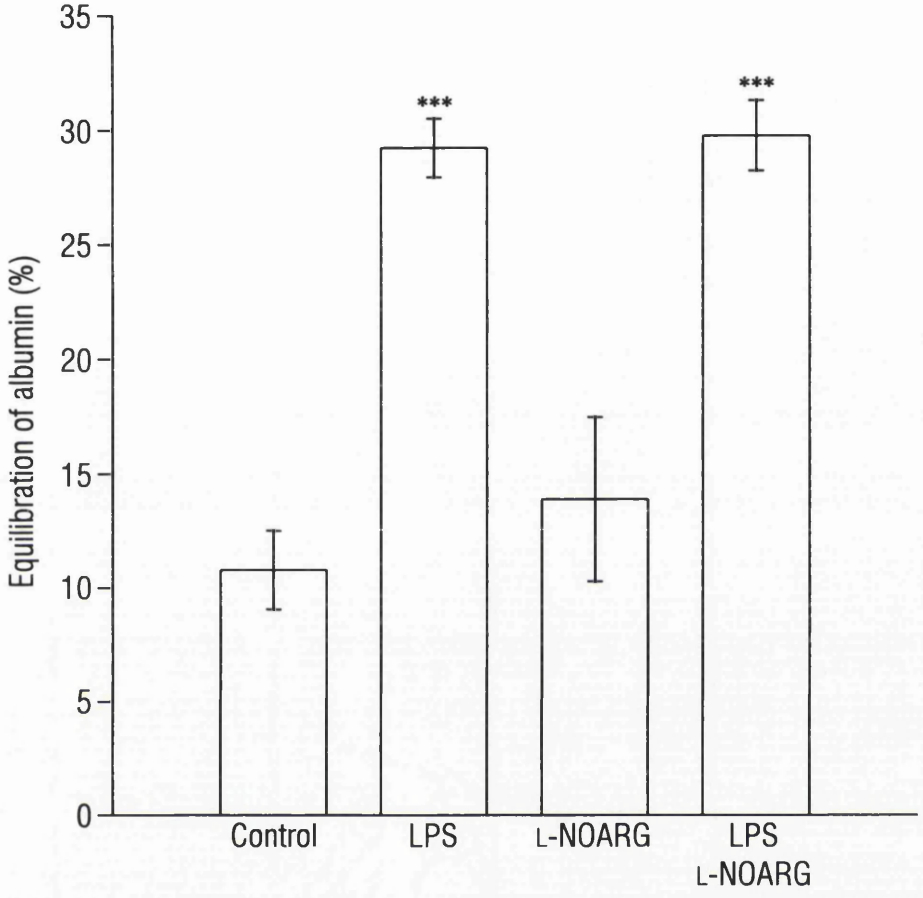


Figure 6.5 Graph showing the effects of 24 hours' exposure to 30 ng ml^{-1} lipopolysaccharide (LPS) on albumin transfer across monolayers of bovine aortic endothelial cells during a 60 minute incubation period. The effects of concomitant addition of 100 μM N^{ω}-nitro-L-arginine (L-NOARG) are also shown. Each column is the mean of 6 observations with vertical bars representing s.e. mean. ***$p<0.001$, indicates a significant difference from Control.

and when it was added in combination with LPS, it showed no inhibitory effect on the stimulated increase in albumin transfer.

Since L-NOARG is a relatively insoluble substance in aqueous solutions, and since it is possible that the reason for its failure to show an inhibition was because it was not added at a sufficiently high concentration, subsequent experiments were carried out using its more soluble methyl ester form, N^{ω}-nitro-L-arginine methyl ester (L-NAME). This therefore allowed higher concentrations of the drug to be studied. Thus, the effects of L-NAME (500 μM) on a 2 hour incubation with LPS (30 ng ml^{-1}) were investigated (Figure 6.6). L-NAME itself did not affect the level of albumin transfer, measured during a 90 minute incubation period, compared to control levels. Also, when it was added concomitantly with LPS, it failed to show any inhibition of the induced increase in albumin transfer.

Similarly, the effects of L-NAME (500 μM) on a 24 hour incubation with LPS (30 ng ml^{-1}) are shown in Figure 6.7. L-NAME itself had no significant effect on albumin transfer across BAEC monolayers, measured during a 90 minute incubation period, relative to control levels. Furthermore, when it was added in combination with LPS, it again failed to show any inhibitory effect on the resultant increase in albumin transfer.

Figure 6.8 shows the effects of another nitric oxide synthase inhibitor, N^{ω}-monomethyl-L-arginine (L-NMMA), added at an even higher concentration of 2 mM, on the increase in albumin transfer across BAEC monolayers stimulated by 2 hours' exposure to LPS (30 ng ml^{-1}). Again, addition of the inhibitor itself did not significantly alter the level of albumin transfer, measured during a 90 minute incubation period, compared to control levels. Also, when it was added in combination with LPS, rather than inhibiting the induced increase in albumin transfer, it was observed

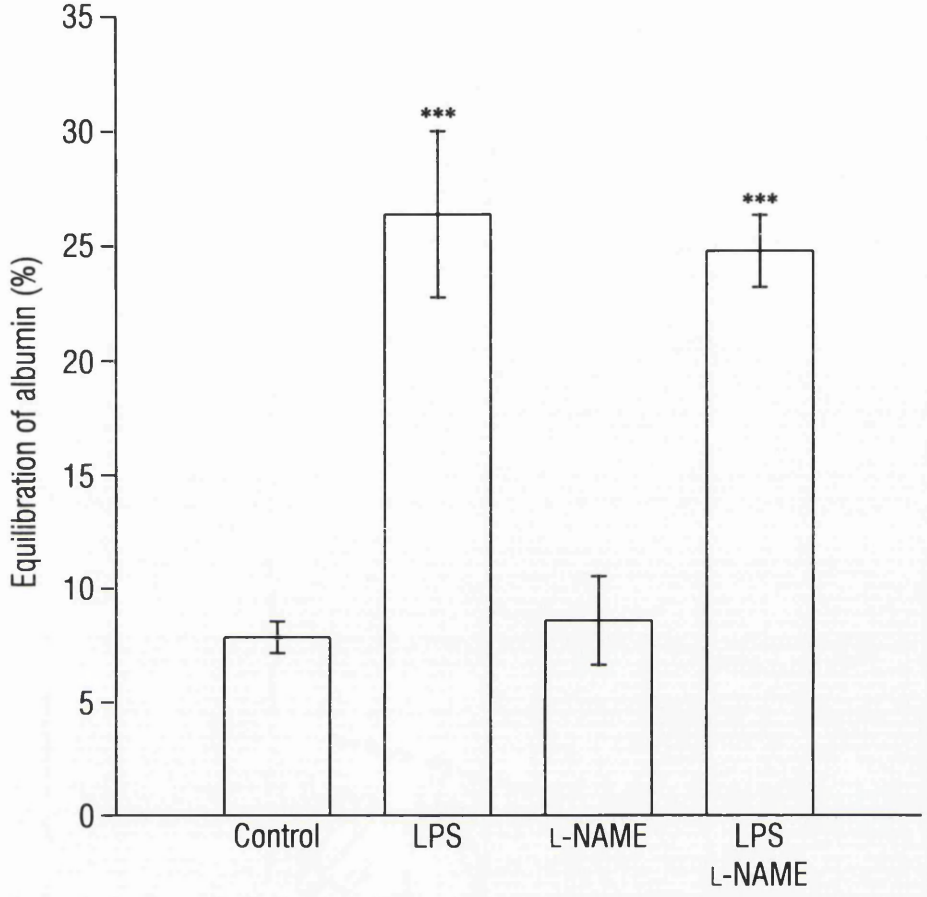


Figure 6.6 Graph showing the effects of 2 hours' exposure to 30 ng ml^{-1} lipopolysaccharide (LPS) on albumin transfer across monolayers of bovine aortic endothelial cells during a 90 minute incubation period. The effects of concomitant addition of 500 μM N^{ω}-nitro-L-arginine methyl ester (L-NAME) are also shown. Each column is the mean of 6 observations with vertical bars representing s.e. mean. ***$p<0.001$, indicates a significant difference from Control.

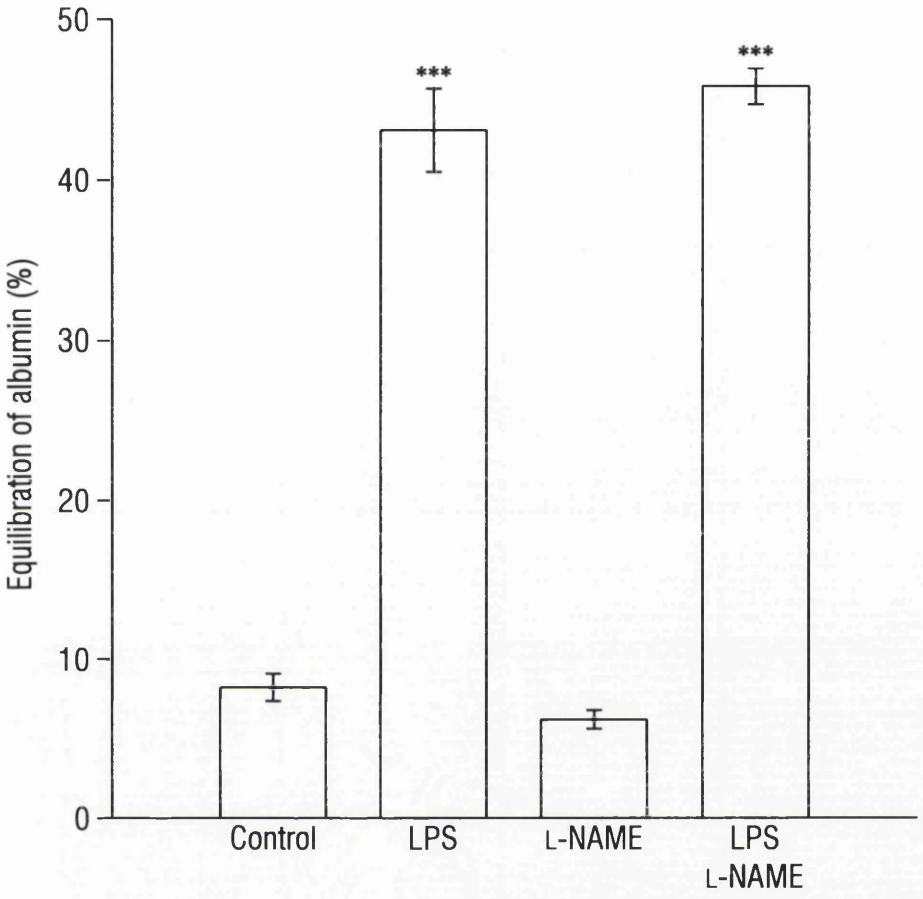


Figure 6.7 Graph showing the effects of 24 hours' exposure to 30 ng ml^{-1} lipopolysaccharide (LPS) on albumin transfer across monolayers of bovine aortic endothelial cells during a 90 minute incubation period. The effects of concomitant addition of 500 μM N^{ω}-nitro-L-arginine methyl ester (L-NAME) are also shown. Each column is the mean of 6 observations with vertical bars representing s.e. mean. ***$p<0.001$, indicates a significant difference from Control.

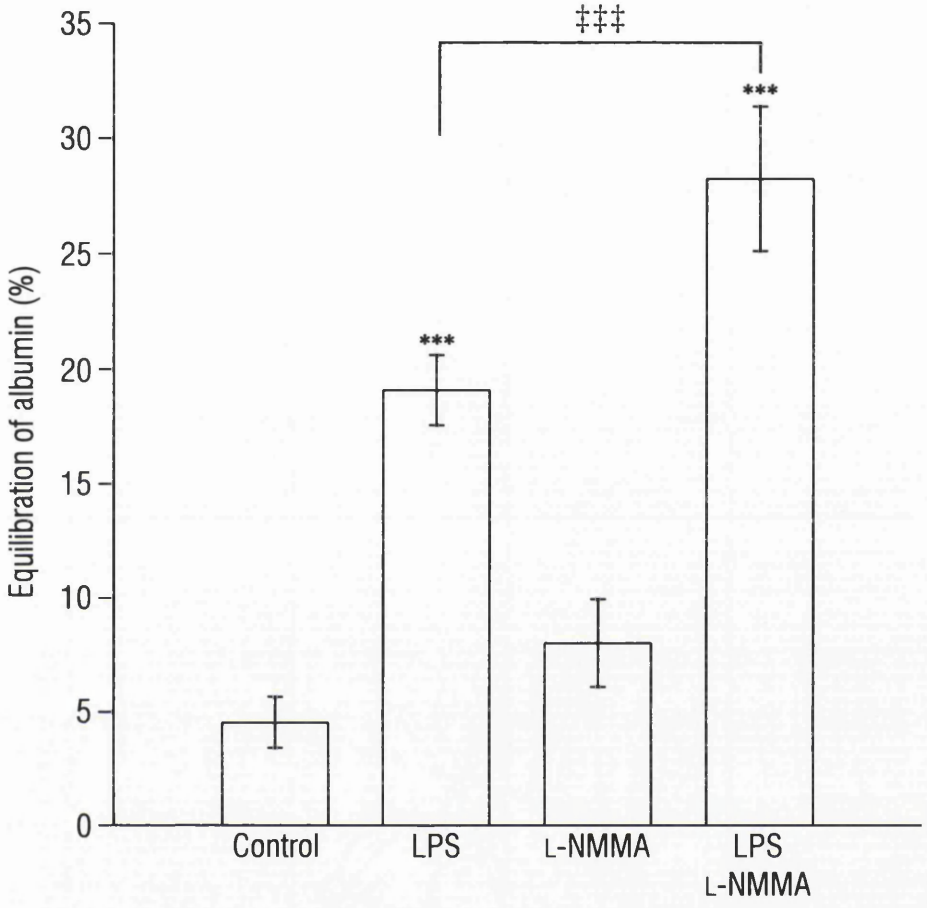


Figure 6.8 Graph showing the effects of 2 hours' exposure to 30 ng ml^{-1} lipopolysaccharide (LPS) on albumin transfer across monolayers of bovine aortic endothelial cells during a 90 minute incubation period. The effects of concomitant addition of 2 mM N^{ω}-monomethyl-L-arginine (L-NMMA) are also shown. Each column is the mean of 6 observations with vertical bars representing s.e. mean. ***$p<0.001$, indicates a significant difference from Control. ‡‡‡$p<0.001$, indicates a significant difference between groups joined by a bracket.

to enhance it. Though this enhancement was small in magnitude, it was found to be statistically significant.

