The effects of inflammatory mediators on endothelial and smooth muscle function *in vivo*

A thesis presented for the degree of Doctor of Philosophy in the Faculty of Medicine of the University of London

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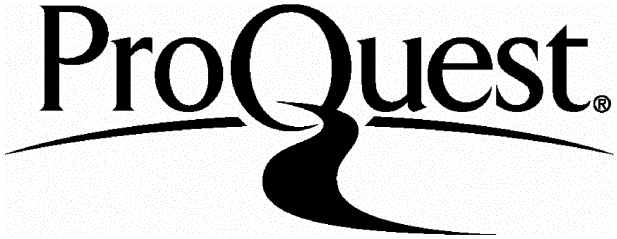

To Valima, Bhagubhai, Jasuben and Nita

For feeling without saying and for saying with feeling...

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Abstract

Inflammation of the blood vessel wall has been implicated in the pathogenesis of diseases as apparently diverse as septic shock, unstable angina and acute myocardial infarction. Most studies exploring the effects of inflammatory signals on vascular reactivity have been undertaken in animals or using human cells or tissues *in vitro*. However, it is recognised that there is considerable species variation in the mechanisms of inflammation and that the results of studies *in vitro* differ from those undertaken *in vivo*. This thesis describes the development and application of a novel approach to administer inflammatory mediators to volunteers *in vivo* in order to determine any changes in vascular reactivity seen. A single vessel was isolated from the rest of the circulation and exposed to inflammatory agents. The change in the reactivity was then assessed with the vessel in its normal physiological environment. Bacterial endotoxin and certain pro-inflammatory cytokines were used to initiate an inflammatory response. Changes in endothelial and smooth muscle function were assessed and the contribution of nitric oxide or prostanoids determined. In some studies surgical biopsies were taken in order to characterise the molecular basis of the pharmacological changes. Parallel experiments were also undertaken to study the effects of inflammation in a variety of *in vitro* systems, using human cells and tissues.

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Chapter 1

Introduction

Our forebearers were well conversant with notion of inflammation and inflammatory disease. An Egyptian inscription allegedly depicting the latter is thought to be derived from the hieroglyph for 'fire' and this similarity between heat and inflammation seems to have been one which impressed itself strongly upon the minds of ancient scientists and physicians (Smith, 1978). For example, in Greek Hippocratic writings the term *phlegomone* - fiery heat - was used when describing disorders thought to be due to an influx of blood into a normally 'bloodless' area. Indeed, the very name 'inflammation' is derived from the Latin word *flamma* for fire. In some cases it was believed that, in filling the vascular tissue, the new blood forced out other substances such as *pneuma* giving rise to oedema or with *black bile* thus giving rise to cancers (Galen). It was in fact a Roman medical encyclopaedist, Celsus, living in 30A.D. who first coined the phrase 'heat, swelling, redness and pain (calor, tumour, rubor and dolor) to describe the cardinal signs of inflammation - a phrase which will be found, in many textbooks of pathology. All of these descriptions have one feature in common: an increase in blood flow due to vasodilatation.

It was probably the English surgeon and naturalist John Hunter who provided us with the first really useful insight into the inflammatory process itself (Jarcho, 1970). His view was that inflammation was the response of the body to a disease or injury and not disease in itself. He believed that inflammation was inseparable in many ways from the healing process. Another major step forward was made by people such as Addison (1802-1881) and Waller (1816-

1870) when they identified the importance of white corpuscles in inflammation - work which was eventually developed by Virchow (1871) and later by Metchnikoff (1892) in their work on phagocytosis (1892).

Features of inflammation

Inflammation is the reaction of the vascular and supporting elements of a tissue to injury, and results in the formation of a protein-rich exudate, provided the injury has not been so severe as to destroy the area. All the changes characteristic of acute inflammation (***pain, swelling, heat and redness***) involve an increase in blood flow into the injured area - vasodilatation. Each of these features is briefly described below.

Pain

Pain is a characteristic (but not universal) feature of inflammation and is thought to be due to activation of sensory nerves, or a change in the threshold for activation of sensory nerves. Sensory fibres also possess other properties.

In 1937 Sir Thomas Lewis described the changes he saw during acute injury to the skin. He showed that spreading hyperalgesia around the skin injury was due to a local nervous mechanism. It could be delayed for some time by the injection of procaine. A contribution of the nerves emerging via the posterior root system were subsequently shown to be involved, but the effect was

independent of the central nervous system. This 'nocisensor' system was conceived to be a system of nerves that was capable of effecting changes in the skin locally and without reference to the central nervous system. It has now been shown that the local nerves are responsible for the release of, agents such as substance P and calcitonin-gene related peptide (CGRP) (Ahluwalia and Cellek, 1997). The release of these neurotransmitters activate the nocisensor system and ultimately result in the pain often seen during the acute inflammatory response. In addition to the hyperalgesia, these neurotransmitters also cause vasodilatation and increased vessel wall permeability, contributing to the swelling and heat of the acute injury.

Swelling

Another characteristic feature of acute inflammation is the formation of an exudate. The exudate results from the accumulation of protein-rich fluid and an inflammatory cell exudate.

• *Protein-rich exudate*

Arteriolar dilatation increases the flow into an inflamed area and this leads to a rise in hydrostatic pressure. The passage of fluid flow through the vessel wall is determined by hydrostatic and osmotic forces, as defined by Starling in 1895. In acute inflammation the hydrostatic pressure in the capillaries even at the venous end may exceed the osmotic pressure of the plasma protein, and therefore fluid and low molecular weight solutes will tend to pass into the

tissue spaces. However, the permeability of vessels to proteins and certain other small molecules varies considerably from one tissue to another. Thus, for example, the vessels of the brain and thymus are less permeable than those elsewhere, even during acute inflammation. Thus in addition to changes in hydrostatic pressure, a crucial factor in the formation of an inflammatory exudate is an increased permeability of vessel walls to plasma proteins. Examination of the endothelial cells of capillaries in acutely inflamed tissues has revealed several changes - increase in the number and size of pinocytic vesicles, blebs under the luminal cell membrane, and projections or spikes arising from the membrane. Gaps appear in the endothelial lining due to separation of adjacent endothelial cells. Whether other changes occur, for example, in the basement membrane is not clear.

• *Cellular exudate*

The inflamed vessel wall also allows white cells to adhere and migrate through it. Endothelial cells participate in the recruitment of leukocytes to sites of inflammation by secreting chemotactic molecules and by regulating the expression of adhesion molecules. Adhesion of white cells to the endothelium is called margination or pavementation. The molecular mechanisms underlying these events are currently under intensive investigation. Adhesion molecules are glycoproteins whose molecular mass varies from 60-120kDa depending on the degree of glycosylation. They have a single membrane-spanning domain. They are not found on unstimulated endothelial cells and their expression is induced during inflammation. *In vitro*

they first become detectable on the surface of the endothelium 30min after an exposure to endotoxin or pro-inflammatory cytokines. Examples of these adhesion molecules include endothelial-leukocyte-adhesion-molecule-I (ELAM-I (Harlan, 1985; Pober and Cotran, 1991)) and intracellular-adhesion-molecule-I (ICAM-I (Dustin and Springer, 1988)).

After adhesion the leukocytes pass actively by amoeboid motion through the vessel wall (emigration). This movement is related to the effect of a chemical gradient - a phenomenon termed chemotaxis. Finally the white cells may ingest foreign material such as bacteria by phagocytosis.

The predominant cell in the early stage of the acute inflammatory reaction is the neutrophil, while in the later stages monocytes and lymphocytes predominate. In the tissues monocytes become large and phagocytic (macrophages). Neutrophils have been shown to be attracted to a number of agents such as endotoxin (described in more detail later), tumour necrosis factor α (TNFα), interleukin 1β (IL-1β) and platelet activating factor (PAF). Both monocytes and macrophages share common morphological and membrane characteristics which contain receptors for IgE, cytokines and growth factors. Both cell types produce reactive oxygen species, and have a spectrum of lysosomal enzymes, which are similar to the neutrophil. The ability of these activated cells to phagocytose is well known, but in addition they also secrete a wide spectrum of pro-inflammatory mediators including IL-

1β and TNFα and PAF, which activate neutrophils and stimulate mast cell degranulation.

Heat and redness

Both the heat and redness are due to vasodilatation. The first change is an increase in blood velocity due to dilatation of the terminal arterioles Subsequent opening of the postcapillary sphincters allows blood to pass into the capillary bed, and vessels which were temporarily shut down now become functional again. The inflamed part therefore appears to contain an increased number of vessels. In addition, their calibre is increased. It appears that dilatation of the arterial and venous vessels is an important part of the inflammatory response and it is the process of inflammatory vasodilatation that is the subject of this thesis.

Stimulants of acute inflammation

Since the inflammatory reaction is a response to injury, its causes are those of cell damage. These may be:

- ***Mechanical trauma*** - For example cutting and crushing
- ***Chemical injury*** - There are numerous chemicals which injure cells. Many, like corrosive acids, alkalis and phenol, are general protoplasmic poisons. Others are more selective in their action: thus mercuric chloride causes renal

tubular necrosis. Certain body fluids e.g. bile and urine, cause damage when they escape into the tissues.

- ***Radiation injury*** - Heat, ultraviolet light, and all forms of ionising radiation both electromagnetic and particulate fall into this category.
- ***Injury associated with necrosis*** - A good example of this is the inflammatory reaction around an early myocardial infarct. Necrosis caused by hormonal changes is also accompanied by acute inflammation, e.g. the physiological monthly necrosis of the endometrium following the withdrawal of progesterone is associated with inflammation of the deeper parts of the endometrium.
- ***Injury due to organisms*** - This includes infection with living organisms such as viruses, bacteria and parasites and inoculation/injection of part of the organism (endotoxin).
- ***Injury due to an immunological mechanism*** - This includes the diseases mediated by antigen-antibody interactions as well as those produced by effector T cells.
- ***Injury due to cold and heat.***

Many of the mediators involved in effecting this acute inflammatory response (e.g. to heat, radiation and immunological) elicit a response through the recruitment of cytokines. Moreover, many of the inflammatory events that occur during an acute bacteraemia or septicaemia are thought to be mediated by the outer wall of these organisms - exotoxin in the case of Gram-positive bacteria and endotoxin in the case of Gram-negative bacteria.

The studies in this thesis explored the mechanism of vasodilatation following exposure of a blood vessel to endotoxin or cytokines *in vivo*. These agents are discussed in greater detail.

Endotoxin and vasodilatation

Endotoxin is one of the integral components of the outer bacterial lipopolysaccharide cell wall of all Gram-negative bacteria (Rietschel et al. 1994). The pure protein-free lipopolysaccharide molecule can be functionally divided into 3 parts: (i) a highly variable O-polysaccharide side chain that provides the heat-stable serologic specificity of Gram-negative bacteria and is the basis for O-, or somatic, antigen typing scheme; (ii) an oligosaccharide or R-core region composed of approximately 10 monosaccharides; (iii) a unique backbone referred to as lipid A (Hitchcock et al. 1986; Luderitz et al. 1966).