The effects of L-NMMA (2 mM) on the increase in albumin transfer induced by a 24 hour incubation with LPS (30 ng ml^{-1}) were also investigated (Figure 6.9). Again, L-NMMA itself was observed to exhibit no significant effect on the level of albumin transfer across monolayers of BAEC, measured during a 90 minute incubation period, compared to control levels. When L-NMMA was added concomitantly with LPS, it was again found to cause a small, but significant enhancement in the induced level of albumin transfer rather than causing an inhibition.

These results (Figures 6.5–6.9) therefore show that blocking the synthesis of nitric oxide, using inhibitors of nitric oxide synthase, does not appear to prevent induction of the increase in albumin transfer across monolayers of BAEC induced by both 2 hours' and 24 hours' exposure to LPS.

To assess independently if the L-arginine-nitric oxide system is involved in mediating the LPS-induced increase in albumin transfer, the effects of dexamethasone, a glucocorticoid which inhibits the induction of the Ca^{2+}-independent, inducible form of nitric oxide synthase (Moncada, 1992), were investigated.

In initial experiments with dexamethasone (1 μM), the drug was added as a pretreatment 30 minutes prior to the addition of LPS. The effects of this 30 minute pretreatment on a 24 hour exposure to LPS (30 ng ml^{-1}) are shown in Figure 6.10. Dexamethasone itself had no significant effect on albumin transfer across BAEC monolayers measured during a subsequent 30 minute incubation period. When dexamethasone was added as a

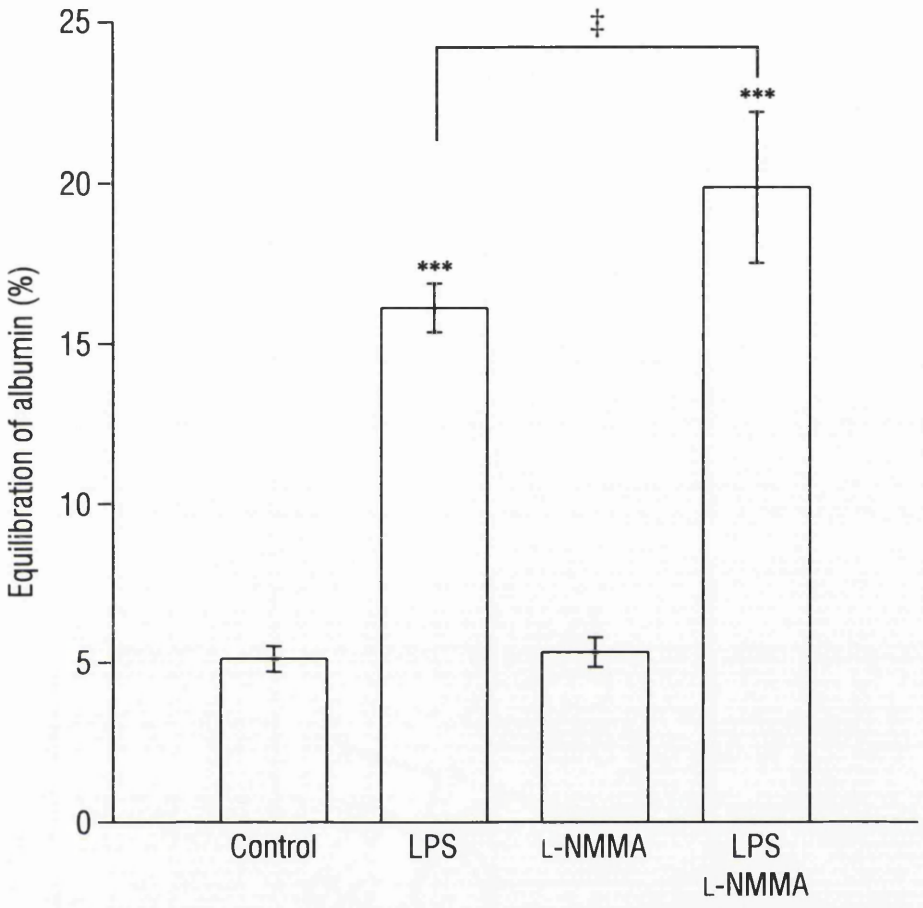


Figure 6.9 Graph showing the effects of 24 hours' exposure to 30 ng ml^{-1} lipopolysaccharide (LPS) on albumin transfer across monolayers of bovine aortic endothelial cells during a 90 minute incubation period. The effects of concomitant addition of 2 mM N^{ω}-monomethyl-L-arginine (L-NMMA) are also shown. Each column is the mean of 6 observations with vertical bars representing s.e. mean. ***$p<0.001$, indicates a significant difference from Control. ‡$p<0.05$, indicates a significant difference between groups joined by a bracket.

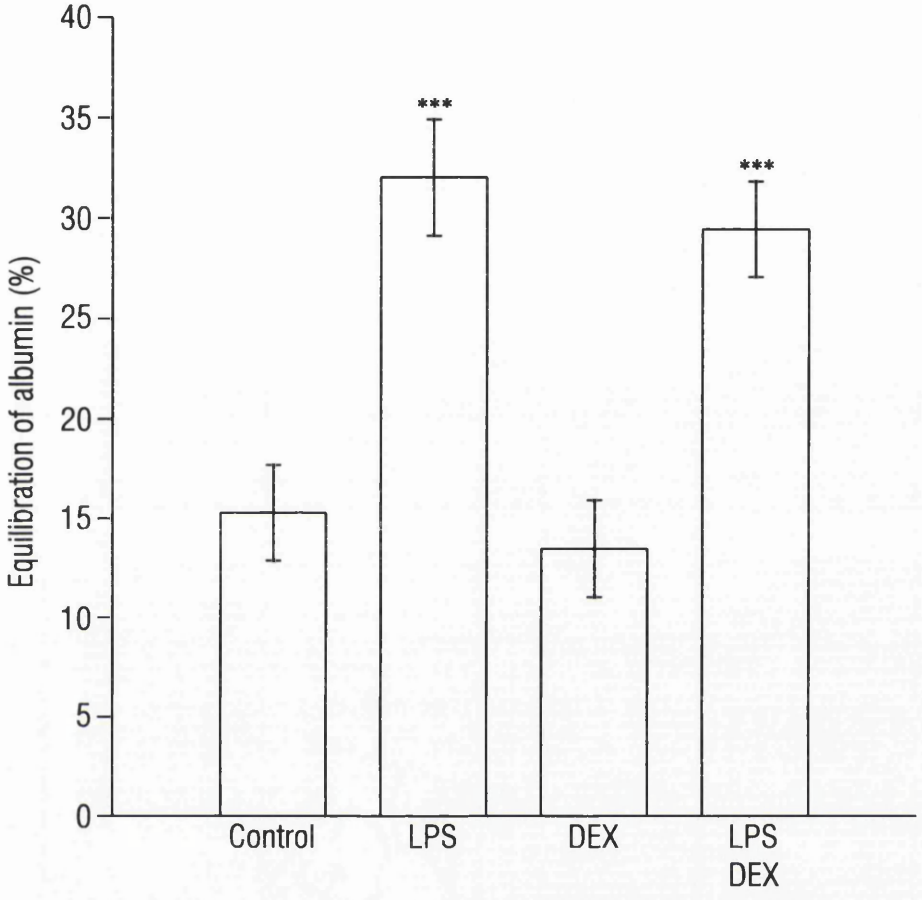


Figure 6.10 Graph showing the effects of 24 hours' exposure to 30 ng ml^{-1} lipopolysaccharide (LPS) on albumin transfer across monolayers of bovine aortic endothelial cells during a 30 minute incubation period. The effects of 30 minutes' pretreatment with 1 μM dexamethasone (DEX) are also shown. Each column is the mean of 6 observations with vertical bars representing s.e. mean. ***$p<0.001$, indicates a significant difference from Control.

pretreatment to LPS, it was found to be without any inhibitory effect on the stimulated increase.

Since it is possible that a 30 minute pretreatment may not be sufficiently long to enable dexamethasone to be fully effective, the duration of the pretreatment was subsequently extended to 20 hours. Figure 6.11 shows the effects of a 20 hour pretreatment with dexamethasone (1 μM) on a subsequent 2 hour incubation with LPS (30 ng ml^{-1}). The dexamethasone pretreatment did not, in itself, affect albumin transfer, measured during a 90 minute incubation period, compared to control levels. Also, when it was added as a pretreatment to LPS, it was found to be without any significant effect on the LPS-induced increase in albumin transfer.

Figure 6.12 shows the effects of a 20 hour pretreatment with dexamethasone (1 μM) on the increase in albumin transfer induced by 24 hours' exposure to LPS (30 ng ml^{-1}) measured during a 90 minute incubation period. Again, the dexamethasone pretreatment itself had no effect, and again when it was added as a pretreatment to the addition of LPS, it showed no inhibitory effect.

These results (Figures 6.10–6.12) show that preventing the induction of nitric oxide synthase does not inhibit the increases in albumin transfer across BAEC monolayers induced by exposure to LPS for either 2 hours or 24 hours. Taking these results together with the results shown in Figures 6.5–6.9, it would appear therefore that nitric oxide does not play a role in mediating the endothelial barrier dysfunction observed on exposure of BAEC to LPS.

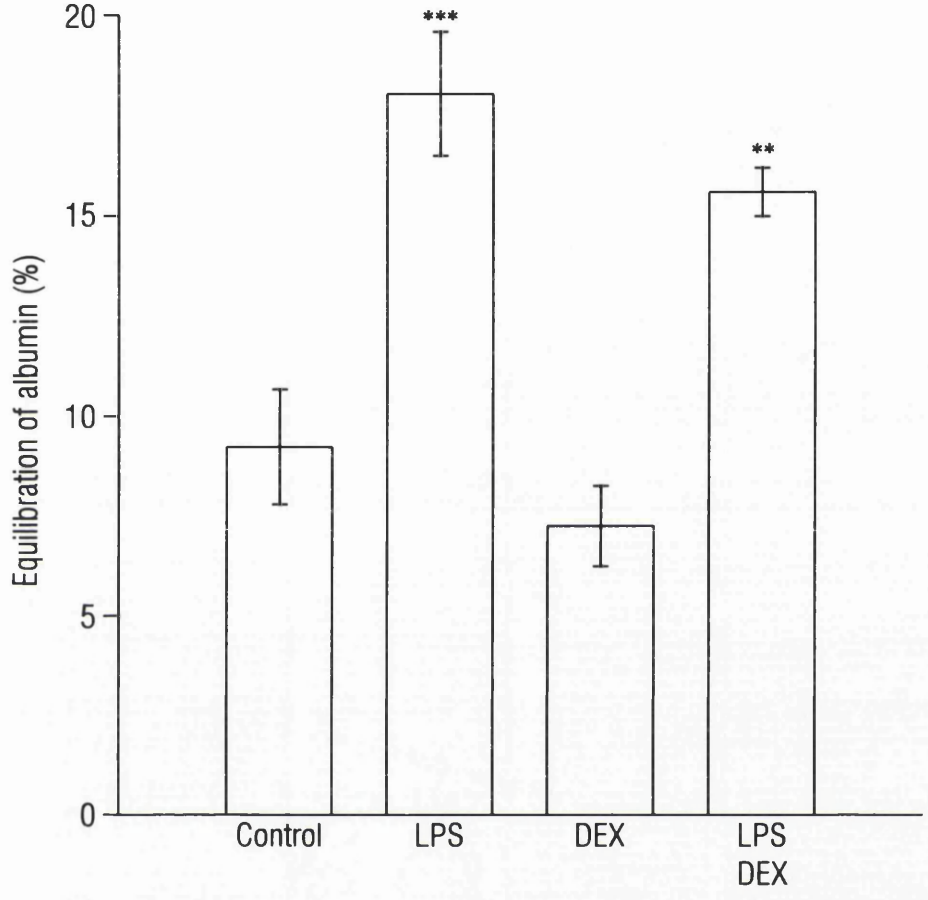


Figure 6.11 Graph showing the effects of 2 hours' exposure to 30 ng ml^{-1} lipopolysaccharide (LPS) on albumin transfer across monolayers of bovine aortic endothelial cells. The effects of 20 hours' pretreatment with 1 μM dexamethasone (DEX) are also shown. Each column is the mean of 6 observations with vertical bars representing s.e. mean. **p<0.01; ***p<0.001, indicates a significant difference from Control.