The lipid A component of endotoxin is responsible for most of the molecule's toxicity. This portion of the molecule is highly conserved and appears to be essentially invariable and present in all forms of endotoxin (Luderitz et al. 1966). Several lines of evidence indicate that endotoxin, and in particular lipid A, is the primary exogenous mediator in the development of vasodilatation associated with Gram-negative bacterial infections. Experimental studies have shown that injection of endotoxin results in a constellation of symptoms almost identical to those observed in Gram-negative vascular inflammation (Braude, 1980). Severe vascular wall inflammation and vasodilatation occurs

about 30 min. after injection of endotoxin in dogs and results in eventual vascular collapse (Snell and Parrillo, 1991).

How does endotoxin mediate its effects? Endotoxin is recognised by defensive cells in essentially all multicellular organisms. After this recognition 2 responses that combat bacterial infections are elicited. In the first, phagocytic cells engulf and kill (if the endotoxin is still part of the whole organism) the bacteria, and cells in the liver and spleen pinocytose fragments of shed membrane. In the second response, leukocytes (as well as other cells) respond to endotoxin by secreting a variety of cytokines that heighten the defensive responses of the host. TNFα, IL-1β, and IL-6 are secreted acutely following stimulation of mononuclear phagocytes with endotoxin (Pugin et al. 1993). These substances induce the acute phase vasodilatory inflammatory response and prime the immune system for rapid activity.

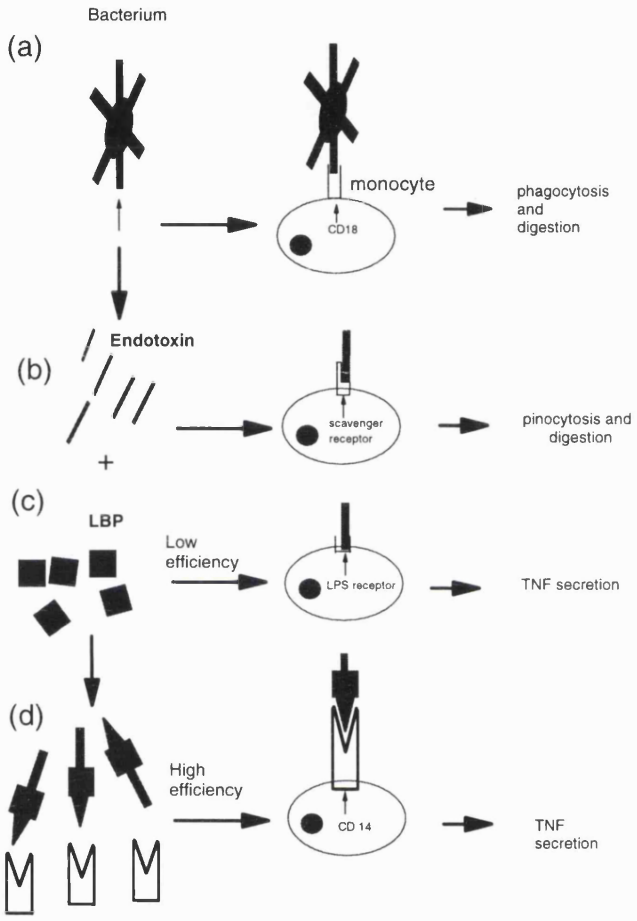
The profile of cytokines secreted in response to endotoxin is now relatively well documented, but the molecules involved in the initial identification of endotoxin are still being discovered. Recent studies have led to the recognition of at least 3 classes of molecules on leukocytes (and in some cases endothelial cells) that are receptors for endotoxin (Figure 1). The CD18 molecules (leukocyte integrins) bind endotoxin and participate in the phagocytic engulfment of bacteria. The scavenger (acetyl-low-density lipoprotein) receptor recognises free circulating endotoxin and mediates its uptake and degradation. A 3rd receptor, CD14, recognises complexes of endotoxin with

the serum protein lipopolysaccharide-binding protein (LBP) (Pearson, 1996; Schletter et al. 1995). CD14 appears to participate in both ingestion of, and synthetic responses to endotoxin because blockade of CD14 with monoclonal antibodies strongly inhibits uptake of endotoxin and secretion of TNFα by human mononuclear cells (Wright, 1991; Pugin et al. 1993). Other endotoxin receptor molecules have also been proposed but further studies are needed to confirm their involvement (Wright, 1991).

The CD14 molecule is also present in the soluble form as it is shedded from cells (Bazil and Strominger, 1991). Soluble CD14 (sCD14) can bind and increase endotoxin-induced activities, such as oxidative burst responses (Schutt et al. 1991) and TNFα production by whole blood cells (Haziot et al. 1994). CD14 may also increase the response to endotoxin by cells that do not normally express this receptor: recent reports have demonstrated that activation of endothelial cells (which do not have cell-bound CD14 receptors) by endotoxin is mediated by sCD14 which can act as a receptor for endotoxin for the endothelial cell (Noel, Jr. et al. 1995). The smooth muscle cell, the other major cell population of the vessel wall also lacks CD14 receptors. Several studies have shown that endotoxin can act directly on smooth muscle cells to result in loss of vascular tone and vasodilatation (Beasley et al. 1990) and again this seems to be due to activation by sCD-14-endotoxin complex (Loppnow et al. 1995).

Figure 1

The role of multiple receptors in the description and response to endotoxin



Endotoxin on the surface of bacteria may be recognised by the CD 18 molecules (a), and dispersions of endotoxin may be recognised by the scavenger receptor (b). These receptors mediate the disposal of endotoxin without initiating synthesis of cytokines such as TNFα or IL-1β. Interaction of endotoxin with lipopolysaccharide binding protein (LBP) yields a complex that can be recognised by CD14 (d). CD14 (which exists as soluble CD14 (sCD14) and cell-membrane bound) mediates both the phagocytic uptake of bacteria and cytokine synthesis in response to very low concentrations of endotoxin-LBP. Because high concentrations of endotoxin have been shown to initiate cytokine synthesis in the absence of LBP or CD14, an additional receptor, apparently of low affinity, may also exist (c).

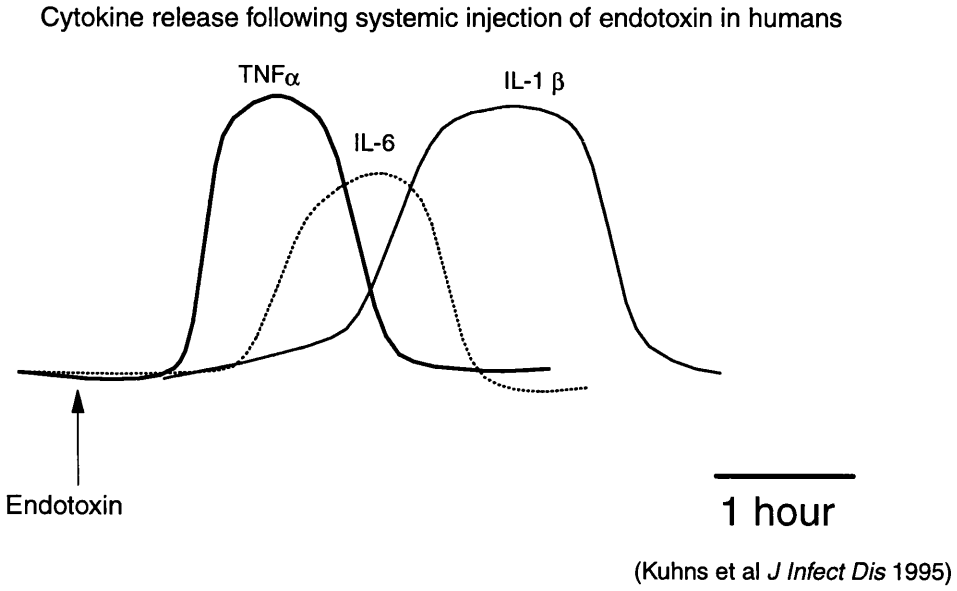
Cytokines and vasodilatation

Cytokines are small proteins, with molecular weights from 8-3KDa, that possess multiple biological activities. They are active in low (picomolar-femptomolar) concentrations. Cytokines are produced primarily in response to external stimuli, for example, to endotoxin and exotoxin. Many pro-inflammatory cytokines are produced in the presence of infection or inflammatory disease and contribute to the immune response, inflammation and endothelial cell activation (Mantovani et al. 1992).

The results of many animal and human studies in which endotoxin has been administered systemically, have characterised the changes in cytokine levels that occur following intravenous injection (Cannon et al. 1990; Hesse et al. 1988; Klosterhalfen et al. 1992; Michie et al. 1988; Kuhns et al. 1995), (Figure 2). During the acute response to endotoxin administration there is a dramatic rise in 3 pro-inflammatory cytokines - TNFα, IL-1β and IL-6 (Hesse et al. 1988; Klosterhalfen et al. 1992; Michie et al. 1988). By 60 min. after endotoxin challenge, levels of TNFα have reached their peak, IL-6 levels increase by 90 min. and IL-1β by 120 min. Using monoclonal antibodies against TNFα, Fong and others (Fong et al. 1989) showed that TNFα is a potent inducer of IL-1β and IL-6 release. Shalaby and others (Shalaby et al. 1989) further suggested that besides endotoxin and TNFα, IL-1β is able to upregulate IL-6. These studies were carried out using a bolus injection of

endotoxin and it is possible, however, that the alterations of cytokine release induced by a persistent septic focus may differ from the changes observed following a bolus injection of endotoxin.

Figure 2



Clinical studies (Hesse et al. 1988; Waage et al. 1989; Waage et al. 1989; Waage et al. 1987) examining serum cytokine levels have demonstrated that TNFα and IL-1β blood levels are significantly elevated in patients with endotoxaemia. In addition, Nijsten and others (Nijsten et al. 1987) described an increase in IL-6 during infectious episodes in humans. However, it should be noted that elevated levels of a particular cytokine in the **systemic** circulation could reflect levels required to induce changes in distal tissues or they may reflect production of cytokine in excess of that needed to initiate physiological responses. Similarly, the lack of detection of a specific cytokine

may result from either a lack of synthesis, the rapid clearance of that cytokine by binding to available receptors in target tissues, rapid renal clearance of cytokine-soluble receptor complexes, or lack of assay sensitivity to detect physiological levels of specific cytokines. This may explain why some studies fail to detect significant levels of IL-1β (or other pro-inflammatory cytokines) in the systemic circulation following injection of endotoxin (Kuhns et al. 1995).

Other changes during a vascular inflammatory event that occur also appear to be associated with a rise in these 3 cytokines. Trauma incurred during major surgical procedures often results in a series of inflammatory responses such as elevation of body temperature, leukocytosis and increased acute phase reactants. Most evidence suggests that these responses are mediated by cytokines such as TNFα, IL-1β and IL-6 (Roumen et al. 1993; Cabie et al. 1993; Baigrie et al. 1992; Baigrie et al. 1991).

When injected into experimental animals, either TNFα or IL-1β induces vasodilatation and shock; moreover, when administered together, this combination acts synergistically (Okusawa et al. 1988). A single intravenous injection of TNFα or IL-1β into patients with cancer induces a sudden fall in blood pressure often requiring treatment (Chapman et al. 1987; Walsh et al. 1992). Healthy volunteers receiving TNFα respond in a similar fashion (van der Poll et al. 1990; van der Poll et al. 1992), whilst an infusion of IL-6 into patients results in fever, chills, and minor fatigue, a significant increase in C-

reactive protein, fibrinogen and platelet counts but no hypotension (van Gameren et al. 1994; Weber et al. 1994; Weber et al. 1993).