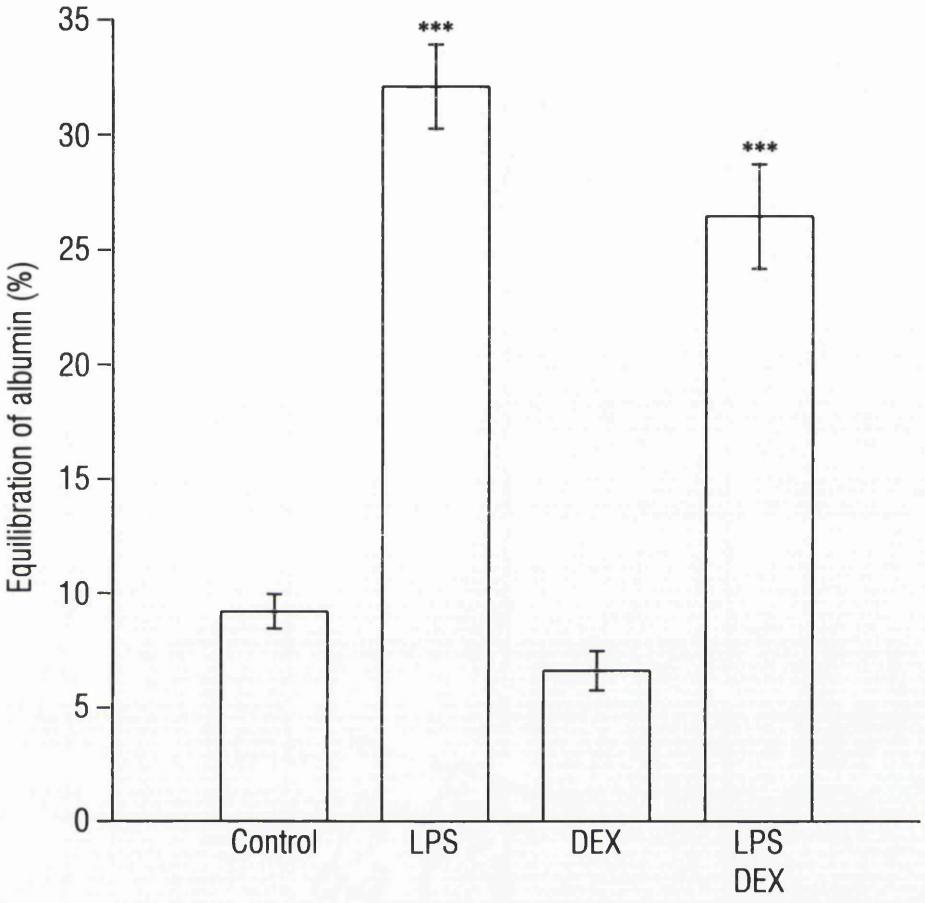


Figure 6.12 Graph showing the effects of 24 hours' exposure to 30 ng ml^{-1} lipopolysaccharide (LPS) on albumin transfer across monolayers of bovine aortic endothelial cells during a 90 minute incubation period. The effects of 20 hours' pretreatment with 1 μM dexamethasone (DEX) are also shown. Each column is the mean of 6 observations with vertical bars representing s.e. mean. ***$p<0.001$, indicates a significant difference from Control.

6.1.3 Involvement of prostaglandins in the LPS-induced increase in albumin transfer

Prostaglandins have been implicated in the development of septic shock (Parratt & Sturgess, 1975). Hence, in order to investigate if prostaglandin production plays a role in LPS-induced endothelial barrier dysfunction, the effects of the flurbiprofen, a cyclo-oxygenase inhibitor, were studied.

Flurbiprofen (30 μM) did not itself effect albumin transfer across BAEC, measured during a 60 minute incubation period, compared to control levels when added alone (Figure 6.13). Also, when flurbiprofen was added concomitantly with a 24 hour exposure to LPS, it was found to be ineffective in inhibiting the induced increase in albumin transfer.

This result would indicate that the production of prostaglandins plays no role in the mediating the endothelial barrier dysfunction induced by a 24 hour incubation with LPS.

6.1.4 Involvement of hydrogen peroxide in the LPS-induced increase in albumin transfer

As was shown in the previous chapter, hydrogen peroxide plays a key role in mediating the endothelial barrier dysfunction induced by both the hypoxanthine-xanthine oxidase and homocysteine-copper free radical generating systems. Thus, in order to determine if hydrogen peroxide is also involved in mediating the barrier dysfunction induced by LPS, the effects of catalase were investigated. The catalase concentration studied was 1000 U ml^{-1}. This concentration was chosen as it is a substantially high concentration which is many-fold in excess of that which was found to be effective in abolishing the endothelial barrier dysfunction induced by

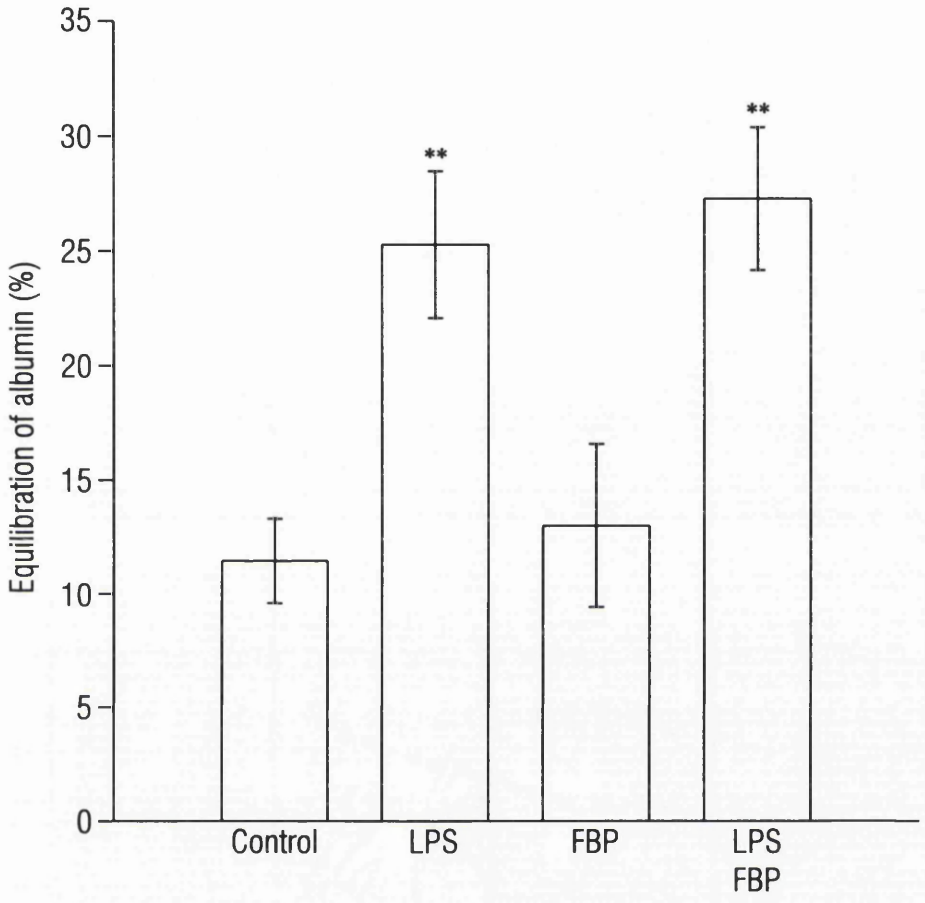


Figure 6.13 Graph showing the effects of 24 hours' exposure to 30 ng ml^{-1} lipopolysaccharide (LPS) on albumin transfer across monolayers of bovine aortic endothelial cells during a 60 minute incubation period. The effects of concomitant addition of 30 μM flurbiprofen (FPB) are also shown. Each column is the mean of 6 observations with vertical bars representing s.e. mean. **$p<0.01$, indicates a significant difference from Control.

either the hypoxanthine-xanthine oxidase system or the homocysteine-copper system.

Catalase (1000 U ml^{-1}) itself was found to have no effect when compared to the control level of albumin transfer across BAEC monolayers measured during a 30 minute incubation period (Figure 6.14). When catalase was added in combination with a 24 hour incubation with LPS (30 ng ml^{-1}), it was found to exhibit no inhibitory action on the resultant increase in albumin transfer.

This result therefore indicates that hydrogen peroxide is not involved in mediating the increase in albumin transfer induced by a 24 hour exposure to LPS.

6.2 Effects of interferon-γ (IFN-γ) on barrier function

Stolpen *et al.* (1986) demonstrated the ability of IFN-γ to alter cell shape and morphology using monolayers of human umbilical vein endothelium. Additionally, IFN-γ expression *in vivo* has been shown in mice to be up-regulated following stimulation with LPS (Cockfield *et al.*, 1993) and thus IFN-γ could conceivably be a mediator of LPS-induced endothelial barrier dysfunction. Consequently, the ability of IFN-γ to induce an increase in albumin transfer was investigated.

The effects of 24 hours' exposure of BAEC monolayers to IFN-γ (0.75–75 U ml^{-1}) are shown in Figure 6.15. At all the concentrations tested, IFN-γ was found to be without effect on the level of albumin transfer, measured during a 90 minute incubation period, compared to basal levels. Thus it would appear that IFN-γ does not directly affect endothelial barrier function over a 24 hour time period within the concentration range tested.

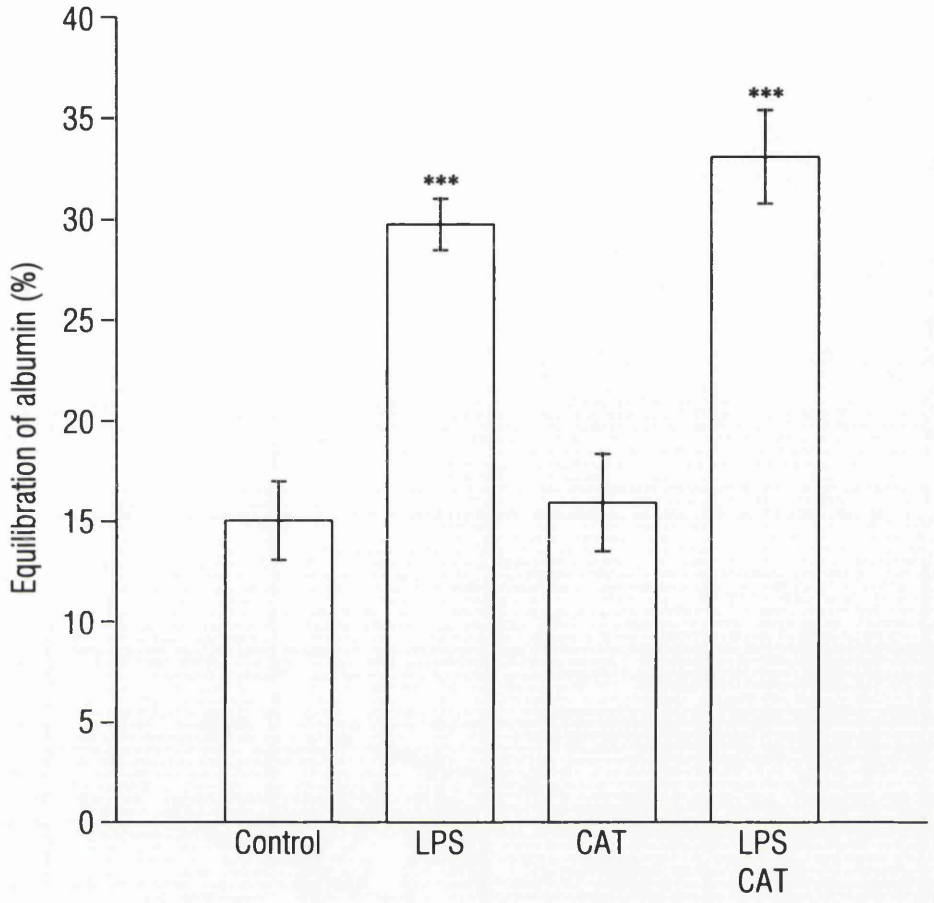


Figure 6.14 Graph showing the effects of 24 hours' exposure to 30 ng ml^{-1} lipopolysaccharide (LPS) on albumin transfer across monolayers of bovine aortic endothelial cells during a 30 minute incubation period. The effects of concomitant addition of 1000 U ml^{-1} catalase (CAT) are also shown. Each column is the mean of 6 observations with vertical bars representing s.e. mean. ***$p<0.001$, indicates a significant difference from Control.

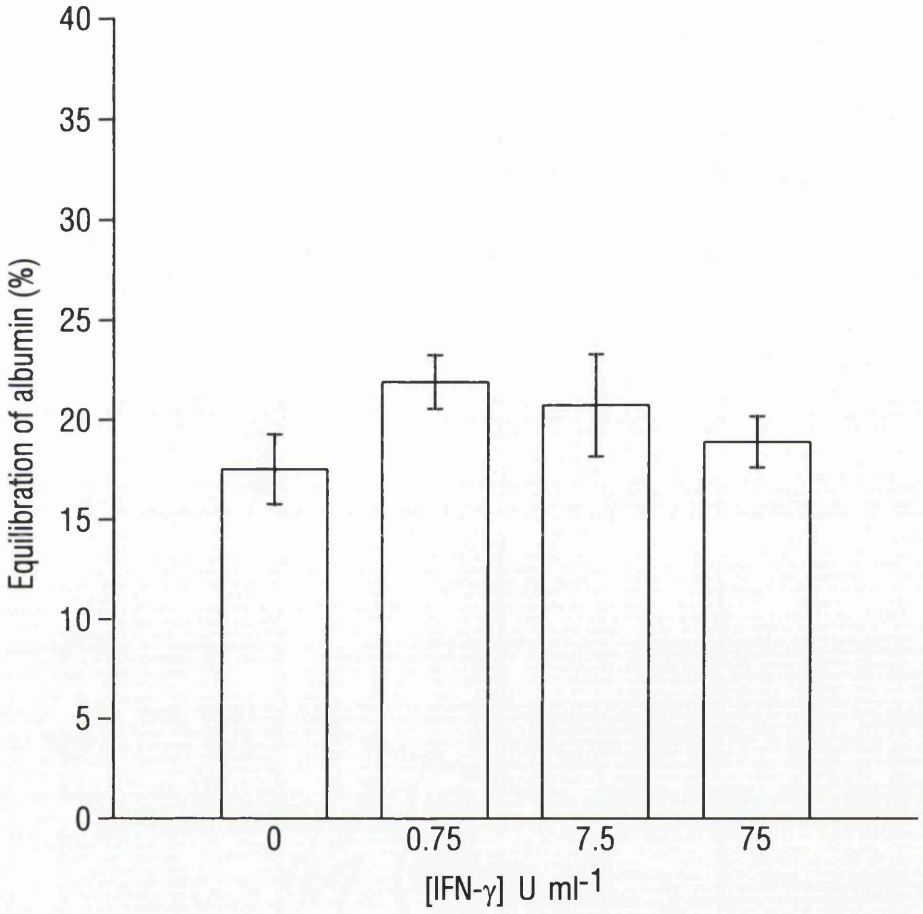
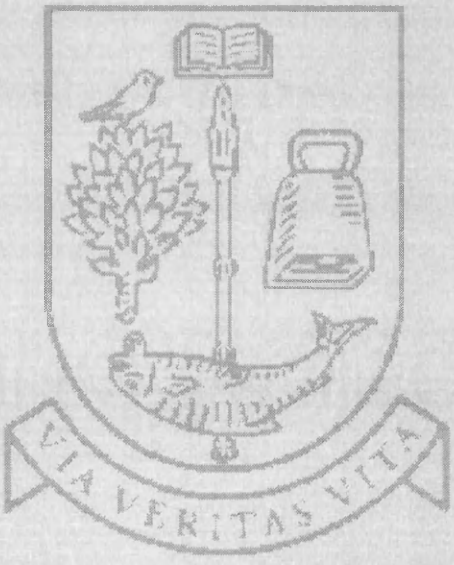


Figure 6.15 Graph showing the effects of 24 hours' exposure to 0–75 U ml^{-1} interferon-γ (IFN-γ) on albumin transfer across monolayers of bovine aortic endothelial cells during a 90 minute incubation period. Each column is the mean of 6 observations with vertical bars representing s.e. mean.

DISCUSSION



CHAPTER 7

7.1 Effects of the hypoxanthine-xanthine oxidase system

7.1.1 Production of reactive oxygen species

The hypoxanthine-xanthine oxidase free radical generating system has been widely implicated as a major cause of the damage which results from ischaemia-reperfusion injury (Jones *et al.*, 1968; Crowell *et al.*, 1969; DeWall *et al.*, 1971; Hansson *et al.*, 1983; Adkison *et al.*, 1986), including damage specifically to the vascular endothelium (Braunwald & Kloner, 1986). However, before investigating the actions of the hypoxanthine-xanthine system on endothelial barrier function in this study, it was necessary to validate the ability of the system to generate reactive oxygen species. This therefore enabled the establishment of suitable reagent concentrations, reaction conditions and an appropriate time-course for the production of reactive oxygen species.

The cytochrome C assay is an established assay which has been used to demonstrate the production of superoxide anion (Fridovich, 1970). In this study, neither hypoxanthine nor xanthine alone were found to have any effect on the oxidation state of an oxidised sample of cytochrome C, but when both were present in combination, an initial rapid reduction of the sample was observed indicating that formation of a reducing species was occurring. Only when both hypoxanthine and xanthine oxidase were present in combination was this reducing species produced, indicating that the complete enzyme-substrate system is needed for this to occur.

In the presence of superoxide dismutase, which rapidly converts superoxide anion to hydrogen peroxide, the initial rapid reduction of cytochrome C was inhibited. This finding therefore confirmed that the reducing species formed by the reaction between hypoxanthine and xanthine oxidase was superoxide anion.

The initial rapid reduction of cytochrome C in the control response to hypoxanthine and xanthine oxidase was found to give way after around 15 minutes to a more slowly developing reoxidation. This reoxidation could be blocked in the presence of catalase, which breaks down hydrogen peroxide to water and oxygen, therefore indicating that it is caused by the production of hydrogen peroxide which can be produced spontaneously from superoxide anion or through the actions of superoxide dismutase.

These results confirm the ability of the hypoxanthine-xanthine oxidase system to generate both superoxide anion and hydrogen peroxide. This finding therefore concurs with previous reports describing the generation of these species by hypoxanthine and xanthine oxidase (McCord & Fridovich, 1968; Fridovich, 1970).

Although it was not shown using this assay, it is possible that other reactive oxygen species may also be produced by the hypoxanthine xanthine oxidase system. Since both superoxide anion and hydrogen peroxide are produced, and provided there is a sufficient source of ferric iron, it is possible that hydroxyl radicals could be formed by the Fenton reaction (Beauchamp & Fridovich, 1970; Repine *et al.*, 1981; Starke & Farber, 1985) as follows:

$$Fe^{3+} + \cdot O_2^- \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^-$$

Also, since endothelial cells are known to release nitric oxide, it is feasible that nitric oxide could react with superoxide anion to generate peroxynitrite anion which itself is a powerful oxidant and which can also give rise to hydroxyl radical independently of a source of iron (Beckman *et al.*, 1990):

$$NO^{\cdot} + {}^{\cdot}O_2^- \rightarrow ONOO^-$$

$$ONOO^- + H^+ \rightarrow ONOOH$$

$$ONOOH \rightarrow {}^{\cdot}OH + {}^{\cdot}NO_2$$

7.1.2 Effects on albumin transfer

The use of a two-chamber model to measure albumin transfer across endothelial monolayers as an indicator of endothelial barrier function is well established (Taylor *et al.*, 1981; Shasby *et al.*, 1982; Rotrosen & Gallin, 1986). Using the particular model employed in this study which was originally developed by Gudgeon & Martin (1989), the effects of the hypoxanthine-xanthine oxidase system on arterial endothelial barrier function of bovine aortic endothelial cell monolayers were studied.

Typically, the basal level of albumin transfer obtained using this system during a 90 minute incubation period following addition of trypan blue-labelled albumin to the upper chamber of each well was between 2 and 25%. Previous experiments in this laboratory had shown that the presence of endothelial monolayers, substantially retarded albumin transfer compared to that obtained across polycarbonate membranes alone, thereby demonstrating the ability of this system to recreate the endothelial barrier *in vitro*. However, since the basal level of albumin transfer varied from one batch of cells to another, it was necessary to perform each experiment

with its own internal controls to enable fair comparison of the effects of agents being tested against basal levels. Thus, in the main, each individual experiment had to be performed using only one batch of cells. Only if basal levels were not statistically different between different cell batches could the results from these different batches be collated.

Addition of either hypoxanthine or xanthine oxidase alone was found to be without effect on the level of albumin transfer obtained compared to control levels, but when both were added in combination a significant increase resulted. As was found to be the case when using the cytochrome C assay to observe the formation of superoxide anion and hydrogen peroxide, this would suggest that the full free radical generating system of both hypoxanthine and xanthine oxidase together was required in order to induce endothelial barrier dysfunction. It would appear, therefore, that this barrier dysfunction is likely to be occurring as a result of the reaction taking place between hypoxanthine and xanthine oxidase. This observation is backed up by the fact that the hypoxanthine-xanthine oxidase-induced increase in albumin transfer across endothelial monolayers was inhibited by concomitant addition of allopurinol (4 mM), a xanthine oxidase inhibitor (Crowell *et al.,* 1969) which inhibits its reaction with hypoxanthine.

The increase above control levels of albumin transfer obtained on addition of the standard concentrations chosen for use in this study, i.e. 0.2 mM hypoxanthine and 20 mU ml^{-1} xanthine oxidase, was not maximal. The concentration-response curved performed in the presence of a hypoxanthine concentration fixed at 0.2 mM showed that greater levels of albumin transfer could be obtained by increasing the concentration of xanthine oxidase (up to 300 mU ml^{-1}). The standard concentrations were, however, sufficient to give a reproducible, sub-maximal increase in albumin transfer against which the effects of potential inhibitors could be gauged.

In vivo studies have demonstrated an impairment of endothelial barrier function caused by experimentally-induced ischaemia and reperfusion. For instance, Svendsen *et al.* (1991) showed that after 20 minutes occlusion of the left anterior descending coronary artery followed by 1 hour of reperfusion in dog hearts, an increased capillary permeability to diethylenetriaminepentaacetic acid was observed. The extent to which activation of the hypoxanthine-xanthine oxidase system contributes to the injury produced on ischaemia followed by reperfusion, including impairment of barrier function, remains, however, to be determined. Other factors, such as neutrophil activation for instance, may play a more important role and, indeed, activated neutrophils have been shown to increase albumin transfer across monolayers of bovine pulmonary artery microvessel endothelial cells (Siflinger-Birnboim & Malik, 1993; Siflinger-Birnboim *et al.*, 1993). The mediator of this neutrophil-induced endothelial barrier dysfunction has, however, not yet been identified. Neutrophils produce many substances including superoxide anion, hypochlorous acid, prostanoids and digestive enzymes, all of which are potentially injurious to the endothelium, and all of which could therefore be responsible for inducing barrier dysfunction. Hydrogen peroxide derived from neutrophil release of superoxide anion, has however, been shown to be a major mediator of neutrophil-induced killing of endothelial cells (Weiss *et al.*, 1981), with hydroxyl radical possibly mediating this action (Varani *et al.*, 1985).

In vitro, the hypoxanthine-xanthine oxidase system has been shown to exert a toxic action on cultured calf pulmonary artery cells measured as ^{51}Cr-release (Kvietys *et al.*, 1989) whilst, *in vivo*, evidence has shown that the hypoxanthine-xanthine oxidase system certainly contributes to injury in ischaemia-reperfusion since allopurinol has been found to inhibit the injury induced by ischaemia-reperfusion. For instance, allopurinol was found

to inhibit the deleterious effects on myocardial function measured by various indices following occlusion of the left anterior descending coronary artery in both dog and sheep (DeWall *et al.*, 1971). Additionally, in rat livers, allopurinol inhibited the increases in enzyme activities and oxygen consumption which were induced by 2 hours of global ischaemia followed by 1 hour of reperfusion (Adkison *et al.*, 1986). Accumulation of hypoxanthine accompanied by an increase in xanthine oxidase activity has also been observed in many tissues during ischaemia (Granger, 1988). This increase in xanthine oxidase activity appears to vary from species to species with activity in humans being considerably less than in other species such as dog and rat (Southard *et al.*, 1987). Evidence has now also shown that both superoxide anion release and an increase in albumin transfer could be induced *in vitro* using monolayers of bovine pulmonary microvessel endothelial cells placed in hypoxic conditions (0.1% O_2) for between 2 and 24 hours, followed by reoxygenation in room air for a period between 16 seconds and 2 hours (Lum *et al.*, 1992). This indicates that impairment of endothelial barrier function can be achieved in the absence of other cells, including neutrophils, and can therefore be mediated by the endothelial cells themselves.

Using xanthine in combination with xanthine oxidase, Shasby *et al.* (1985) demonstrated an increase in albumin transfer across monolayers of porcine pulmonary artery endothelial cells on addition of these agents. This concurs with findings in this study in which it was found that substituting xanthine for hypoxanthine at the same concentration elicited a similar increase in albumin transfer above basal when added in combination with xanthine oxidase. Since superoxide anion is produced on both the conversions of hypoxanthine to xanthine and xanthine to uric acid, it is conceivable to suppose that when using xanthine, it is possible to generate only half the amount of superoxide anion compared to that which can be produced

using hypoxanthine. However, as the concentration response-curve to xanthine oxidase performed in this study showed, at the concentration used, there is an excess of hypoxanthine present and the rate of reaction is limited only by the amount of xanthine oxidase present. Since a similar magnitude of response was obtained using xanthine in place of hypoxanthine, it would appear that at the concentrations used there is still an excess of substrate and so no difference is seen. It would appear, therefore, that xanthine and hypoxanthine can equally well be used as substrates for xanthine oxidase, when added at these concentrations, in terms of their ability to induce superoxide anion production and endothelial barrier dysfunction.

7.1.3 Role of superoxide anion

In order to attempt in this study to establish the nature of the species produced by the hypoxanthine-xanthine oxidase system responsible for mediating its effects on barrier function, various potential inhibitors were co-incubated with hypoxanthine and xanthine oxidase. The first of these, superoxide dismutase, was however found to be ineffective in inhibiting the hypoxanthine-xanthine oxidase-induced increase in albumin transfer either when added at an initial concentration of 30 U ml^{-1}, or when added at a substantially higher concentration of 6000 U ml^{-1}. This latter concentration, in particular, should have been more than sufficient to abolish any effects produced directly by superoxide anion production and indeed using the cytochrome C assay it had been found to almost completely abolish the reduction of cytochrome C by hypoxanthine and xanthine oxidase. From these findings, it can clearly be concluded that superoxide anion is not the damaging species produced by the hypoxanthine-xanthine oxidase system responsible for mediating the system's effects on endothelial barrier function.

These findings, however, would appear to conflict with *in vivo* reports of the actions of superoxide dismutase during ischaemia-reperfusion-induced increased capillary permeability in both feline intestine (Granger *et al.*, 1981; Granger, 1988) and canine coronary vasculature (Dauber. *et al.*, 1991; Svendsen *et al.*, 1991). In each of these studies, superoxide dismutase was found to cause a reduction in the extent of the increase in capillary permeability observed. Moreover, superoxide dismutase was found to inhibit the increase in capillary permeability seen *in vivo* in feline intestine following intra-arterial infusion of hypoxanthine and xanthine oxidase (Granger, 1988). Superoxide dismutase has also been found to be effective *in vivo* in inhibiting ischaemia-reperfusion-induced cell necrosis in feline intestine (Parks *et al.*, 1982) and, *in vitro,* in inhibiting both the cytotoxic effects of ischaemia-reperfusion on bovine coronary microvascular endothelial cells, determined by ^{51}Cr release (Inaunen *et al.*, 1988), and the increase in albumin transfer across monolayers of bovine pulmonary microvessel endothelial cells induced by hypoxia followed by reoxygenation (Lum *et al.*, 1992).

In contrast, Ager & Gordon (1984) found that superoxide dismutase did not inhibit the injurious effects of xanthine and xanthine oxidase on cell function of porcine aortic endothelial cells measured by K^+ efflux, prostaglandin production and the release of cytoplasmic purines. Additionally, Link & Riley (1988) found that superoxide dismutase had no effect on xanthine-xanthine oxidase-induced cell death in a mammalian epithelial cell line, GPK. Furthermore, Kvietys *et al.* (1989) found that superoxide dismutase was ineffective in inhibiting the actions of hypoxanthine and xanthine oxidase as determined by the induction of ^{51}Cr release from calf pulmonary artery endothelial cells.