The studies in this thesis explored the effects these 3 cytokines on blood vessels *in vivo*. These are discussed in greater detail.

TNFα and vasodilatation

CELLULAR RESPONSE

TNFα is principally a macrophage/lymphocyte-derived cytokine with a broad spectrum of immunoregulatory, metabolic and pro-inflammatory activities (Beutler et al. 1985; Bazzoni and Beutler, 1996). Although other cells (such as hepatic, smooth muscle, microglial and endothelial cells) have been shown to be capable of TNFα production, the major source appears to be the macrophage/lymphocyte cells (Beutler et al. 1985). The native structure of TNFα is a trimer with a total molecular weight of 52 kDa. It interacts with 2 receptors - R1 and R2. Essentially, interaction with R1 mediates cytotoxic responses whereas binding to R2 mediates proliferative responses. These receptors are present on nearly all cell types with the exception of erythrocytes and unstimulated T-lymphocytes. Although the presence of the receptor appears to be a prerequisite for a biological effect, there does not seem to be a correlation between the number of receptors and the magnitude of the response (Vassalli, 1992; Bazzoni and Beutler, 1996). Soluble TNFα binding

proteins have also recently been characterised (Spinas et al. 1992). There are 2 types, antigenically distinguishable and corresponding to the shedded extracellular domains of the 2 species of cell-bound receptor. The presence of soluble TNF-R in the serum may compete and inhibit the binding of TNFα action on cells in addition to affecting the pharmacokinetics and stability of TNFα (Suffredini et al. 1995).

On many cell types, even in the absence of protein production, TNFα causes the release of arachidonic acid and this leads to secretion of prostanoids (Fiers, 1991). Moreover, treatment of endothelial cells with TNFα induces excessive production of PGI_2 and PGE_2, platelet activating factor (PAF) and nitric oxide (NO) (McKenna, 1990; Lamas et al. 1991), all potential vasodilators both *in vitro* and *in vivo*. The addition of TNFα to many cell types induces protein synthesis following gene activation. This has been studied in detail in a number of cell types, including endothelial cells, polymorphonuclear cells, monocytes and lymphocytes, and these cell types are thought to be the main targets when TNFα appears in the circulation (Fiers, 1991; Old, 1985).

VESSEL WALL RESPONSE *IN VITRO* AND *IN VIVO* IN ANIMALS

In vitro studies have shown that vascular aortic rings incubated with TNFα result in increased vasodilatation and hyporesponsiveness to vasoconstrictor agents including α-adrenoceptor agonists (Hollenberg et al. 1991; McKenna et al. 1988). Administration of TNFα to experimental animals in sufficient doses

(dependent on species) leads to generalised effects that include pyrexia, systemic hypotension, pulmonary hypertension and endothelial activation. Characteristically, the haemodynamic response to an infusion of TNFα when administered in quantitites similar to those produced endogenously in reposne to endotoxin is rapid and involves a rapid drop in arterial pressure that often leads to vascular collapse (Mitaka et al. 1994; Tracey et al. 1986; Redl et al. 1993).

STUDIES IN HUMANS

Phase I studies in cancer patients demonstrated that an acute bolus injection of TNFα resulted in flu like symptoms within 60-90 min. (Saks and Rosenblum, 1992). Chronic infusion of this cytokine resulted in anorexia and a leucopaenia (Saks and Rosenblum, 1992). The doses used by Van der Poll and others in healthy volunteers did not appear to cause acute haemodynamic changes although a single injection of TNFα elicited rapid and sustained activation of the common pathway of coagulation, probably induced through the extrinsic route (van der Poll et al. 1990; van der Poll et al. 1991). In addition, Derkx and others in 1995 showed that there appeared to be familial differences in endotoxin-induced TNFα release from circulating blood mononuclear cells following systemic injection of endotoxin in healthy volunteers (Derkx et al. 1995). This suggests the possibility of TNFα gene polymorphism which may influence the inter-individual response to endotoxin challenge.

Studies in which anti-TNFα antibody or TNF-receptor blockade have been used in order to improve the outcome in sepsis have been disappointing. Human trials of TNFα antagonists in sepsis have shown no overall survival benefit (Fisher, Jr. et al. 1993; Reinhart et al. 1996) and one recent trial unexpectedly showed increased mortality (Fisher, Jr. et al. 1996). The lack of efficacy of these inhibitors has not been fully explained but these findings question the concept that therapy directed against TNFα will limit the harmful inflammatory responses that occur during septic shock. Perhaps these studies reflect the complexity and unexpected interactions that may occur following inhibition of a single arm of the inflammatory response and underscores the potential for such approaches in septic shock to be ineffective or harmful (Natanson et al. 1994). They also suggest that failure of these therapies in septic shock could relate to redundant TNFα-independent inflammatory pathways, impairment of protective host inflammatory response, or disruption of the normal sequence of immune activation during an inflammatory response. The results do not exclude the possibility that TNFα antagonism may have beneficial effects in noninfectious inflammatory diseases such as rheumatoid arthritis (Elliott et al. 1994) or may be of use as a prophylactic agent in preventing septic shock. However, the results in healthy volunteers and patients suggests that TNFα may not be a major cytokine inducing vascular collapse in humans.

IL-1β and vasodilatation

CELLULAR RESPONSE

The IL-1 gene family is comprised of IL-1α, IL-1β and IL-1 receptor antagonist (IL-1Ra (Dinarello, 1994)). Each member is synthesised as a precursor protein; the precursors for IL-1 (pro-IL-1α and proIL-1β) have molecular weights of 31 kDa. The proIL-1α and mature 17 kDa IL-1α are both biologically active whereas the proIL-1β requires cleavage to a 17 kDa peptide for optimal biological activity. The IL-1Ra precursor is cleaved to its mature form and secreted like most proteins. IL-1α remains cytosolic in nearly all cells, but unlike IL-1β, IL-1α is rarely found in the circulation or in inflammatory fluids. There is evidence that IL-1α functions as an autocrine, intracellular messenger, particularly in cultured endothelial cells and fibroblasts (Dinarello, 1994). IL-1β also remains cytosolic in nonphagocytic cells. In mononuclear cells, however, between 40-60% is transported out of the cell. Unlike IL-1α, the IL-1β precursor requires cleavage for optimal secretion and activity. The enzyme responsible for this is known as the IL-1β converting enzyme (ICE).

In addition to monocytes, several other nucleated cells have been shown to synthesise IL-1β and these include tissue macrophages, microglia, astrocytes, endothelial cells, smooth muscle cells and synovial cells. A fundamental property of IL-1β, like TNFα, is its ability to induce gene transcription of its

own gene (Dinarello et al. 1987) in addition to a wide variety of other genes. Cultured endothelial cells exposed to IL-1β increase the expression of adhesion molecules, which leads to the adherence of leukocytes to endothelial surfaces. These treated endothelial cells also increase production of prostaglandins, PAF, NO and synthesis of other cytokines, all of which may contribute to the acute vasodilatation seen during the acute inflammatory response. Similarly, IL-1β inhibits smooth muscle contraction and this effect appears to be largely dependent on NO production leading to increased guanylate cyclase activity (O'Neill, 1995; Bankers Fulbright et al. 1996; Beasley and McGuiggin, 1994).

There appear to be at least two defined IL-1 cell-mediated receptors. Type I and II. In general, IL-1α binds to type I and IL-1β to type II (O'Neill, 1995; Bankers Fulbright et al. 1996). Following receptor binding IL-1β has been shown to increase protein phosphorylation in cells, and much effort has been made to identify the protein kinases responsible. IL-1β causes rapid induction of a wide variety of genes that encode proinflammatory proteins as well as cytokines that initiate or augment inflammatory cell activation. The induction of these genes is regulated by IL-1β inducible transcription factors that are members of the immediate-early gene response family, including activating factor-1 (AP-1) and nuclear factor κB (NFκB), (O'Neill, 1995). These transcription factors can be activated within minutes of IL-1β receptor ligation independent of *de novo* protein synthesis. IL-1β can also induce the synthesis

of components of these transcription factors later in the activation program. The mechanisms responsible for mediating synergistic functions by interaction of different IL-1β inducible transcription factors or other transcription factors is incompletely understood (O'Neill, 1995; Bankers Fulbright et al. 1996).

VESSEL WALL RESPONSE *IN VITRO* AND *IN VIVO* IN ANIMALS

Diminished vascular contractility of rat aorta is seen *in vitro* when the tissue is incubated with IL-1β (Beasley and McGuiggin, 1994; Beasley and Eldridge, 1994; Beasley et al. 1991; McKenna et al. 1988). In some studies chronic incubation with IL-1β results in a biphasic reduction in contractility (McKenna et al. 1989) suggestive of the same changes that occur *in vivo* in animals and healthy volunteers injected with endotoxin (see later).

In animal studies injection of high dose IL-1β (>1μg/kg) results in acute vasodilatation, decreased systemic vascular resistance, depressed myocardial function, vascular leak and pulmonary congestion (Dinarello, 1994).

In a recent study looking at the inflammatory response in IL-1β deficient mice, the knockout mice responded normally to the systemic administration of endotoxin with no improvement in terms of mortality when compared to wild mice. However, the **local** acute phase tissue response to the inflammatory stimulus was absent when compared to IL-1β competent mice (Fantuzzi and Dinarello, 1996). This suggests that the **systemic** response to endotoxin may

involve other cytokines with overlapping activities but that IL-1β appears to play a key role in the **local** inflammatory response.

STUDIES IN HUMANS

At the time of writing this thesis no studies using IL-1β in healthy volunteers have been performed. However, administration of IL-1β into cancer patients results in chills and hypotension. Hypotensive effects were observed at doses less that 1μg/kg and hypotension appears to be the major clinical response for limiting the maximum clinically dose tolerated dose of 300ng/kg (Dinarello, 1994). Moreover, a recent clinical trial with IL-1Ra demonstrated a 22% reduction of mortality in patients defined as having septic shock syndrome (Fisher, Jr. et al. 1994); however, the overall mortality in all patients randomised to the treatment wing of the study was unchanged and the beneficial effects of IL-Ra were only shown to be significant in a subset retrospective analysis. In addition, a recent study exploring the role of IL-1-Ra blockade on the human response to endotoxaemia (in healthy volunteers) failed to show any benefit (Preas Ii et al. 1996). In sum, these studies appear to suggest, as with other anti-cytokine strategies, that the host systemic responses is more likely to be dependent upon the appearance of other pro-inflammatory cytokines in addition to IL-1β, as well as components of the macroendocrine stress hormone response (such as cortisol, ACTH, and noradrenaline), all of which are unaffected by IL-1R blockade. The studies are also consistent with the evolving consensus that several members of the inflammatory cytokine family, including TNFα and IL-6, share overlapping biological functions and

also confirm the inherent difficulties of extrapolating the results of animal models of septic shock (in which many single anti-cytokine strategies show remarkable efficacy) to the human condition (Ohlsson et al. 1990; Alexander et al. 1991). Nonetheless, together the data suggest that IL-1β may be an important cytokine for mediating vascular collapse during acute systemic inflammation.