7.1.4 Role of hydrogen peroxide

In contrast to the effects of superoxide dismutase, catalase was found in this study to abolish the increase in albumin transfer induced by hypoxanthine and xanthine oxidase, thereby strongly implicating hydrogen peroxide in mediating the actions of this system on endothelial barrier function. This inhibitory effect of catalase appeared to be an all-or-nothing response as indicated by the catalase concentration-response curve which demonstrated a switch from no inhibitory effect to total abolition of the hypoxanthine-xanthine oxidase-induced response on increasing the catalase concentration tested from 1 U ml^{-1} to 3 U ml^{-1}.

This observed inhibitory effect of catalase would appear to be in agreement with reports from other workers. It has been shown, for instance, that catalase inhibited xanthine-xanthine oxidase-induced ^{51}Cr release from bovine pulmonary artery endothelial cells (Martin, 1984), inhibited hypoxanthine-xanthine oxidase-induced ^{51}Cr release from calf pulmonary artery endothelial cells (Kvietys *et al.,* 1989) and prevented cell death induced by xanthine and xanthine oxidase in the GPK epithelial cell line (Link & Riley, 1988). Catalase was also found to inhibit the increase in capillary permeability in dog skeletal muscle induced by ischaemia-reperfusion (Korthuis *et al.,* 1985) as well as the increase in albumin transfer across monolayers of bovine pulmonary microvessel endothelium induced by hypoxia and reoxygenation (Lum *et al.,* 1992). Also, when added in combination with superoxide dismutase, catalase was found to reduce ischaemia-reperfusion-induced infarct size in both canine heart (Jolly *et al.,* 1984) and rat brain (Liu *et al.,* 1989). Since in both these studies, neither catalase nor superoxide dismutase were tested on their own, it is impossible to say, however, whether both were in fact required to reduce infarct size or whether either would have been effective on its own.

The effects of catalase on the increase in albumin transfer induced by xanthine and xanthine oxidase were also investigated in this study. Catalase was found to exhibit a very similar profile of response when inhibiting this increase in albumin transfer compared to that obtained when using hypoxanthine as a substrate for xanthine oxidase, and was found to be active over precisely the same concentration-range (1–3 U ml^{-1}). This therefore confirms the earlier conclusion that xanthine and hypoxanthine are both equally valid substrates for use with xanthine oxidase when investigating its effects on endothelial barrier function.

Several studies have demonstrated cytotoxic actions of hydrogen peroxide on endothelial cells. For instance, Ager & Gordon (1984) showed that hydrogen peroxide stimulated ^{51}Cr release, adenine uptake, K^+ efflux, prostaglandin production and the release of cytoplasmic purines in porcine aortic endothelial cells. Additionally, hydrogen peroxide has been shown to induce cells death in cultured mammalian epithelial cells (Link & Riley, 1988), ^{51}Cr release from rat pulmonary artery endothelial cells (Varani *et al.,* 1990), and both lactate dehydrogenase release and 5-HT uptake in porcine pulmonary artery endothelial cells (Block, 1991). Moreover, hydrogen peroxide was found to increase macromolecular transfer across monolayers of canine kidney epithelial cells (Welsh *et al.,* 1985), as well as porcine pulmonary artery (Wilson *et al.,* 1990), human umbilical vein (Yamada *et al.,* 1990b) and bovine pulmonary microvessel (Siflinger-Birnboim *et al.,* 1992) endothelial cells. Having then established in this study, through the effectiveness of catalase, that hydrogen peroxide is implicated in mediating the effects of the hypoxanthine-xanthine oxidase system on endothelial barrier function, the effect of adding exogenous hydrogen peroxide was investigated to confirm its ability to increase albumin transfer in this system. This was indeed found to be the case, with hydrogen

peroxide eliciting a biphasic concentration response curve, showing a lower peak at around 0.1–0.3 mM and a higher peak at around 10–30 mM.

The lower peak of this curve correlates with the theoretical maximum concentration of hydrogen peroxide, i.e. 0.2 mM, which could be formed from the chosen standard concentrations of hypoxanthine and xanthine oxidase employed in this study. (This is based on the fact that complete conversion of hypoxanthine to xanthine and then to uric acid will produce two molecules of superoxide anion which gives rise to one molecule of hydrogen peroxide for every one molecule of hypoxanthine initially present—see Introduction.) It is therefore quite conceivable that the hypoxanthine-xanthine oxidase-induced increase in albumin transfer across monolayers of BAEC is, in fact, mediated by hydrogen peroxide.

The precise reason for the biphasic-nature of the hydrogen peroxide concentration-response curve which was obtained is unclear. One possible explanation is that at low concentrations (corresponding to the lower peak at 0.1–0.3 mM), sufficient hydrogen peroxide is present to cause impairment of the barrier function of the endothelial monolayer, but when this concentration is increased, some form of protective mechanism (e.g. release of intracellular catalase) is triggered within the cells and this is able to counter the effects of hydrogen peroxide on barrier function. As the concentration of hydrogen peroxide is further increased (corresponding to the higher peak at 10–30 mM), then enough hydrogen peroxide becomes available to overcome this protective effect and so a second wave of endothelial barrier dysfunction is observed. An alternative explanation is that hydrogen peroxide may be causing its effects on barrier function at two distinct target sites in the endothelial cell thereby acting by two distinct mechanisms, one of which has a low sensitivity to hydrogen peroxide and

one of which has a higher sensitivity, with each of these corresponding to a peak in the concentration-response curve.

The findings of Siflinger-Birnboim *et al.* (1992) perhaps back up the latter of these two hypotheses. They showed that protein kinase C was involved in mediating the hydrogen peroxide-induced increase in albumin transfer across monolayers of bovine pulmonary microvessel endothelial cells as shown by a partial inhibition of the actions of hydrogen peroxide using two protein kinase C inhibitors, H7 and calphotsin C. This inhibition, however, was at its highest against a hydrogen peroxide concentration of 0.1 mM and was found to diminish as the hydrogen peroxide concentration was increased. From this they concluded that at low concentrations (around 0.1 mM) the effects of hydrogen peroxide on endothelial barrier function were mediated, at least in part, by protein kinase C but at higher concentrations some other mechanism might be involved such as release of prostanoids or PAF.

As would be expected, catalase was found in this study to abolish the increase in albumin transfer induced across monolayers of BAEC by hydrogen peroxide. This inhibitory action of catalase was tested against hydrogen peroxide concentrations of both 0.1 and 10 mM, each of these concentrations representing a peak in the hydrogen peroxide concentration-response curve, respectively. To abolish the increase in albumin transfer induced by 10 mM hydrogen peroxide, a correspondingly high concentration of catalase was required, namely around 3000 U ml^{-1}. In contrast, the increase in albumin transfer induced by 0.1 mM hydrogen peroxide was found to be abolished using only 0.3 U ml^{-1} catalase which is nearer in magnitude to the catalase concentration (3 U ml^{-1}) found in this study to be required to abolish the increase induced by the hypoxanthine-xanthine oxidase system. However, lower concentrations of catalase were not tested

against the effects of 0.1 mM hydrogen peroxide, so it is conceivable that even lower concentrations of catalase would also have been effective.

Since hydrogen peroxide production is clearly required for the endothelial barrier dysfunction induced by the hypoxanthine-xanthine oxidase system, it would perhaps have been expected that addition of superoxide dismutase would have enhanced the level of albumin transfer obtained. However, as has already been described, this was not in fact the case with superoxide dismutase being found to have no effect on the actions of the hypoxanthine-xanthine oxidase system. The likeliest explanation for this observation is that conversion of superoxide anion to hydrogen peroxide is already occurring sufficiently rapidly in the absence of exogenously added superoxide dismutase to yield an increase in albumin transfer which is maximal for the concentrations of hypoxanthine and xanthine oxidase used. This conversion of superoxide anion to hydrogen peroxide may either be occurring spontaneously or may be being catalysed by endogenous superoxide dismutase present in the endothelial cells.

7.1.5 Role of hydroxyl radicals

Whilst the results so far discussed would indicate that hydrogen peroxide is clearly an important mediator of the hypoxanthine-xanthine oxidase-induced increase in albumin transfer, it may, however, not be the sole damaging species produced which is responsible for this increase. It is quite possible, for example, that hydrogen peroxide is merely acting as a precursor for the formation of some other mediator of endothelial barrier dysfunction. One such possible mediator is the hydroxyl radical (Beauchamp & Fridovich, 1970) and in a number of studies, hydroxyl radicals have been implicated in causing damage to both endothelium and other tissues. For instance, hydroxyl radical scavengers have been shown

to inhibit neutrophil-induced killing of bovine pulmonary artery endothelial cells (Varani *et al.,* 1985), and to inhibit both ischaemia-reperfusion-induced contractile dysfunction (Bolli *et al.,* 1989) and ischaemia-reperfusion-induced increased coronary microvascular permeability (Dauber. *et al.,* 1991) in dog hearts. Hydroxyl radical scavengers have also proved effective in inhibiting ischaemia-reperfusion-induced lactate dehydrogenase release and haemodynamic dysfunction in isolated perfused rat hearts (Karwatowska-Prokopczuk & Beresewicz, 1992) and, additionally, have been shown to inhibit hypoxanthine-xanthine oxidase-induced ^{51}Cr release from calf pulmonary artery endothelial cells (Kvietys *et al.,* 1989). Moreover, intracellular formation of hydroxyl radicals following exposure of porcine pulmonary artery endothelial cells to hydrogen peroxide has been demonstrated using spin trapping techniques (Britigan *et al.,* 1992).

In order, therefore, to investigate if hydroxyl radicals are involved in mediating the endothelial barrier dysfunction induced in this study by the hypoxanthine-xanthine oxidase system, the effects of co-incubating various hydroxyl radical scavengers with hypoxanthine and xanthine oxidase were studied. The first hydroxyl radical scavenger to be studied was mannitol which is a membrane-impermeant scavenger (Kvietys *et al.,* 1989). Mannitol which did no affect albumin transfer in itself was, however, found to be totally ineffective in abolishing the increase in albumin transfer across monolayers of BAEC which was induced by incubation with the hypoxanthine-xanthine oxidase system. However, since mannitol is unable to cross the endothelial cell membrane, it is only able to scavenge, and therefore inhibit the effects of, any hydroxyl radicals which are produced extracellularly. So whilst it would seem likely from this result that extracellular generation of hydroxyl radicals is not involved in mediating the effects of the hypoxanthine-xanthine oxidase system on endothelial

barrier function, the possibility still exists that intracellular generation of hydroxyl radicals is involved.

To investigate any involvement of intracellular hydroxyl radical generation in mediating the increase in albumin transfer induced by hypoxanthine and xanthine oxidase, the effects of comcomitant addition of two membrane-permeant hydroxyl radical scavengers, namely dimethylthiourea (Kvietys *et al.,* 1989) and N-(2-mercaptopropionyl)-glycine (MPG) (Bolli *et al.,* 1989), were therefore studied. Neither dimethylthiourea (10 mM), nor MPG (1 mM) had any effect on the basal level of albumin transfer and, additionally, neither agent was found to affect the increase in albumin transfer induced by the hypoxanthine-xanthine oxidase system. This therefore indicates that hydroxyl radicals, whether generated extracellularly or intracellularly, do not appear to be responsible for mediating the effects of the hypoxanthine-xanthine oxidase system on endothelial barrier function.

The use of higher concentrations of both dimethylthiourea and MPG than those tested in this study, in order to ensure that the ineffectiveness of each was not merely due to the agents being added at an insufficiently high concentration, was not possible. When the concentration of dimethylthiourea was increased above 10 mM, it was found by itself to induce an increase in albumin transfer. This was presumably due to a cytotoxic action of the agent on the endothelial cell monolayers. In the case of MPG, any concentration tested above 1 mM was found to induce some form of irreversible block on the transfer of albumin. The cause of this effect was unclear but it may have resulted from an interaction taking place between MPG and some component of the endothelial cell monolayers. Alternatively, it could have been due to an interaction occurring between MPG and either the polycarbonate filters on which the cell

monolayers were grown or a component of the trypan-blue labelled albumin complex which was used as a marker of endothelial barrier function.

Since hydroxyl radicals have been shown to be produced from superoxide anion and hydrogen peroxide, in the presence of a source of ferric iron through the Fenton reaction (Beauchamp & Fridovich, 1970), the effects of both removing and augmenting available ferric iron were investigated in this study. This allowed an independent assessment to be made of the involvement of hydroxyl radical production in mediating the increase in albumin transfer across monolayers of BAEC stimulated by the hypo-xanthine-xanthine oxidase system.

Removal of ferric iron was achieved by using the iron chelator, deferoxamine. Initial experiments in which deferoxamine (500 μM) was added concomitantly with hypoxanthine and xanthine oxidase showed no effect of the iron chelator. Starke & Farber (1985) suggested, however, that cells must be pretreated with deferoxamine for several hours in order for it to be able to enter the cells and chelate intracellular iron, including iron stored as ferritin, in addition to merely chelating any iron present in the surrounding medium. Thus, in order to ensure chelation of both intracellular and extracellular iron was occurring in this study, the effects of pretreating endothelial cell monolayers overnight with deferoxamine (500 μM), prior to the addition of hypoxanthine and xanthine oxidase, was subsequently investigated. However, even when added as an overnight pretreatment, deferoxamine was found to have no inhibitory effect on the increase in albumin transfer induced by the hypoxanthine-xanthine oxidase system.

Additionally, augmenting available ferric iron, by concomitant addition of ferric chloride (50 μM) with hypoxanthine and xanthine oxidase, failed to generate an enhancement of the resultant increase in albumin transfer.

Taking this finding together with those obtained in this study for deferoxamine, it would clearly appear that the endothelial barrier dysfunction induced by the hypoxanthine-xanthine oxidase system is not dependent on the availability of ferric iron and therefore the Fenton reaction would appear not to be involved. Moreover, when the results for mannitol, dimethylthiourea and MPG are also taken into account, it seems clear that production of hydroxyl radicals is not necessary to obtain an increase in albumin transfer in response to addition of the hypoxanthine-xanthine oxidase system. It can be concluded, therefore, that the effects of this system on endothelial barrier function are not mediated by hydroxyl radicals.

These findings for the effects of hypoxanthine and xanthine oxidase on albumin transfer would appear to conflict with previous reports on the cytotoxic actions of both the hypoxanthine-xanthine oxidase system and hydrogen peroxide in which hydroxyl radicals have been implicated as the mediating the effects of these agents. For instance, Starke & Farber (1985) demonstrated that hydrogen peroxide-induced killing of hepatocytes required the presence of both superoxide anion and ferric iron suggesting that these effects of hydrogen peroxide were mediated by production of hydroxyl radicals by way of the Fenton reaction. Additionally, when measuring ^{51}Cr release from bovine pulmonary artery cells as an indicator of cytotoxicity induced by neutrophils, which also generate superoxide anion and hydrogen peroxide, these effects were found to be inhibited by the hydroxyl radical scavengers, dimethylthiourea and mannitol, as well as by deferoxamine (Varani *et al.*, 1985). Moreover, Kvietys *et al.* (1989) showed that ^{51}Cr release induced by the hypoxanthine-xanthine oxidase system from calf pulmonary artery endothelial cells was inhibited by the membrane-permeant hydroxyl radical scavengers, dimethylthiourea and dimethyl sulphoxide, as well as by a 5 hour pretreatment with deferoxamine.

In all these studies, however, cell death or ^{51}Cr release was taken as an indicator of the effects of the agents being tested whereas in this study endothelial barrier function, a physiological function, was measured. Furthermore, cell death was measured after a 24 hour period, whilst ^{51} Cr release was only detectable at significant levels after around at least 4 hours. In contrast, an increase in albumin transfer, measured in this study as an indicator of endothelial barrier function, was observable following a standard 90 minute incubation period, and indeed could be seen after around only 30 minutes. Hence, it would seem that whilst hydroxyl radical production might be necessary to cause cytotoxicity/cell death in endothelial and other cells, it is not necessary to cause endothelial barrier dysfunction in BAEC which develops over a much shorter period of time. Ager & Gordon (1984) demonstrated that ^{51}Cr release is a substantially less sensitive index of the actions of the xanthine-xanthine oxidase system on vascular endothelial cells than other indices such as prostaglandin production, purine release and, in particular, K^+ efflux. Endothelial barrier function, as measured in this study, would appear also to be a much more sensitive indicator of the effects of the hypoxanthine-xanthine oxidase free radical generating system than ^{51}Cr release, and, additionally, is perhaps more physiologically relevant.

7.1.6 Role of peroxynitrite anion

It has been recognised for some years that both EDRF (which may be nitric oxide or a related substance) and authentic nitric oxide can be inactivated by superoxide anion (Gryglewski *et al.*, 1986; Rubanyi & Vanhoutte, 1986). In 1990, Beckman *et al.* speculated that the reaction which takes place between nitric oxide and superoxide anion gives rise to the production of peroxynitrite anion ($ONOO^-$), a powerful oxidant in itself which additionally gives rise to the formation of hydroxyl radicals in

an iron-independent manner. Since endothelial cells are known to release nitric oxide (or a related substance which gives rise to nitric oxide), it is conceivable that this nitric oxide could react with superoxide anion, generated on addition of the hypoxanthine-xanthine oxidase system, to yield peroxynitrite anion. Additionally, endothelial cells have been shown to release superoxide anion (Matsubara & Ziff, 1986) which could react with nitric oxide. Thus, it is possible that peroxynitrite anion can contribute to the actions of the hypoxanthine-xanthine oxidase system on endothelial barrier function, either in itself or through peroxynitrite anion-derived formation of hydroxyl radicals.

In order to test this hypothesis, the nitric oxide synthase inhibitor, N^{ω}-nitro-L-arginine (L-NOARG) was co-incubated with hypoxanthine and xanthine oxidase in order to block any production of nitric oxide by the endothelial cells and therefore prevent any possible formation of peroxynitrite anion. The concentration of L-NOARG used was 30 μM which is sufficient, for instance, to block nitric oxide production in the endothelium of both rabbit aortic rings and rat perfused mesentery (Moore *et al.*, 1990). L-NOARG was found, however, to have no effect on the resultant increase in albumin transfer across BAEC monolayers, indicating that formation of nitric oxide, and therefore by implication formation of peroxynitrite anion through an interaction of nitric oxide with superoxide anion, does not contribute to the endothelial barrier dysfunction induced by the hypoxanthine-xanthine oxidase system. Additionally, L-NOARG was found to have no effect in itself on the basal level of albumin transfer, thereby indicating that nitric oxide plays no role in the regulation of endothelial barrier function.

This conclusion is backed up by the fact that removing the source of available superoxide anion using superoxide dismutase did not inhibit the increase in albumin transfer induced by the hypoxanthine-xanthine oxidase

system, and also by the fact that catalase did inhibit this increase since if peroxynitrite anion were the damaging species produced responsible for the system's effects on endothelial barrier function, then formation of hydrogen peroxide would not be necessary. Also, if peroxynitrite anion-derived formation of hydroxyl radical was responsible for inducing the endothelial barrier dysfunction, then hydroxyl radical scavengers would have been effective and, as has already been described, they were not.

The inability of L-NOARG to inhibit the increase in albumin transfer induced by the hypoxanthine-xanthine oxidase system is therefore internally consistent with previous results in this study. Overall, it can been clearly seen that peroxynitrite, formed through an interaction between endothelium-derived nitric oxide and superoxide anion, does not play a role in mediating the effects of the hypoxanthine-xanthine oxidase system on the endothelial barrier function of BAEC.

7.1.7 Role of hypochlorous acid

Hypochlorous acid is a reactive oxygen species which is a powerful oxidant and which is known to be produced during ischaemia-reperfusion injury by activated neutrophils (Harrison & Schultz, 1976). Neutrophils contain the enzyme myeloperoxidase which catalyses the formation of hypochlorous acid from hydrogen peroxide and chloride ions. It has been shown, additionally, that dithiothreitol can reverse the damaging effects of hypochlorous acid on contractile function of rat cardiac muscle (Eley *et al.*, 1989) and on calcium homeostasis in rabbit ventricular myocytes (Eley *et al.*, 1991).

Since hypochlorous acid represents another mediator which hydrogen peroxide could potentially give rise to, dithiothreitol (3 mM) was co-

incubated with hypoxanthine and xanthine oxidase in order to assess if hypochlorous acid is in any way involved in mediating the actions of the hypoxanthine-xanthine oxidase system on the endothelial barrier function of BAEC. Dithiothreitol, which had no effect on basal levels, was similarly found to have no effect on the level of albumin transfer obtained on addition of hypoxanthine and xanthine oxidase to BAEC monolayers. Therefore, it can be concluded that hypochlorous acid plays no role in mediating the effects of the hypoxanthine-xanthine oxidase system on endothelial barrier function.

7.2 Effects of homocysteine

A high circulating level of homocysteine, as seen in sufferers of homocystinuria, is a recognised risk factor for the development of atherosclerosis (Malinow, 1990). Moreover, various studies have revealed that homocysteine can induce endothelial injury. For instance, visible patches of destruction of aortic endothelium accompanied by the formation of atherosclerotic lesions could be observed following three months of continuous infusion of homocysteine in baboons (Harker *et al.*, 1976; Harker *et al.*, 1983). The development of endothelial barrier dysfunction has been suggested to be a key step in the development of atherosclerosis (Ross, 1986). However, it has not been established whether the ability of homocysteine to cause an increase in the incidence of atherosclerosis in sufferers of homocystinuria is linked to an ability to induce endothelial barrier dysfunction.

7.2.1 Effects on albumin transfer

When added alone to monolayers of BAEC, homocysteine was found to have no effect on the level of albumin transfer obtained. Thus,

homocysteine, itself, would appear not to directly induce endothelial barrier dysfunction.

Starkebaum & Harlan (1986) demonstrated, however, that homocysteine-induced cytotoxicity, determined by ^{51}Cr release from both human umbilical vein and bovine aortic endothelial cells, was dependent on the presence of copper to catalyse the oxidation of homocysteine to its dimeric form, homocystine. They also showed that this oxidation could be catalysed using caeruloplasmin, the form in which copper is predominately found in the circulation, as well as by normal human serum which contains caeruloplasmin. These results indicate, therefore, that it is perfectly conceivable that this oxidation of homocysteine can occur readily in the circulation *in vivo*.

Thus, the effects of homocysteine, in the presence of copper added conveniently in the form of copper sulphate, were subsequently investigated in this study. When copper sulphate (5 and 50 μM), which itself did not affect albumin transfer, was co-incubated with homocysteine (0.5 and 1.5 mM), a significant increase in albumin transfer across monolayers of BAEC was indeed observed to result. Thus, it would appear that endothelial barrier dysfunction is induced by the copper-catalysed oxidation of homocysteine, as opposed to occurring through a direct action of homocysteine itself.

Within the limited concentration ranges tested in this study, the induction of endothelial barrier dysfunction by the copper-catalysed oxidation of homocysteine was found to be dependent on the concentrations of both homocysteine and copper sulphate used. Standard concentrations of 1.5 mM homocysteine and 50 μM copper sulphate were chosen for subsequent experiments against which the effects of various potential inhibitors were

assessed. In conjunction with the chosen standard concentration of copper sulphate, this concentration of homocysteine afforded a reasonable compromise in that it was sufficient to yield a reproducible response, in terms of its ability to induce an increase in albumin transfer, and yet was not too far removed from the concentration of homocysteine which has been observed in the circulation of homozygotic homocystinuria sufferers, i.e. up to 250 μM (Perry *et al.*, 1967). Whilst this concentration is a around six times less than that used as a standard in this study, an increase in albumin transfer was also observed using a homocysteine concentration as low as 500 μM, only around twice that which has been observed clinically. Also, in this study, the effects of homocysteine on endothelial barrier function were assessed using only a 90 minute incubation with homocysteine. In the *in vivo* situation where homocysteine is continually present within the circulation, it is quite conceivable that a lower concentration, such as that in fact observed of 250 μM or less, would be sufficient over a longer time period to induce a similar effect on endothelial barrier function to that observed in this study *in vitro*.

7.2.2 Role of superoxide anion

Having established that, in the presence of copper sulphate, homocysteine can induce endothelial barrier dysfunction, an investigation was then conducted in an attempt to establish the nature of the damaging species produced on the oxidation of homocysteine to homocystine. The first possible species to be investigated was superoxide anion since it is conceivable that this anion is produced during the oxidation of homocysteine in a similar fashion to its production by the hypoxanthine-xanthine oxidase free radical generating system. Hence, superoxide dismutase was co-incubated with homocysteine and copper sulphate to establish any inhibitory actions it might have. However, when superoxide

dismutase was added, initially at a concentration of 3 U ml^{-1} and subsequently at 6000 U ml^{-1} to fully ensure that any effects of superoxide anion production were eliminated, no inhibition of the homocysteine-copper sulphate-induced increase in albumin transfer was observed.

This would indicate, therefore, that superoxide anion production does not mediate the endothelial barrier dysfunction which is induced on addition of homocysteine and copper sulphate to BAEC monolayers. Starkebaum & Harlan (1986) found that superoxide dismutase was similarly ineffective in inhibiting ^{51}Cr release from BAEC monolayers induced by homocysteine and copper sulphate, thereby agreeing with the conclusion from this study that superoxide anion does not mediate the effects of oxidation of homocysteine.

7.2.3 Role of hydrogen peroxide

Using a previously described assay for the production of hydrogen peroxide assessed by its ability to stimulate the horseradish peroxidase-mediated oxidation of fluorescent scopoletin (Root *et al.*, 1975), Starkebaum & Harlan (1986) demonstrated the production of hydrogen peroxide from homocysteine and copper sulphate. Additionally, it has already been established in this study and in others (Wilson *et al.*, 1990; Yamada *et al.*, 1990b; Siflinger-Birnboim *et al.*, 1992) that hydrogen peroxide can induce an increase in macromolecular transfer across endothelial cell monolayers. In order, therefore, to investigate in this study if the endothelial barrier dysfunction induced by homocysteine and copper sulphate occurs as a consequence of the production of hydrogen peroxide, catalase was co-incubated with homocysteine and copper sulphate to investigate its effects on the increase in albumin transfer across BAEC monolayers.

Catalase (1–100 U ml^{-1}) was found to abolish the increase in albumin transfer induced by homocysteine and copper sulphate thereby suggesting, as with the hypoxanthine-xanthine oxidase system, that hydrogen peroxide plays a key role in mediating the system's effects on endothelial barrier function. This finding is in agreement with the observations of Starkebaum & Harlan (1986) who found that catalase inhibited the ^{51}Cr release which was induced by homocysteine and copper sulphate from monolayers of BAEC.

7.2.4 Role of hydroxyl radicals

As with the hypoxanthine-xanthine oxidase system, the possibility existed that hydrogen peroxide was not the sole damaging species produced which mediated the actions of the copper-catalysed oxidation of homocysteine on endothelial barrier function. Hydroxyl radicals which have been implicated in previous studies as mediators of the injurious actions of hydrogen peroxide (Starke & Farber, 1985; Britigan *et al.*, 1992), in the presence of a source of both superoxide anion and ferric iron (Beauchamp & Fridovich, 1970), may again be the damaging species responsible for inducing endothelial barrier dysfunction. Their involvement in the actions of the copper-catalysed oxidation of homocysteine had, however, not been previously investigated. In order, therefore, to investigate an involvement of hydroxyl radicals in this study, the effects of co-incubating homocysteine and copper sulphate with both hydroxyl radical scavengers and with the iron-chelator, deferoxamine, were studied.