IL-6

CELLULAR RESPONSE

IL-6 is a 23 kDa protein that is produced by almost all cell types in response to a variety of different stimuli including endotoxin or cytokines (such as IL-1β (Content et al. 1985) and TNFα (Jablons et al. 1989)). The gene for IL-6 contains consensus sites for ubiquitous transcription factors such as AP-1, NF-κB, a c-fos serum-responsive element, and a cyclic AMP-responsive element (Scholz, 1996). IL-6 has a high affinity for its cell-bound and soluble form of receptor - IL-6R expressed in lymphoid and nonlymphoid cells. A number of studies have shown IL-6 to be important in the regulatory production of acute phase proteins during an inflammatory response (Rusconi et al. 1991; Helfgott et al. 1989; Ulich et al. 1991; Furukawa et al. 1992). In cell culture, IL-6 does not appear to affect the prothrombotic or proinflammatory effects of IL-1β on vascular cells and it has been suggested that the key role for IL-6 is in activating T and B lymphocytes (in addition to the systemic production of acute phase reactants). The production of IL-6 by endothelial cells supports

the notion that these cells are involved in immunological pathways and in the regulation of the acute-phase response.

VESSEL WALL RESPONSE *IN VITRO* AND *IN VIVO* IN ANIMALS

Incubation of vascular tissue with IL-6 appears to cause an impairment on contractility in several animal studies (Ohkawa et al. 1995); however, *in vitro* studies using human vessels fail to produce any changes in vessel tone (Beasley and McGuiggin, 1994). This may be due to the absence of circulating cells or intraluminal factors that may be important for IL-6 action *in vivo.* In animal studies injection of IL-6 does not appear to cause any acute haemodynamic changes and its principal role appears to be in the immune response, haemopoiesis, and host defence. In animal models of septic shock the use of IL-6 antagonists alone did not protect against death (Libert et al. 1992) although a recent study showed an improved survival during gut-derived sepsis in a murine model of sepsis (Genari and Alexander, 1995).

HUMAN STUDIES

There have been no studies using IL-6 in healthy volunteers. However, use of IL-6 in cancer patients resulted in fever and chills in most patients and was associated with mild renal and liver function abnormalities at higher doses. Dose-limiting toxicity was reached at 30 micrograms/kg i.v. every 8h due to reversible neurotoxicity, but significant rapidly reversible anaemia and hyperglycaemia were seen at lower doses. Platelet counts, white blood cell counts, and acute phase reactant levels were substantially elevated but were

associated with minimal haemodynamic changes (Stouthard et al. 1996; van Gameren et al. 1994; Weber et al. 1994; Weber et al. 1993). At the time of writing this thesis, there have been no trials looking at the effects of IL-6 receptor blockade during endotoxaemia (either in healthy volunteers or during sepsis), but in both the clinical trials using anti-TNF blockade or IL-1Ra high circulating concentrations of IL-6 predicted a worse outcome irrespective of treatment (Fisher, Jr. et al. 1996; Fisher, Jr. et al. 1994). Recent investigations have also suggested a relationship between blood concentrations of IL-6 and poor outcome during several inflammatory conditions, including acute myocardial infarction (Neumann et al. 1995), major surgical procedures (Scholz, 1996) and Kawasaki disease (Furukawa et al. 1992). It remains to be determined whether IL-6 antagonism in other non-infective inflammatory diseases such as unstable angina and myocardial infarction confers any benefit in terms of outcome. In summary, the data suggest that IL-6 might not be involved in vascular collapse during infection, but might mediate other changes in the vessel wall that affect vascular tone or behaviour.

Mechanism of vasodilatation in response to endotoxin or cytokines

The administration of endotoxin to experimental animals and to healthy volunteers results in a biphasic cardiovascular response (Wurster et al. 1994; Suffredini et al. 1989). Administration of TNFα or IL-1β in animals appears to result in a slower more sustained drop in blood pressure. The effects of endotoxin can be therefore be divided into 2 phases - acute and chronic. The

initial rapid and transient fall in mean blood pressure usually recovers within 30min. This is followed over the next 2-3h by a slow decrease in blood pressure which is due to a fall in peripheral vascular resistance. Of significance is the observation that the acute dilator effects are not seen *in vitro*. The effects of endotoxin, TNFα, IL-1β and IL-6 *in vitro* appear to occur earliest 2-3 h following exposure of the vessel to these agents (Hollenberg et al. 1991; Ohkawa et al. 1995; Beasley and McGuiggin, 1994; Beasley et al. 1990). The vasodilatation seen might be due to changes in neural activity or to changes within the vessel wall itself.

Neural mechanisms

Innervation of blood vessels by the sympathetic nervous system is an important means by which blood vessels maintain their physiological constrictor tone. Several studies in animals and in humans have demonstrated that blockade of the sympathetic nervous system using α-blockers results in significant vasodilatation (Whelan, 1967). Similarly, sympathetic ganglionectomy results in vasodilatation and considerable loss of tone in superficial vessels (Lewis and Landis, 1935). The sympathetic nervous system plays an even greater role in the regulation of venous tone and indeed is believed to be the most important neural influence in this vascular bed (Sivo J et al. 1994; Hooker, 1918; Donegan, 1921). During acute vascular inflammation the loss of vessel tone is thought to be due predominantly to the release of vasodilator factors from the endothelium which act directly on

smooth muscle or interfere with sympathetic constrictor activity. However, recent studies suggest that sympathetic nerves and other nerves fibres (such as the non-adrenergic-non-cholinergic nerves -NANC fibres) may also be important in contributing to the release of vasodilator substances (Rubanyi, 1993; Ahluwalia and Cellek, 1997). Indeed, levels CGRP (released from NANC fibres) increase after endotoxin administration in rats and prior administration of a CGRP-antagonist inhibits the vasodilatation (Huttemeier et al. 1993). However, the role of changes in constrictor or dilator neural activity during inflammation in humans remains to be clarified.

Endothelium and smooth muscle

Acute effects

Endothelial cells are strategically located at the interface between blood and tissues. Therefore it is not surprising that these cells which are involved in the regulation of molecules and cells across the vessel wall play an active role in inflammatory reactions. In addition to responding rapidly (seconds to minutes) to inflammatory agonists such as bradykinin and histamine, endothelial cells, upon exposure to endotoxin or cytokines, undergo profound alterations of function that involve gene expression and *de novo* protein synthesis (Pober and Cotran, 1990; Introna et al. 1994; Mantovani et al. 1992).

The endothelial cell responds to endotoxin via a direct, lipopolysaccharide binding protein (LBP) and a soluble CD-14 (sCD-14)-dependent pathway

(Kielian and Blecha, 1995); inhibition of this binding by monoclonal antibodies against CD-14 reduces the production of cytokines by endothelial cells *in vitro* as well as the hypotension and end organ dysfunction in primate models of endotoxaemia (Leturcq et al. 1996). Most of the metabolic effects of endotoxin are mediated by endothelial and smooth muscle production of cytokines. The pro-inflammatory cytokines IL-1β, TNFα and interferon γ have been showed to be synthesised by cultured human endothelial cells *in vitro* (Pober and Cotran, 1990; Introna et al. 1994).

The mechanisms underlying the initial dilator effect of endotoxin appear to vary between species and between vascular beds. Endotoxin stimulates the immediate release of a NO-like factor from cultured bovine aortic endothelial cells (Salvemini et al. 1990) and produces an endothelium-dependent fall in resistance in the rat isolated perfused heart (Baydoun et al. 1993). However, these effects appear to be variable (Myers et al. 1992) and inhibition of NO synthesis does not affect the acute transient fall in blood pressure produced by administration of endotoxin to rabbits (Marceau et al. 1991). Moreover, the rapid relaxation of rabbit mesenteric artery produced by IL-1β is not dependent on NO synthesis but blocked by the cyclo-oxygenase (COX) inhibitor indomethacin (Marceau et al. 1991), as is the initial rapid decrease in blood pressure following endotoxin challenge in cats (Parratt and Sturgess, 1975; Parratt and Sturgess, 1974). One possibility is that endotoxin or cytokines stimulate the immediate release of mediators such as PAF and or kinins from endothelial cells and these act to stimulate production of NO or

vasodilator prostanoids depending on the receptor present and the coupling of these receptors to their respective effector systems (Baydoun et al. 1993; Katori et al. 1989; Fleming et al. 1992). Denudation of the endothelium *in vitro* results in a reduction in the hyporesponsiveness of vascular tissue exposed to endotoxin or cytokines, emphasising the importance of this monolayer in the production of vasodilator factors (Beasley and McGuiggin, 1994; Beasley et al. 1990; Hollenberg et al. 1991; Ohkawa et al. 1995). Nevertheless, these studies also indicate the importance of inducible vasodilator enzymes in the smooth muscle that contribute to the vascular hyporesponsiveness even in the absence of the endothelium.

Delayed effects

Chronic hypotension develops 2-3h after administration of endotoxin or certain cytokines and this is seen in animals and humans. One of the features of hypotension during this phase is that it is accompanied by the loss of vascular hyporesponsiveness to vasoconstrictor agents including α-adrenoceptor agonists (Parratt, 1973). This delayed hyporesponsiveness can be demonstrated *in vitro* and *in vivo,* and vessels isolated from endotoxin-treated animals demonstrate impaired vasoconstrictor responses to noradrenaline or phenylepherine. This delayed effect appears to be endothelium independent (Julou Schaeffer et al. 1990). The response is impaired to a wide variety of agents which act through different receptors and second messenger systems, and the most straightforward explanation is that there is increased production

of a dilator substance within the smooth muscle itself that opposes vasoconstriction.

The lag before the onset of persistent hypotension suggests that endotoxin or cytokines mediate their effects indirectly. This idea is strengthened by the observation that antibodies that neutralise endotoxins or cytokines have little effect on vascular reactivity or hypotension if given after the hypotension has developed. Moreover, the changes can be prevented *in vitro* by cyclohexamide and glucocorticoids, suggesting that during the delay protein synthesis occurs and the products of this synthesis affect vascular reactivity.

Chemical mediators involved in locally mediated vasodilatation

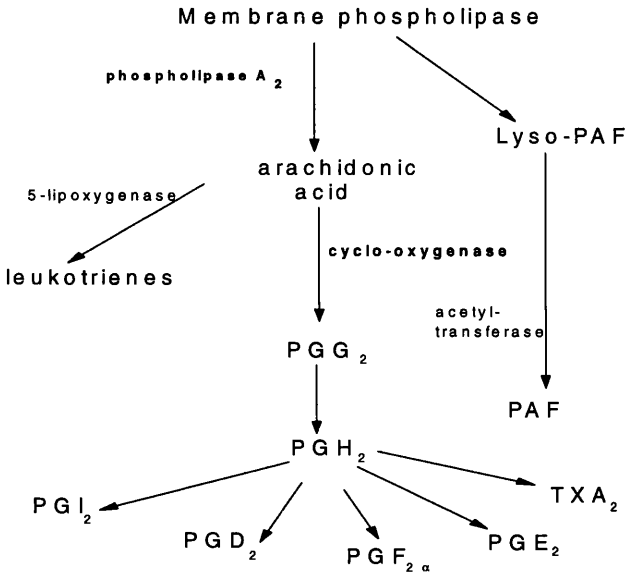
Exposure of the vessel wall to endotoxin or cytokine *in vitro* and *in vivo* results in the cellular release of chemical mediators of the vasodilatory response. These mediators may be released from activated circulating white cells and platelets or by the endothelium and smooth muscle of the vessel wall.