The first hydroxyl radical scavenger to be investigated, mannitol (15 mM), was found to be without effect on the increase in albumin transfer across monolayers of BAEC induced by incubation with homocysteine and copper sulphate. Since mannitol is a membrane-impermeant scavenger, this would

therefore indicate that extracellularly produced hydroxyl radicals do not contribute to the development of the endothelial barrier dysfunction induced by homocysteine and copper sulphate.

When the membrane-permeant hydroxyl radical scavenger, dimethylthiourea (1 mM), was similarly co-incubated with homocysteine and copper sulphate, it too was found to have no effect on the resultant increase in albumin transfer. This would therefore confirm the result obtained for mannitol, and would further suggest that, in addition to there being no role played by extracellular generation of hydroxyl radicals, there is also no role played by intracellular generation of these radicals in mediating the endothelial barrier dysfunction induced by the actions of homocysteine and copper sulphate.

When independently assessing a role played by hydroxyl radicals, by employing an overnight pretreatment with the iron chelator, deferoxamine (500 μM), to inhibit any production of hydroxyl radicals occurring through the ferric-iron catalysed Fenton reaction, there was again found to be no resultant inhibition of the increase in albumin transfer induced by the copper-catalysed oxidation of homocysteine. This therefore backs up the two previous findings for mannitol and dimethylthiourea in indicating that there would appear to be no involvement of hydroxyl radical production in the generation of the effects of homocysteine and copper sulphate on endothelial barrier function.

7.3 Conclusions

In conclusion, the ability of the hypoxanthine-xanthine oxidase system to generate endothelial barrier dysfunction has been confirmed in this study using an *in vitro* model of the endothelial barrier in which albumin

transfer across monolayers of BAEC was measured. Since both activation of the hypoxanthine-xanthine oxidase system and the induction of endothelial barrier dysfunction has been observed *in vivo* following ischaemia and reperfusion, it is therefore conceivable that the system is a major cause of endothelial barrier dysfunction following ischaemia-reperfusion injury.

When certain experiments were repeated using xanthine in place of hypoxanthine when added in combination with xanthine oxidase, similar profiles of response were obtained. Although using xanthine potentially only offers half the opportunity for superoxide anion and hydrogen peroxide production as that afforded by hypoxanthine, no difference was in fact observed since at the concentrations used both xanthine and hypoxanthine were present in excess. Therefore, it would seem that at the concentrations employed, both xanthine and hypoxanthine may equally well be used as substrates for xanthine oxidase when assessing the effects of the system on endothelial barrier function *in vitro*.

It has been shown, in this study, that the endothelial barrier dysfunction induced by the hypoxanthine-xanthine oxidase system does not arise as a consequence of the production of superoxide anion, hydroxyl radical, peroxynitrite anion or hypochlorous acid, but that there is a clear requirement for the generation of hydrogen peroxide. This requirement was revealed by the ability of catalase to inhibit the endothelial barrier dysfunction induced by hypoxanthine and xanthine oxidase. It is likely, therefore, that hydrogen peroxide is the sole mediator of this endothelial barrier dysfunction.

In confirmation of this conclusion, exogenously added hydrogen peroxide was also found to be able to induce endothelial barrier dysfunction as

shown by an increase in albumin transfer across monolayers of BAEC. In particular, a concentration of hydrogen peroxide equivalent to that which could theoretically be generated by hypoxanthine and xanthine oxidase, when added at the concentrations used in this study, was found to induce an increase in albumin transfer which could similarly be inhibited by catalase.

Whilst the injurious effects on endothelial cells of copper-catalysed oxidation of homocysteine have been previously demonstrated (Starkebaum & Harlan, 1986), the effects of this oxidation on endothelial barrier function have not. In this study, however, it has been shown that the copper-catalysed oxidation of homocysteine, rather than a direct action of homocysteine itself, can indeed lead to the induction of endothelial barrier dysfunction as shown by an increase in albumin transfer across monolayers of BAEC. It is possible that dysfunction also occurs *in vivo* in sufferers of homocystinuria and therefore contributes to the increased incidence of atherosclerosis which is observed.

The endothelial barrier dysfunction induced by the copper-catalysed oxidation of homocysteine was observed not to arise as a consequence of the production of superoxide anion or hydroxyl radical, but the production of hydrogen peroxide was clearly required. As with the hypoxanthine-xanthine oxidase system, it is likely that hydrogen peroxide is the sole damaging species produced on the oxidation of homocysteine and is therefore the sole mediator of the endothelial barrier dysfunction which is observed to result.

CHAPTER 8

8.1 Effects of lipopolysaccharide (LPS)

Lipopolysaccharide (LPS), also known as endotoxin, derived from gram-negative bacteria is a common cause of septic shock. Amongst other symptoms, such as most notably profound hypotension and hyporeactivity to vasoconstrictors, septic shock is characterised by profound vascular leakage resulting from the development of endothelial barrier dysfunction. Whilst increasing evidence has indicated that both the hypotension (Rees *et al.*, 1990; Thiemermann & Vane, 1990; Kilbourn *et al.*, 1990b; Nava *et al.*, 1991; Wright *et al.*, 1992) and hyporeactivity to vasoconstrictors (Fleming *et al.*, 1991; Gray *et al.*, 1991; Vallance *et al.*, 1992; Szabó *et al.*, 1993) result from the generation of nitric oxide, the mediator of the endothelial barrier dysfunction of septic shock has not as yet been identified. The possibility, however, that this mediator might also be nitric oxide has not, so far, been investigated.

8.1.1 Effects on albumin transfer

Following a 24 hour incubation period, after which the transfer of albumin across monolayers of BAEC was measured during 90 minutes, LPS (0.1–1000 ng ml^{-1}) was found to stimulate an increase in albumin transfer in a concentration-dependent manner. This would indicate that the ability of LPS to induce endothelial barrier dysfunction can be demonstrated *in vitro* using the model of the endothelial barrier which was employed in this study. Additionally, this finding demonstrates that LPS can cause endothelial barrier dysfunction through a direct action on the endothelial cells themselves. The presence of other cell types is therefore not necessary

to facilitate the mediation of the effects of LPS on endothelial barrier function, however, *in vivo*, other cell types may play a contributory role.

This ability of LPS to induce endothelial barrier dysfunction is in accord with previous findings of other workers. Meyrick *et al.* (1986) showed, for instance, that following 24 hours' exposure, LPS similarly caused a concentration-dependent increase in both the water and albumin permeability of cultured monolayers of bovine pulmonary artery endothelial cells. Induction of endothelial barrier dysfunction by LPS has also been observed *in vivo*. Using intravenously administered horseradish peroxidase as an indicator of endothelial barrier function of rats which had previously been injected with LPS for a specific time period, Penn & Chisolm (1991) demonstrated that LPS induced a significant increase in the accumulation of horseradish peroxidase within the intima and media of the aorta. This implied that LPS had induced dysfunction of the endothelial barrier which had therefore enabled the horseradish peroxidase to cross the endothelial layer and accumulate within the underlying intima and media. Kang & Williams (1991) similarly demonstrated the ability of LPS to increase endothelial permeability in rat aortas *in vivo* using the horseradish peroxidase assay.

In order to determine the time-course of the development of the increase in albumin transfer observed in this study in response to incubation with LPS, a standard concentration of 30 ng ml^{-1} was chosen for subsequent investigation. This concentration of LPS was found to generate a reproducible increase in albumin transfer and was also sufficiently sub-maximal so as to allow the effects of various potential inhibitors of its action to be observed in subsequent experiments.

When albumin transfer across monolayers of BAEC was measured following incubation with this chosen standard concentration of LPS for various time intervals, a biphasic time-course was revealed with an early, transient peak being observed that was maximal around after 2 hours' exposure to LPS. A later, sustained peak was also observed and this appeared to near a maximum by 24 hours. Incubation periods longer than 24 hours were not, however, investigated in order to in fact confirm this.

Co-incubation with polymixin B, which inhibits the actions of LPS by binding the Lipid A portion of the LPS molecule with high affinity (Morrison & Jacobs, 1976), was found, as would be expected, to abolish the increases in albumin transfer which were induced by LPS following both 2 hours' and 24 hours' incubation. Previous investigations have revealed that the immunological activity of LPS is mediated by the Lipid A portion of the molecule (Lüderitz *et al.,* 1973). The findings in this study for the actions of polymixin B would therefore suggest that the Lipid A portion of the LPS molecule is also responsible for mediating its effects on endothelial barrier function.

Interestingly, the biphasic nature of the time-course of the development of endothelial barrier dysfunction induced by LPS, in many ways parallels the time-course which has been observed *in vivo* for the development of the hypotension also induced by LPS. Wright *et al.* (1992) showed, for instance, that administration of LPS to the anaesthetised rabbit produced a biphasic fall in both mean arterial pressure and regional blood flow. The initial, transient phase of the response was observed to occur immediately following injection of LPS and recovery was achieved within a 10 minute period. The second phase of the response began to develop approximately 30 minutes following injection and consisted of a slow progressive fall in mean arterial pressure which was sustained throughout the duration of the experiment.

Additionally, Szabó *et al.* (1993) demonstrated, that whilst the hyporeactivity to the vasoconstrictor actions of noradrenaline in LPS treated rats was evident within 1 hour of LPS administration, the regulation of this hyporeactivity at 1 hour differed from the regulation of the hyporeactivity which was observed after 3 hours. Although both components were found to be dependent on the generation of nitric oxide, results showed that at 1 hour this was due to stimulation of a constitutive isoform of nitric oxide synthase whilst at 3 hours, nitric oxide production occurred as a result of the induction of an inducible isoform. This was indicated by the ability of dexamethasone to abolish the hyporeactivity observed at 3 hours but not that observed at 1 hour.

8.1.2 Role of the L-arginine-nitric oxide system

Since activation of the L-arginine-nitric oxide system has been shown to be responsible for mediating both the hypotension and the hyporeactivity to vasoconstrictors which is induced by LPS in animal models of septic shock, the possibility that activation of the L-arginine-nitric oxide system is also responsible for the endothelial barrier dysfunction induced by LPS was therefore investigated in this study. Initial investigation involved co-incubation of LPS with 3 different inhibitors of nitric oxide synthase to establish their ability to inhibit the effects of LPS on albumin transfer across monolayers of BAEC.

The first of these nitric oxide synthase inhibitors, N^{ω}-nitro-L-arginine (L-NOARG), was assessed for its ability to inhibit the increase in albumin transfer which was stimulated by 24 hours' incubation with LPS. The concentration of L-NOARG used was 100 μM which is sufficient, for instance, to block nitric oxide production in the endothelium of both rabbit aortic rings and rat perfused mesentery (Moore *et al.*, 1990). This concentration

of L-NOARG was, however, found to have no effect on the increase in albumin transfer which was induced by LPS in this study.

Since L-NOARG is relatively insoluble in aqueous solution, it could only be tested at a maximum concentration of 100 μM. In order, therefore, to assess the effects of higher concentrations so as to rule out the possibility that its ineffectiveness was due to the fact that it had not been added at a sufficiently high concentration, further experiments were conducted using its more soluble methyl ester form, N^{ω}-nitro-L-arginine methyl ester (L-NAME). This enabled the concentration tested to be increased to 500 μM. Additionally, the effects of L-NAME were assessed against the increase in albumin transfer induced by 2 hours' exposure to LPS as well as that induced by 24 hours' exposure. L-NAME was, however, found to be similarly ineffective to L-NOARG in terms of its ability to inhibit the increase in albumin transfer induced by LPS at both these time-points.

The third nitric oxide synthase inhibitor studied, N^{ω}-monomethyl-L-arginine (L-NMMA), was added at an even higher concentration of 2 mM. It also did not inhibit the increases in albumin transfer induced by incubation with LPS for either 2 hours or 24 hours and, indeed, it was found to induce a statistically significant enhancement of the level of albumin transfer obtained at both time-points.

Taking the results for all three nitric oxide synthase inhibitors together, it would quite clearly appear that generation of nitric oxide is not responsible for mediating the effects of LPS on endothelial barrier function at either of the two time-points investigated, i.e. 2 hours and 24 hours. Indeed, when taking into account the results obtained for L-NMMA, it is possible that basal nitric oxide production might play a role in inhibiting the LPS-induced endothelial barrier dysfunction. However, the fact that this enhancement

was only observed using L-NMMA, and not when using L-NOARG or L-NAME, might suggest that it occurs through some action other than inhibition of nitric oxide synthase which is specific to L-NMMA. Another possible explanation for the differential effects of L-NMMA compared to the effects of both L-NOARG and L-NAME is that the enhancement is concentration-dependent with the concentrations of L-NOARG and L-NAME, which were considerably less than that employed for L-NMMA, being insufficient for this effect to be observed.

These results clearly conflict with the observed effects of the actions of nitric oxide synthase inhibitors on the hypotension induced by LPS both *in vivo* and *in vitro*. L-NMMA has been shown, for instance, to reduce the hypotension induced by LPS in both rat (Thiemermann & Vane, 1990; Nava *et al.*, 1991; Nava *et al.*, 1992) and dog (Kilbourn *et al.*, 1990b). Additionally, both L-NMMA and L-NAME were observed to increase blood pressure in human sufferers of septic shock after conventional therapy for hypotension had failed (Petros *et al.*, 1991).

Nitric oxide synthase inhibitors have also been found to be effective in inhibiting LPS-induced hyporeactivity to vasoconstrictors. L-NAME was observed to inhibit the hyporeactivity to noradrenaline which was observed *in vitro* in aortic rings obtained from LPS-treated rats (Fleming *et al.*, 1991) and *in vivo* in anaesthetised LPS-treated rats (Gray *et al.*, 1991; Szabó *et al.*, 1993). Also, L-NMMA was found to inhibit the hyporeactivity to the thromboxane-mimetic, U-46619, which was induced *in vitro* in rings of jugular veins obtained from LPS-treated rabbits (Vallance *et al.*, 1992) as well as the hyporeactivity to noradrenaline which was observed *in vivo* in anaesthetised rats (Gray *et al.*, 1991).