The list of possible chemical mediators of acute inflammation is long. However, for the pathogenesis of vasodilatation, 2 proteins induced by endotoxin and cytokines are of particular interest: nitric oxide synthase and cyclo-oxygenase. These 2 mediators of vasodilatation will be discussed.

Derivatives of arachidonic acid

Arachidonic acid is formed from cell-membrane phospholipid when the membrane is disturbed; this disturbance leads to the activation of phospholipase A_2 (Flower et al. 1976). Arachidonic acid is then available for the formation of secondary metabolites (Figure 2).

Figure 3



The lipid mediator cascade following phospholipase A_2 activation

One class of products of the arachidonic acid cascade is the prostanoids (PGs). The rate limiting enzyme for prostanoid production from free arachidonic acid is cyclo-oxygenase (COX) (Hemler and Lands, 1976). At least 2 isoforms of COX are now known to exist, one which is present as a normal constituent of healthy endothelium (COX-I) and the other which is induced in response to endotoxin and cytokines (COX-II (Hla and Neilson, 1992)). The constitutive

form of COX is thought to mediate many physiological functions. Its activation leads, for instance, to the production of prostacyclin which when released by the endothelium is anti-thrombogenic and vasodilatory (Moncada et al. 1976). Both the smooth muscle and the endothelium have been shown to express COX-I and to generate both constrictor and dilator prostanoids. While most cells have the capacity to generate many different prostanoids there is some selectivity, with platelets and macrophages producing predominantly thromboxane A_2 (TXA_2) and endothelial cells PGI_2. PGD_2 is the principal prostanoid produced by mast cells and PGE_2 by the microvasculature (Moncada and Vane, 1976, 1977, 1978).

COX-II is induced in many cells including the endothelium and smooth muscle by pro-inflammatory stimuli (O'Neill and Ford Hutchinson, 1993). Its induction is inhibited by glucocorticoids and associated with *de novo* protein synthesis. Ferreira and others showed using specific antisera, and COX inhibitors, that IL-1β is a key cytokine for the release of COX-II metabolites (Poole et al. 1992). The role of PGs at the site of inflammation are multiple, but their major specific action is one of vasodilatation. This is attributed particularly to PGE_2 and PGI_2. In addition, although individually each agent produces little or no vessel leakage, in combination with substances which increase vessel permeability, these prostanoids will markedly potentiate the formation of the resultant oedema (Williams and Morley, 1973). A role for COX-II and its products in the pathogenesis of septic shock is supported by

the finding that COX inhibitors restore blood pressure in certain animal models of shock (Parratt and Sturgess, 1974).

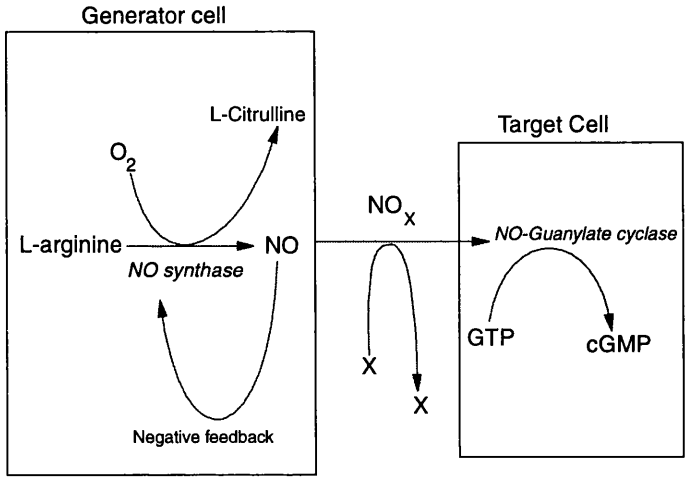
In vivo human studies in which endotoxin has been injected as a bolus dose have noted acute activation of the kallikrein-kinin system and changes in the circulating prostanoid levels (DeLa Cadena et al. 1993). Prior administration of a COX-inhibitor markedly reduced the acute pyrexia and malaise, but did not affect the early or late cardiovascular and haemodynamic changes associated with endotoxaemia (Martich et al. 1992; Godin et al. 1996).

Nitric oxide

There is considerable interest in the role of NO as a mediator of physiological and pathophysiological vasodilatation. Three NO synthases have been described: an endothelial isoform (eNOS), a neuronal isoform (nNOS) in 'nitrergic' nerves and a macrophage or inducible isoform (iNOS). NOSs cleave a nitrogen from one of the guanidino nitrogens of the semi-essential amino acid L-arginine, and combine it with oxygen from molecular oxygen to form NO. The by-product of this reaction is the amino acid L-citrulline (Figure 3). The genes encoding for the 3 isoforms of NO synthase have been located to human chromosome 7 (eNOS), 12 (nNOS) and 17(iNOS) (Bloch et al. 1995; Marsden et al. 1993; Nadaud et al. 1994), and the enzymes show sequence homology with each other and with cytochrome P450 reductase (White and Marletta, 1992) (although NOS is not a member of the cP450 supergene family).

Figure 3

The L-arginine-nitric oxide pathway



Synthesis of nitric oxide and production of physiological effects. Nitric oxide synthase catalyses synthesis of nitric oxide from L-arginine and molecular oxygen. L-citrulline is the by product. Nitric oxide itself might inhibit the activity of nitric oxide synthase by interacting with the haem moiety of this enzyme. Physiological effects are produced after nitric oxide binds to the haem moiety of guanylate cyclase and activates this enzyme to produce cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP) in target and generator cells. Carrier molecules (X) that stabilise nitric oxide have been proposed.

The chemistry of the 5 electron oxidation of L-arginine to form NO and citrulline is unusual and involves a series of co-factors including flavins, tetrahydrobiopterin, NADPH and calmodulin. Calmodulin appears to be important in activating electron transfer from flavins to the haem moiety of NOS; this determines the rate of flavin reduction, an important step in the biosynthetic pathway. Two of the isoforms (eNOS and nNOS) are calcium-calmodulin sensitive and the binding of the calcium-calmodulin to the enzyme complex determines the rate of NO generation. In contrast, iNOS binds

calmodulin tightly, thus allowing maximum flavin reduction and rendering the enzyme activity effectively independent of the prevailing concentration of calcium/calmodulin.

In healthy vessels, production of NO in the cardiovascular system occurs mainly from endothelial cells expressing eNOS. However, endotoxin, cytokines including IL-1β and TNFα, and products of Gram-positive bacteria induce the expression in endothelial and smooth muscle cells of iNOS. The induction of NO synthase involves protein synthesis and is inhibited by glucocorticoids (Rees et al. 1990). Interestingly, the production of NO synthase by the constitutive NOS appears to be inhibited following induction of the inducible NOS and this effect appears to be mediated by a decrease in the stability of the mRNA encoding for constitutive NOS messenger (Yoshizumi et al. 1993). Thus whereas in the healthy vessel the endothelium is the major vascular source of NO, during inflammation the whole vessel synthesises this mediator.

It is now clear that activated macrophages express high levels of NOS and produce large amounts of NO (Schoedon et al. 1993). Cytokines that induce the expression of iNOS in macrophages include IL-1β, IFNγ, TNFα and migration inhibitory factor. The NO generated contributes to the role of macrophages as highly effective killers of intra- and extra-cellular pathogens (although NO itself is probably not the cytotoxic molecule, rather it may be peroxynitrite ($ONOO^{\cdot}$) a product of the interaction between NO and oxygen). A number of cytokines, including IL-4, IL-10 (Cunha et al. 1992; Liew et al.

1989) and transforming growth factor-β, can down regulate the induction of NO synthase in macrophages. In addition, macrophages exposed to endotoxin alone and then stimulated with a mix of IFN-γ and endotoxin express significantly lower levels of NO synthase than cells stimulated without pre-exposure to endotoxin (ie they exhibit tolerance). NO itself can reduce the activity of NO synthase by feedback inhibition, and also inhibit the production of IFN-γ by Th1 cells (thus turning off its own synthesis from upstream in the inflammation cascade).

In vivo studies in humans have also shown increased production of NO following cytokine administration. Hibbs and others documented increased endogenous nitrate synthesis in patients receiving IL-2 demonstrating that a cytokine-inducible, high-output L-arginine/NO pathway exists in humans (Hibbs, Jr. et al. 1992). What was not clear from these studies was whether the the increased nitrate was of endothelial or smooth muscle origin or generated from other circulating inflammatory cell types. Similarly, studies in patients confirm that NO appears to be generated in excess quantities during sepsis and that inhibition of NOS with N^G-monomethyl-L-arginine (L-NMMA) will restore blood pressure (Petros et al. 1991). However, L-NMMA inhibits all 3 isoforms of NOS and the precise role of iNOS in human inflammatory conditions remains uncertain. Moreover, although there is considerable evidence that NO plays a central role in the hypotension that characterises endotoxic shock, it is still not clear which isoenzyme is responsible for its production. Many *in vitro* and *in vivo* studies in animal models of septic shock have documented and attribute

the presence of iNOS in endothelial and vascular smooth muscle cells for the excessive production of NO (Moncada and Higgs, 1993). However, there are other reports that show conflicting results. Cook and others (Cook et al. 1994) localised the production of iNOS to infiltrating monocytes and macrophages within the vascular wall but failed to detect the presence of iNOS within smooth muscle cells. Similarly, Robinson and others failed to demonstrate the presence of iNOS in cardiomyocytes following endotoxin administration in the rat, but were able to detect iNOS positive cells in the infiltrating macrophages and fibroblasts (Robinson, M et al. 1996). Furthermore, many studies have failed to demonstrate high output iNOS activity in human mononuclear phagocytes or neutrophils (Yan et al. 1994; Schneemann et al. 1993), and it has been difficult to induce expression of iNOS in human blood vessels *in vitro.* Indeed, whilst a role for iNOS has clearly been established in animal studies, this is not the case for humans. Such disparities may exist because of inter-species variation in the response to acute inflammation but clearly the mechanism and source of the excessive NO production that occurs in humans during administration of endotoxin or cytokine requires further investigation. In view of the difficulties in expressing iNOS *in vitro* it is probable that studies need to be undertaken *in vivo* where a particular combination of cytokines might be important.

Exploring the effects of endotoxin and cytokines on blood vessels

Studies in animals

Most *in vitro* and *in vivo* models of vascular inflammation investigate several key process that occur during its development:

1) The primary induction (e.g. by endotoxin or cytokines)

2) The intermediate mediator level (e.g. endothelial and smooth muscle response)

3) The final mediator level (e.g. NO or prostanoid production, membrane destabilisation and free radical formation).

IN VITRO STUDIES

The advantage of *in vitro* studies is that it allows a carefully controlled examination of one or several aspects of the inflammatory process. For example, nitrate (or prostanoid) measurements in murine macrophages incubated with endotoxin or cytokines (Bogle et al. 1992), or the measurement of messenger RNA for the induction of new enzymes in cells following exposure to an inflammatory stimulus (Bogle et al. 1994). Moreover, *in vitro* organ bath pharmacology permits a controlled investigation using highly selective and specific agonists/antagonists (which may not be suitable for *in vivo* use) to probe different enzyme pathways or receptor populations. In this way, vascular tissue taken from animals injected with endotoxin/cytokines or

human septic vessels can be examined in a more systematic and controlled environment than is possible *in vivo*.

However, inflammation like many other diseases, is a multifactorial event affecting and affected by many complementary systems *in vivo*. Using an *in vitro* based system it is not possible to examine the vessel, or constituents thereof, in its pathophysiological environment. This may result in difficulties in reproducing a vascular event or eliciting a pharmacological response *in vitro* as a result of absent circulating factors (such as sCD14 for endotoxin presentation) in the blood or the absence of active neural pathways that contribute to the acute inflammatory effects *in vivo*. Furthermore, it is a well described phenomenon for cells grown in culture to begin to lose surface receptors on repeated cell-passage (Traish et al. 1995). This may clearly lead to artifactual responses not relevant to the *in vivo* situation.

IN VIVO STUDIES

Most investigators use a rodent model. These are inexpensive, widely available, can be obtained genetically identical, the same age and sex, on the same diet, and specific pathogen free. Such models have given important insights into mechanisms. However, in terms of the relevance to human disease, there are several clear limitations when assessing animal models. Many animal models utilise systemic injection of endotoxin or cytokine to reproduce the acute inflammatory event that occurs during sepsis. This poses 2 major problems. First, in most cases patients are not exposed to endotoxin in a

single large bolus. Second, and perhaps more importantly, is the fact that the amount of endotoxin that is administered to the animal is usually very large and often sufficient to eventually kill the animal. As a result, the outcome of many such studies lead to controversial results, both in the haemodynamic and in the metabolic spheres. In addition, the haemodynamic response to endotoxin or cytokines is species specific. In the dog for example, intravenous endotoxin causes hypotension within the first minute, the apparent result of hepatic and venous vasodilatation and decreased venous return. In contrast, in the subhuman primate the infusion of endotoxin evokes quite a different haemodynamic response. Circulatory failure evolves over 3-4 h without a precipitous early decline in blood pressure and the picture is often one of a hypodynamic circulation - whereas human sepsis is a hyperdynamic condition. In addition, administration of endotoxin to healthy volunteers results in a biphasic reduction in blood pressure very similar to the rodent model. In terms of outcome studies in animal models of endotoxaemia and cytokinaemia, short-term treatment benefits are not valid with regards to survival, as long term treatment may not be improved. For example, in endotoxaemic rats, PAF failed to increase long-term survival, whereas short-term survival was increased (Bahrami et al. 1989; Salari et al. 1990). Furthermore, although many agents may prove to be effective in the treatment of sepsis in animal experiments, this has not been translated into clinical benefit. It is clear that fundamental mechanisms (e.g. induction of iNOS) may differ markedly between animals and humans.

Studies in humans

Investigation of the initial vascular response to sepsis in humans is difficult because of practical problems in studying patients at the onset of sepsis. Animal models based on the administration of endotoxin (or cytokines) have been used to study these early vascular events but as discussed above, these studies are limited by variation in species and end organ damage to endotoxin (Redl et al. 1996).

It was Bradley and others in 1945 who first documented the haemodynamic alterations that occurred in normotensive and hypertensive subjects following injection of endotoxin (Bradley et al. 1945). This important paper clearly documented the arterial and venous dilatation that occurs in endotoxaemia in humans. Gilbert and others made similar and more extensive observations comparing humans with other animals (Gilbert RP, 1960), and a more systematic study of the metabolic and haemodynamic changes was made by Suffredini and others in 1989 (Suffredini et al. 1989). These investigators studied the temporal response of cytokine elaboration that occur after endotoxin administration as well as documenting the haemodynamic changes that ensued.

Since then several studies looking at the systemic effects of cytokine administration on the cardiovascular system have also been performed (van der Poll et al. 1990; van der Poll et al. 1990; van der Poll et al. 1991; van der

Poll et al. 1992). Pharmacological studies have looked at the effects of prior administration of steroids, NSAIDs, cytokine antagonists and other agents on the response of the cardiovascular system to the effects of endotoxin and cytokines (van der Poll et al. 1995; Preas Ii et al. 1996; Suffredini et al. 1995; Martich et al. 1992). However, these studies are limited by several factors associated with performing studies in healthy volunteers. The administration of any systemic inflammatory agent into healthy volunteers is always constrained by the amount of active agent that can be safely administered. Furthermore, systemic administration of endotoxin or cytokine (or any other agent) evokes a systemic neurohumoral reflex response that confounds any attempt to study the mechanisms of changes occurring in the vasculature. To explore the cellular and mechanistic events that occur (*in vivo*) as a direct result of the local interaction of the inflammatory agent with the vessel wall, experimental models need to be developed that remove the systemic component of the acute inflammatory event.

Aims and objectives of this thesis

The aims of this thesis were to develop in humans a model to investigate the **local** changes that occur in vessel wall reactivity following **direct** exposure to inflammatory agents.

Using this model the objectives were to:

- Investigate the local changes that occur in a vessel **perfused with blood** in its normal physiological environment.

- To administer these inflammatory agents individually or together, in order to reproduce local changes that occur during an acute inflammatory event.

- To examine the effects of repeated administration of an inflammatory agent. This would determine the reproducibility of the model in the same individual, as well as allow a greater opportunity to investigate the effects of different pharmacological agents on the same individual on multiple occasions.

- To explore the cellular events occurring in the vessel wall and thus determine the endothelial, smooth muscle and neural response to acute inflammation.

- To explore these cellular changes *ex vivo* in order to examine microanatomical and molecular biological changes that might be associated with an acute inflammatory response.

Chapter 2

Materials and methods

Introduction

A variety of methods were used in this thesis. However, the central aim was to explore the effects of inflammation on vascular reactivity *in vivo* and the principal method used was the dorsal hand vein technique. Additional methods were used in an attempt to reproduce and explore *in vitro* the mechanism of effects seen *in vivo*.

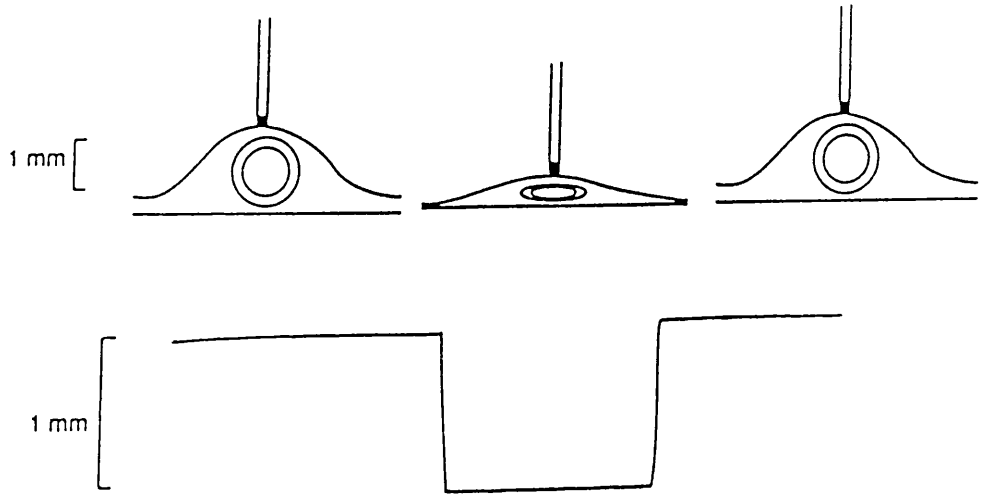
Method for the study of the superficial hand veins

The pharmacological responses of the superficial dorsal veins were studied using the technique of Nachev and others (Robinson et al. 1970), with the modification of Aellig (Aellig, 1981). This technique allows the measurement of vein size in response to the local infusion of drugs into a vein on the back of the hand, and so enables the direct effects of drugs on venous smooth muscle tone to be studied and quantified (Aellig, 1981; Robinson et al. 1973; Robinson, 1990; Aellig and Robinson, 1978).

The principle of the method is to measure the internal diameter of a single vein on the back of the hand, at a constant distension pressure (Figure 1). At a constant distension pressure, changes in the internal diameter of the vein reflect changes in the venous tone or contractile state of the smooth muscle being measured; contraction results in a reduction in vein diameter, while

relaxation produces an increase in venous diameter (Aellig and Robinson, 1978).

Figure 1



The internal diameter of a single hand vein is recorded by measuring the linear displacement of a light weight probe placed on the skin overlying the summit of the vessel when the pressure in a congesting cuff placed around the upper arm is reduced from 40 to 0mmHg. The downward movement of the probe (upper trace) is recorded as a downward movement on the pen recorder (lower trace).

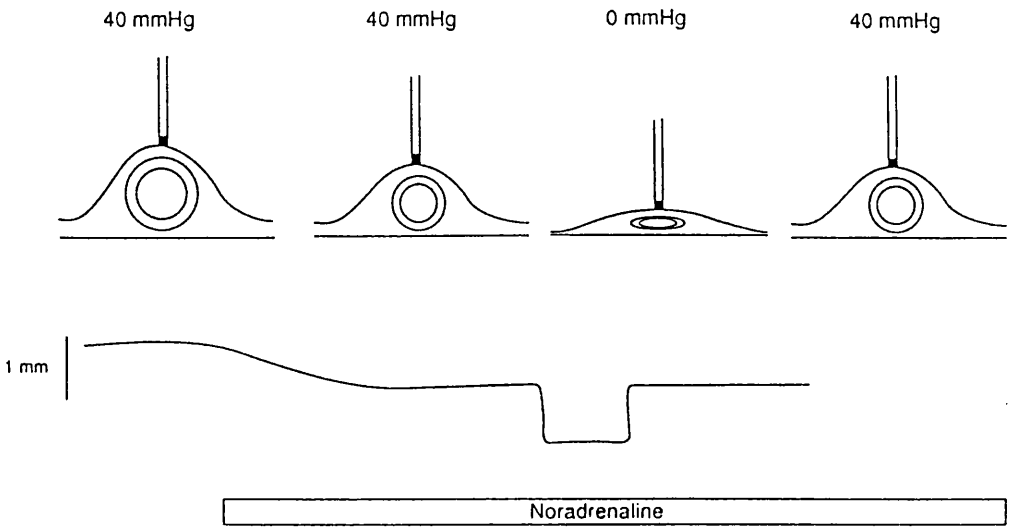
The internal diameter of a single vein was assessed by measuring the linear displacement of a light-weight probe, which rested on the skin overlying the summit of the vein, when the pressure in a congesting cuff placed around the upper arm was reduced to from 40mmHg to 0mmHg. Studies were performed with the subject lying supine with the forearm and hand supported at an angle of approximately 30^0 on a rigid padded support. The arm was positioned so that it lay above the level of the central venous pressure, so that the vein

emptied completely when the distension pressure was reduced to zero (Figure 1). A constant distension pressure was provided by placing a standard sphygmomanometer around the upper arm and inflating it to 40mmHg. This manoeuvre provides a pressure in the vein of approximately 30-35mmHg. When the congesting cuff is inflated and the vein distended, the pressure within the vein is constant (Collier, 1975; Zitnik and Lorenz, 1969; Burch and Murthadha, 1956; Donegan, 1921). It has been demonstrated previously that provided the subjects are relaxed, warm and comfortable, resting internal venous diameter remains constant over several hours and days (Collier, 1975; Burch and Murthadha, 1956; Donegan, 1921).