Also, in contrast to the results observed in this study, Palmer *et al.* (1992) found that LPS-induced cell death of porcine aortic endothelial cells was inhibited by incubation with L-NMMA. This would suggest that LPS-induced cytotoxicity of endothelial cells, measured as cell death, is mediated by nitric oxide. However, this cytotoxic response was only evident using a standard stimulus of 10 μg ml^{-1} LPS which was more than 300 times greater than the standard concentration of LPS, i.e. 30 ng ml^{-1}, used in this study to induced endothelial barrier dysfunction. Cytotoxicity is perhaps a less physiologically relevant means by which to assess the effects of LPS on the vascular endothelium.

In order to independently investigate the involvement of nitric oxide production through stimulation of an inducible isoform of nitric oxide synthase in the actions of LPS on endothelial barrier function, the effects of the glucocorticoid, dexamethasone were subsequently studied. Dexamethasone has been shown to prevent the induction of Ca^{2+}-independent inducible isoforms of nitric oxide synthase (Knowles *et al.*, 1990; Rees *et al.*, 1990), but has no effect on the activity of either constitutive or inducible isoforms (Moncada, 1992).

In initial experiments, dexamethasone (1 μM) was added as a 30 minute pretreatment prior to addition of LPS and was found to have no effect on the increase in albumin transfer across monolayers of BAEC stimulated by 24 hours' incubation with LPS. Since it was possible, however, that a longer pretreatment was necessary in order for dexamethasone to establish its effects, further experiments were conducted using a 20 hour pretreatment prior to the addition of LPS. Despite this substantially longer pretreatment, however, dexamethasone was still found to be ineffective in inhibiting the increase in albumin transfer induced by a 24 hour incubation with LPS and was, additionally, also found to have no effect on the increase in albumin

transfer induced by a 2 hour incubation with LPS. These results would therefore indicate that induction of an inducible isoform of nitric oxide synthase is not required in order to establish the effects of LPS on endothelial barrier function.

As with the findings for the nitric oxide synthase inhibitors tested in this study, these findings for the actions of dexamethasone would appear to be in conflict with previously reported effects of the glucocorticoid on both the hypotension and the hyporeactivity to vasoconstrictors induced by LPS. For instance, Rees *et al.* (1990) showed that the gradual loss of tone which was observed in rat aortic rings, and which they attributed to LPS present in the buffer in which the rings had been placed, was prevented by prior addition of dexamethasone. The hyporeactivity to U46619 observed in hearts obtained from LPS-treated rabbits was similarly prevented by prior treatment with dexamethasone (Smith *et al.,* 1991), as was the hyporeactivity to noradrenaline observed in anaesthetised rats 60 minutes after treatment with LPS (Szabó *et al.,* 1993). The hyporeactivity induced immediately following administration of LPS to anaesthetised rats was, however, unaffected by dexamethasone treatment suggesting that only the late phase of the hyporeactivity response is mediated by induction of an inducible isoform of nitric oxide synthase. Dexamethasone was also found to inhibit LPS-induced killing of porcine aortic endothelial cells (Palmer *et al.,* 1992) suggesting that induction of an inducible isoform of nitric oxide synthase is also involved in mediating the cytotoxic actions of LPS on the vascular endothelium.

Taking the results obtained in this study for the nitric oxide synthase inhibitors and dexamethasone together, it would seem clear that nitric oxide production does not mediate the endothelial barrier dysfunction which is induced by LPS. This would appear to be in contrast to LPS-

induced hypotension and hyporeactivity to vasoconstrictors, both of which have been shown to depend upon the production of nitric oxide. Moreover, LPS has been shown to stimulate the immediate release of nitric oxide from BAEC (Salvemini *et al.*, 1990) and it is therefore likely that generation of nitric oxide through activation of a constitutive nitric oxide synthase in the vascular endothelium mediates the immediate phase of both the hypotension and the hyporeactivity to vasoconstrictors induced by LPS. LPS has also been shown to induce nitric oxide production through stimulation of an inducible nitric oxide synthase in the smooth muscle of the vascular wall (Fleming *et al.*, 1990; Knowles *et al.*, 1990) and therefore it is likely that this nitric oxide production mediates the dexamethasone-sensitive late, sustained phases of both the hypotension and the hyporeactivity to vasoconstrictors which are induced by LPS. Since the increases in albumin transfer across BAEC observed in this study were found, in contrast, to be unaffected by both nitric oxide synthase inhibitors and pretreatment with dexamethasone, it would appear that nitric oxide production, through either a constitutive or an inducible nitric oxide synthase, is not in any way responsible for the effects of LPS on endothelial barrier function. Furthermore, other work in this laboratory using a chemiluminescence technique to measure nitrite accumulation as an indicator of nitric oxide production, showed no evidence of the induction of nitric oxide synthase in BAEC following 24 hours' incubation with LPS (Berman *et al.*, 1993). LPS-induced endothelial barrier dysfunction would therefore appear to be mediated by a different mechanism from that which mediates both the hypotension and the hyporeactivity to vasoconstrictors which are also induced by LPS.

8.1.3 Role of prostaglandins

Prostaglandin release, particularly release of prostaglandin E_2 and prostacyclin, has been observed *in vivo* during septic shock (Petrak *et al.,* 1989) and prostaglandins have been shown to contribute to the pathogenesis of septic shock (Parratt & Sturgess, 1975). Additionally, vasodilator prostaglandins including prostaglandins E_1, E_2 and G_2 have been shown *in vivo* to enhance both histamine- and bradykinin-induced plasma exudation in rabbit skin (Williams & Peck, 1977). This effect was linked to their vasodilator actions which result in an increased hydrostatic pressure and an increase in the area of vessel wall available for plasma exudation. Another cyclo-oxygenase product, thromboxane A_2, has also been shown to increase endothelial permeability *in vivo* (Petrak *et al.,* 1989).

In order, therefore, to establish if prostaglandin production plays a role in mediating the increase in albumin transfer across BAEC induced by 24 hours' incubation with LPS, the effect of co-incubation of LPS with the cyclo-oxygenase inhibitor, flurbiprofen (30 μM), was investigated. Flurbiprofen was, however, found to have no effect on the increase in albumin transfer stimulated by LPS. This indicates that production of prostaglandins and other cyclo-oxygenase products is not responsible for mediating the effects of LPS on endothelial barrier function following a 24 hour incubation with LPS.

8.1.4 Role of hydrogen peroxide

Hydrogen peroxide has been shown, in this study and in others (Wilson *et al.,* 1990; Yamada *et al.,* 1990b; Siflinger-Birnboim *et al.,* 1992), to have the ability to induce endothelial barrier dysfunction as

shown by an increase in macromolecular transport across endothelial monolayers. Additionally, it has been shown in this study to be a key mediator of the endothelial barrier dysfunction induced by both the hypoxanthine-xanthine oxidase system and the copper-catalysed oxidation of homocysteine. In order, therefore, to investigate the possibility that hydrogen peroxide is also the mediator of the endothelial barrier dysfunction induced by LPS, the effect of catalase on the LPS-stimulated increase in albumin transfer across monolayers of BAEC was investigated.

Catalase which was added at a concentration 1000 U ml^{-1} was found to have no effect on the increase in albumin transfer obtained following incubation of BAEC monolayers with LPS for 24 hours. This concentration of catalase had previously been found in this study to be sufficient to abolish the increase in albumin transfer induced by both the hypoxanthine-xanthine oxidase system and by either concentration (0.1 mM and 10 mM) of hydrogen peroxide tested. Therefore, it would appear that hydrogen peroxide is not responsible for mediating the observed effects of LPS on endothelial barrier function at the later time-point which was investigated in this study.

8.2 Effects of interferon-γ (IFN-γ)

Various cytokines including interleukins, TNF-α and IFN-γ are released during septic shock (Bone, 1991a) and many of these have been observed to increase endothelial permeability both *in vivo* and *in vitro.* For example, interleukin-2 was found to increase albumin transfer across ovine aortic endothelial cells (Bechard *et al.,* 1990), whilst interleukins-1α and -1β have been shown to increase albumin transfer across monolayers of both bovine pulmonary artery endothelial cells (Campbell *et al.,* 1992) and human umbilical vein endothelial cells (Burke-Gaffney & Keenan,

1993a). TNF-α has also been observed to increase albumin, inulin and sorbitol transfer across monolayers of BAEC (Brett *et al.*, 1989) as well as albumin transfer across monolayers of bovine pulmonary artery (Goldblum & Sun, 1990; Wheatley *et al.*, 1993) and human umbilical vein (Camussi *et al.*, 1991; Burke-Gaffney & Keenan, 1993a; Burke-Gaffney & Keenan, 1993b) endothelial cells.

IFN-γ has been shown to alter cell shape and morphology when incubated with monolayers of human umbilical vein endothelial cells (Stolpen *et al.*, 1986). Additionally, LPS has been shown to induce an up-regulation of IFN-γ expression in mice (Cockfield *et al.*, 1993). Thus it is possible that IFN-γ in fact mediates the endothelial barrier dysfunction which is induced by LPS and, hence, the effects of IFN-γ on albumin transfer across monolayers of BAEC were investigated in this study.

When albumin transfer was measured across BAEC monolayers subsequent to 24 hours' incubation with human recombinant IFN-γ (0.75–75 U ml^{-1}) no increase above control levels was, however, observed. This finding would seem to indicate, therefore, that IFN-γ does not act directly on endothelial cell monolayers to induce endothelial barrier dysfunction.

Beynon *et al.* (1993) similarly found that human recombinant IFN-γ (125 U ml^{-1}) did not induce endothelial barrier dysfunction using human umbilical vein endothelial cells across which the transfer of anti-FITC antibody was measured. However when IFN-γ was subsequently added in combination with interleukin-4, which itself did not affect anti-FITC antibody transfer, a significant increase in transfer was found to result, thus showing that IFN-γ can act synergistically with interleukin-4 to induce endothelial barrier dysfunction.

In contrast to these findings, as well as those in this study, Burke-Gaffney & Keenan (1993a) demonstrated the ability of human recombinant IFN-γ to stimulate an increase in albumin transfer across monolayers of human umbilical vein endothelial cells. This increase was attained following an incubation period with IFN-γ of 20 hours and could be observed using an IFN-γ concentration of 25 U ml^{-1} which was within the concentration-range investigated in this study, i.e. 0.75–75 U ml^{-1}. The only major difference between their study and this was the fact that they used human venous endothelial cell monolayers to assay the effects of human recombinant IFN-γ, whereas in this study bovine arterial endothelial cell monolayers were employed to assay the effects of human recombinant IFN-γ. Thus, it is possible that the reason for the failure of IFN-γ to increase albumin transfer across BAEC monolayers in this study, was due to an insufficiency in species morphology between the human recombinant IFN-γ and the bovine endothelial cells used. Alternatively, it is possible that IFN-γ is only able to increase the albumin transfer across venous endothelium and not across arterial endothelium. More simply, this difference might occur because IFN-γ receptors are present in human umbilical vein endothelial cells whilst they may be lacking in BAEC. These suggestions, however, do not explain the fact that Beynon *et al.* (1993) failed to observe an ability of IFN-γ to induce endothelial barrier dysfunction in human umbilical vein endothelial cell monolayers when added alone.

8.3 Conclusions

In conclusion, the ability of LPS to induce endothelial barrier dysfunction through a direct action on the vascular endothelium has been confirmed in this study by the ability of LPS to stimulate an increase in albumin transfer across monolayers of BAEC. This increase in albumin transfer was found to be concentration-dependent and exhibited a biphasic time-course

which in many ways parallels the profiles of both the hypotension and the hyporeactivity to vasoconstrictors which have been demonstrated to be induced by LPS in both *in vivo* and *in vitro* models of septic shock.

Evidence now suggests that the early phases of both the hypotension and the hyporeactivity to vasoconstrictors which are induced by LPS are mediated by nitric oxide production through stimulation of a constitutive nitric oxide synthase in the vascular endothelium, whilst in both cases, the late phase would appear to be mediated by nitric oxide production through induction of an inducible nitric oxide synthase in the smooth muscle of the vascular wall. In contrast, no evidence was found in this study for the involvement of nitric oxide production in either the early or late phase of the endothelial barrier dysfunction induced by LPS in BAEC monolayers. This was demonstrated by the inability of three nitric oxide synthase inhibitors, as well as dexamethasone, to inhibit either phase of the LPS-induced endothelial barrier dysfunction. It would therefore, appear that this endothelial barrier dysfunction is mediated by a different mechanism from that which mediates both the hypotension and the hyporeactivity to vasoconstrictors which are induced by LPS.

Additionally, it was observed that the late phase of the endothelial barrier dysfunction induced by LPS was not mediated by either a cyclo-oxygenase product, or by hydrogen peroxide. This was indicated by the inability of both flurbiprofen and catalase to inhibit the LPS-induced endothelial barrier dysfunction which was obtained following 24 hours' incubation with LPS.

It is also unlikely that the late phase of the response to LPS observed in this study is mediated by IFN-γ. When the effects of IFN-γ were independently investigated, it was found to have no effect on the endothelial barrier function of BAEC monolayers following a 24 hour incubation.

In addition to IFN-γ, many substances which may affect endothelial barrier function have been shown to be released during the sepsis cascade including TNF-α, interleukins, PAF, leukotrienes and thromboxane A_2 (Bone, 1991a). Further investigation is therefore required in order to establish in any of these agents, or indeed any other agents, are involved in mediating the observed effects of LPS on endothelial barrier function and which may therefore be responsible for the production of the profound vascular leakage which is observed in septic shock.

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