Assessing drug effects

The technique allows the study of dose-response relationships for vasoconstrictors or vasodilators given directly into the vein. Drugs are infused directly into the vein, 10-15 mmHg upstream from the point of measurement so that their direct effect on the venous smooth muscle can be quantified, free from interference by possible reflex cardiovascular changes that may result from systemic administration of drugs (Aellig and Robinson, 1978). An effective local concentration of a vasoactive drug can be achieved by giving doses that are 100 times below a systemically effective dose (Figure 2).

Figure 2



Drugs are infused through a 23SWG needle placed with its tip 10-15mm upstream from the point of measurement of venous diameter. The figure shows a diagrammatic representation of venoconstriction. Noradrenaline infused into the vein causes a reduction in internal diameter which is reflected in a downward movement of the probe resting on the summit of the vein. The new internal diameter of the vessel is recorded by deflating and then re-inflating the upper arm congesting cuff.

The response to venoconstrictor agents can be measured directly, the drug is infused into the vein and the vein size measured after a given time. The measurement of the responses to vasodilator substances is more complex. In relaxed subjects, in a warm environment, superficial hand veins have no tone and are fully dilated (Collier, 1975). In order to observe a dilator response the veins must be partially preconstricted (as in the organ bath). This is achieved by a continuous local infusion of noradrenaline or other constrictor drugs, at a

dose sufficient to reduce the diameter of the vein to approximately 50% of its resting control size (Aellig and Robinson, 1978). The response to the vasodilator drugs is then studied by **co-infusion** of the vasodilator together with the vasoconstrictor.

Expression of results

Responses can either be expressed as absolute or percentage changes in vein size. Venoconstriction may be expressed as the percentage reduction in vein diameter from the fully relaxed state, while venodilatation can be expressed as either the percentage increase in size from the preconstricted vein or as percentage reversal of the noradrenaline-induced preconstriction (Aellig and Robinson, 1978; Robinson, 1990).

Most investigators express their results in terms of a dose-response curve. The stimulus is taken as the rate of drug infusion as this can be measured precisely. The concentration of drug reaching the venous smooth muscle cannot be known accurately but blood flow in a single dorsal hand vein has been estimated at 0.5-1ml/min (Aellig and Robinson, 1978; Collier, 1975) and consequently, an approximate plasma concentration of drug reaching the venous smooth muscle can be estimated from the dose and rate of each drug infusion.

Experimental details

The studies described in this thesis were performed in a temperature controlled laboratory (28-30^{0}C), with the ambient temperature kept constant within ±1^{0}C during each study. Drugs or physiological saline were infused continuously into the vein, using a constant rate infusion pump (Harvard apparatus, USA) at 0.25ml/min, via a 23SWG "butterfly" needle (Abbott Laboratories Ltd, Queensborough, UK) placed with its tip 10-15mm upstream from the point of measurement. The needle was connected to the pump by an epidural catheter (Portex, Hythe, UK). Vein size was measured at 5 minute intervals. The upper arm congesting cuff was inflated to 40mmHg and the vein diameter was measured using a Schaevitz linear variable differential transformer mounted on a tripod, connected to a Lectromed amplifier and pen chart recorder. Vein size was measured directly in arbitrary units and was converted to mm after calibration of the linear variable differential transformer at the end of each experiment. Results were expressed as the percentage reduction in venous diameter from the initial basal diameter during the noradrenaline-induced constriction, and percentage increase in diameter from the pre-constricted state during co-infusion of vasodilators drugs under study.

$$\textbf{Percentage dilatation} = \frac{Vd - Vc}{Vb - Vc} \times 100$$

where **Vd = diameter during infusion of noradrenaline and dilator**
Vc = diameter during infusion of noradrenaline alone
Vb = basal vein diameter

In each study, before measurements were made in response to the drugs under study, stable baseline measurements of the vein size were made for 10-15 min (during infusion of saline alone). The dose of noradrenaline necessary to constrict the vein to approximately 50% of its resting size was determined for each subject, and the vein size during constant infusion of this dose of noradrenaline was measured for a further 10-15 min to ensure a stable constricted state before infusion of vasodilator drugs.

Calculations and Statistics

The results were expressed as mean ± SEM and compared using the Students' t-test or analysis of variance as appropriate for paired and unpaired observations, where $p<0.05$ was considered statistically significant.

Validation of dorsal hand vein technique *in vivo*

The technique of assessing venous tone as described makes a number of assumptions:

a) the pressure in the vein remains constant throughout the inflation of the cuff

b) the vein is circular in cross section when distended

c) the vein collapses fully when the cuff is deflated

d) at 28°C (laboratory temperature) the vein expresses no intrinsic tone and is fully dilated

e) the diameter of the vein will reflect the contractile state, or tone, of the circular smooth muscle in the vein wall; contraction therefore resulting in a decrease in vein diameter and relaxation an increase. (Robinson, 1978).

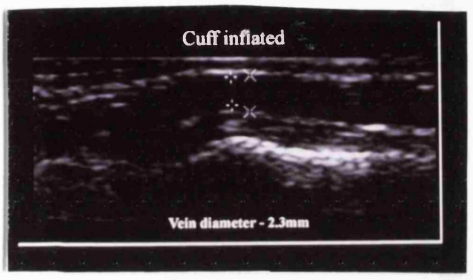
The 1st assumption was tested by Birch in 1956 and later by Robinson and others (Robinson et al. 1970; Collier, 1975; Burch and Murthadha, 1956; Shepherd and Vanhoutte, 1975). Birch inserted an intravenous needle inserted into a hand vein and connected the distal end to a manometer. He documented the constancy of the pressure within the vein under a constant distention pressure. Collier and others performed similar such studies and confirmed that at 40mmHg, the intravenous pressure remained at 30-35mmHg during a 1h infusion of saline. Assumptions (b), (c), (d) and (e) are widely accepted but have not been proven. Therefore in order to examine some of the real-time changes that occur in the dorsal hand vein a series of transdermal ultrasound echocardiographic images were taken during inflation and deflation of the cuff and during infusion of dilator and constrictor drugs.

In these studies, subjects lay on a bed in the usual manner, with hand placed above the level of the heart. After insertion of a 23 gauge needle, a 7 MHz paediatric transducer (Acuson computed technology, Uxbridge, UK) was placed 5-10mmHg upstream from the tip of the needle. After the cuff was inflated, a longitudinal and cross-sectional cross view of the vessel was generated (Acuson 128XP ultrasound hardware). Measurement of the internal diameter was made, during infusion of saline for 15 min followed by a 5min

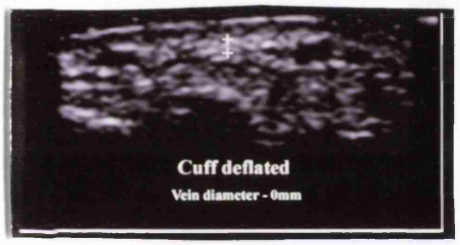
infusion of GTN (4pmol/min). The cuff was then deflated and a recording of the vein collapsing were made. Finally, recordings were made of the vessel constricting during a continuous infusion of noradrenaline.

Figure 3

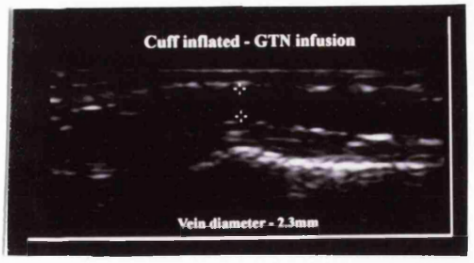
Panel A



Panel B



Panel C

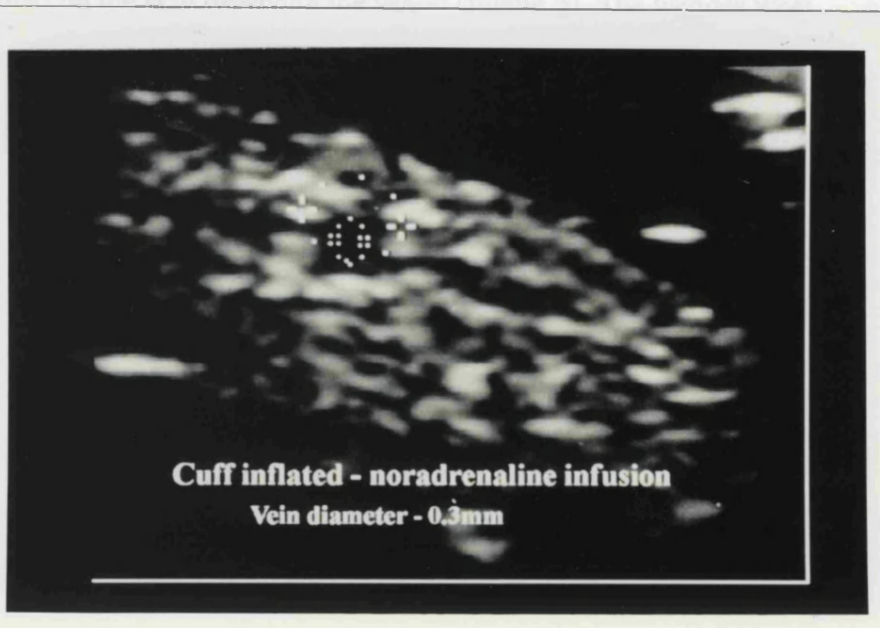


Panel A shows the vessel in logitunidnal section fully distended after inflation of the cuff to 40mmHg and during a saline infusion. Panel B shows the vessel collapsed fully after the cuff has been deflated. Panel C shows the same vessel after a 5min infusion of GTN (4pmol/min). There was no change in the internal diameter recordings as measured by the ultrasound calipers during infusion of GTN.

Figure 4

Panel A

Panel B



Panel A shows a cross-sectional view of the same vessel as in Figure 3 during an infusion of saline.

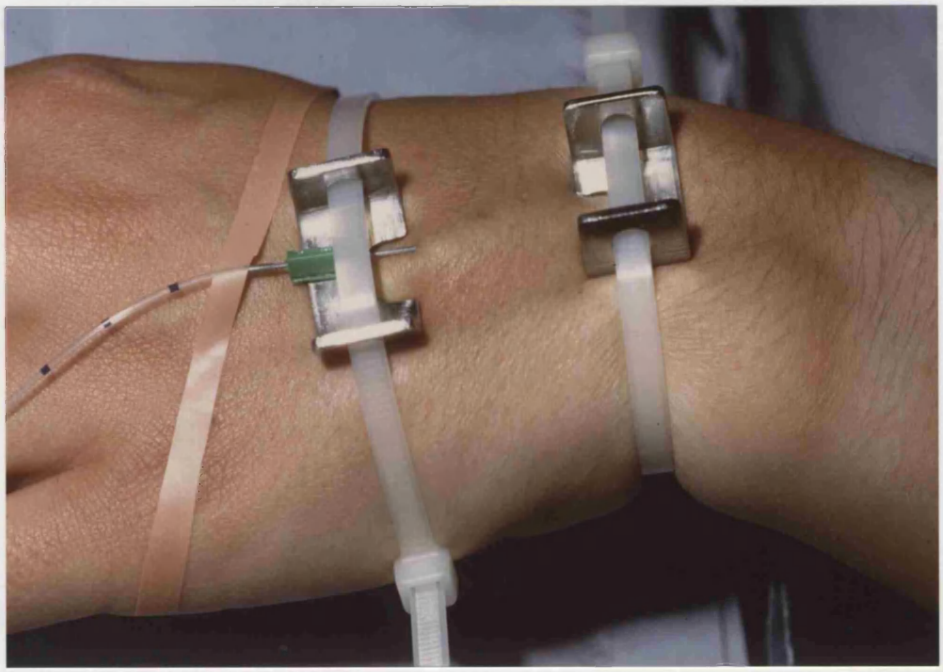
Panel B shows a cross-sectional view of the vessel following a 10 min infusion of noradrenaline (160 pmol/min).

Isolation and instillation of endotoxin and cytokines

In this thesis, experiments have been described which involved the use of potentially toxic substances (bacterial endotoxin and pro-inflammatory cytokines). In order to use these in a safe and reproducible manner and without causing any systemic adverse effects, a model was developed that allowed the investigation of the local effects of these substances on vascular reactivity.

To instill endotoxin, cytokines or control solution (saline), a length of the vein under study was isolated from the circulation by means of two steel wedges placed 2-3cm apart on the skin overlying the vessel (Figure 5). The wedges were weighted to occlude the inflow and outflow to the isolated segment leaving the study vessel distended during the period of the instillation. A solution of endotoxin (1ml), cytokine (1ml) or saline (1ml) was injected into the isolated segment. One hour later the contents of the segment were aspirated and the wedges removed so that the circulation of the blood through the vessel was re-established. Although it was not assessed formally, there appeared to be no significant leakage during the process of instillation since the vessel stayed distended despite deflation of the upper arm cuff.

Figure 5



A length of the vein under study was isolated from the circulation by means of two steel wedges placed 2-3cm apart on the skin overlying the vessel. The wedges were weighted to occlude the inflow and outflow to the isolated segment leaving the study vessel distended during the period of the instillation

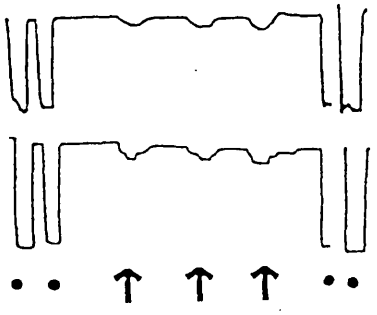
Single deep breath as a venoconstrictor stimulus

Taking a deep breath causes a transient reflex constriction of the superficial veins. The response usually begins within 2-4 sec, reaches its maximum in 20-40 sec, and returns to the control value within about 2 min. Duggan and Lyons and later Samuleoff, Bevegard and Shepherd showed the pathway of the reflex is the sympathetic nervous system (Samueloff et al. 1966; Duggan et al. 1953) and that with repeated deep breaths at rapid intervals, the constriction of the

superficial veins was not sustained. They also showed that venoconstriction was due to a spinal reflex because it was present in a tetraplegic with complete spinal cord section (Browse and Hardwick, 1969). The venoconstrictor response is abolished by infiltrating procaine around the vein (Burch and Murthadha, 1956) or by infusing phentolamine whilst performing the manoeuvre, suggesting that it is sympathetically-mediated through an action on vascular α-receptors (Benjamin et al. 1988).

In the studies performed in this thesis, sympathetic venoconstriction was induced by asking subjects to take a single deep inspiration over a period of 5 sec, to hold this for a comfortable period (approximately 10 sec), and then to breathe out slowly before returning to normal breathing. This venoconstrictor response varied between individuals and between occasions that the manoeuvre is performed. However, the constrictor response was of the same magnitude in adjacent vessels on each occasion. In the studies using this manoeuvre, 2 adjacent vessels were used simultaneously in order to get reproducible and meaningful results. One vessel was used as the study vein whilst the adjacent parallel vein was used a control (Figure 6).

Figure 6



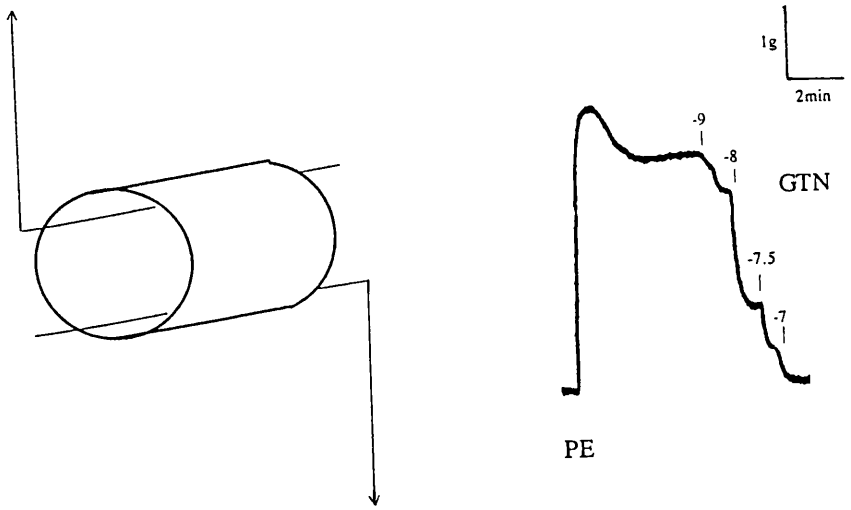
Two adjacent vessels were studied simultaneously. The arrows indicate the time points at which the subject was asked to perform the deep-breath manoeuvre. The ● indicates deflation of the cuff.

Method for the measurement of vascular tone *in vitro*

Standard techniques were used for the measurement of isometric tension in isolated vessels mounted in an organ bath. Blood vessels mounted in an organ bath *in vitro* are denervated and have no basal tone but respond to a number of different contractile agents. In this thesis the α-adrenoceptor agonist phenylepherine was used to generate active tone in isolated veins (Figure 7).

Specimens of saphenous vein (from patients undergoing coronary artery by-pass surgery) were collected in cold Krebs' solution of the following composition (mM): NaCl 118, KCl 4.7, CaCl 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 11. Each vessel was freed of connective tissue, cut into rings 3-5mm long and suspended between two hooks connected to a transducer (Statham UC3, Gould Inc; Cleveland, Ohio, USA) for the measurement of isometric tension. The preparations were suspended in 10ml organ baths filled with oxygenated ($95\%O_2/5\%CO_2$) warmed (37^0C) Krebs' solution and vessels were preconstricted submaximally with phenylephrine (0.9-1.0μM); the vessels were equilibrated for 1-3h at a basal tension of 2g. Endothelial integrity was assessed following submaximal preconstriction with phenylephrine (0.9-1.0 μM) by relaxation to bradykinin (1μM); only vessels that produced a relaxation greater than 40% were used.

Figure 7



Left panel: schematic representation of a blood vessel prepared for the measurement of isometric tension. The vessel is mounted between two hooks; one hook is fixed and the other is connected to a force transducer which measures chamges in tension. The preparation can be maintained in an organ bath for several hours.

Right panel: representative from a segment of human saphenous vein with phenylepherine (PE:1mM) and relaxed with increasing concentrations of GTN (concentrations expressed as log molar).

Method of platelet aggregometry

An optical method of measuring platelet aggregation was used. Venous blood drawn into a syringe containing 3.15% (w/v) trisodium citrate was centrifuged at 200G for 20 min to obtain platelet-rich plasma (PRP). 1ml aliquots of PRP was placed in cuvettes and the response to 10μl collagen (or other aggregating agents) determined using a dual channel optical aggregometer. When PRP is stirred and a suspension of collagen added to it, some of the platelets adhere to the collagen coating the fibres. After this adherence, the free platelets begin to swell and stick together. The reaction accelerates until large platelet masses form, and these fall to

the bottom of the cuvette. This reduces the optical density of the PRP allowing more light (infra-red) to pass through. If all of the platelets clump, the resultant optical density is the equivalent of platelet poor plasma (PPP). The light transmittance through PPP represents 100% aggregation and that through PRP represents 0% aggregation. The aggregometer develops a voltage proportional to the transmittance of light through the plasma. This voltage is recorded on a strip chart recorder as a function of time.

Blood was drawn for platelet aggregometry in subjects taking part in the studies on oral aspirin or paracetamol therapy. Immediately prior to taking the drug, and 2h later 15ml of venous blood was drawn into a syringe containing 2.5ml of 3.15% (w/v) trisodium citrate. Blood was centrifuged at 200G for 20min to obtain platelet rich plasma. 1ml aliquots of platelet-rich plasma were placed in cuvettes and the responses to 10µl of collagen and 300µM arachidonic acid were determined using a dual channel optical aggregometer (Chronolog Corporation, Havertown, PA, USA). 1ml of platelet rich plasma was placed in an eppendorf microtube and centrifuged at 1300G for 5 min to produce a supernatant of platelet- poor plasma which was used as a control. The maximal extent of aggregation was used for data analysis and results after drug administration were compared with control (pre-drug) values.

Drugs

The following drugs were used in studies described in this thesis. Sodium arachidonate (5mg/vial) stored under nitrogen was obtained from Sigma (Dorset, UK). Vials were stored at -20°C and a single vial was used for each study. Sodium arachidonate (5mg) was dissolved in 154µl absolute alcohol to produce a stock solution of 1mM. Subsequent dilution was in saline and the final concentration of alcohol in the infusate was 0.0001%. Arachidonic acid was always used within 6h of preparation. Lysine acetylsalicylate (1g/vial) was obtained from Synthe Labo (Le Plessis Robinson, France), paracetamol (500mg) and noradrenaline (2mg/vial) from Sanofi Winthrop (Guilford, Surrey, UK), dispersible aspirin from Aspar Pharmaceuticals Ltd (London, UK), glyceryl trinitrate (5 mg/vial) from Dupont Pharma (Hertfordshire, UK), ascorbic acid (100mg/ml) from Evans Medical Ltd (Horsham, UK) and collagen reagent from Chrono-Log (Havertown, PA, USA) Ascorbic acid 0.5 ml was added to noradrenaline stock solutions to prevent auto-oxidation. Endotoxin (EC-5, 10 000 EU/vial) was obtained from USP (Rockville, MD, USA). Vials were stored at -4^{0}C. The endotoxin was rehydrated with 20 ml of sterile saline (0.9%) to give a solution of 1000 EU/ml. The vial was then shaken for a minimum of 20 min. The solution was divided into aliquots and stored at -20^{0}C for up to 10 weeks. To determine activity of the endotoxin, at 4 weekly intervals dose response curves to endotoxin were constructed in J774 murine macrophage cell lines and nitrite production determined (Bogle et al. 1992). All endotoxin was filtered through a 0.2µm bacterial filter (Acrodisc PF, from Gelman Sciences, Ann Arbor, Michigan, USA). L-NMMA was from The Welcome Foundation Limited (Beckenham, Kent, UK), bradykinin from Clinalpha AG (Laufelfingen, Germany),

hydrocortisone (20mg) from MSD (Hertfordshire, UK), heparin (100U/ml) from CP Pharmaceuticals Ltd (Wrexham, UK). Heparin (100U) was added to the endotoxin solution before administration to prevent thrombus formation. Hydrocortisone (20mg) from MSD (Hertfordshire, UK), human recombinant tumour necrosis factor-α (10μg/vial), human recombinant interleukin-1β (10μg/vial) and human recombinant interleukin-6 (10μg/vial) from Bachem California (Walden, UK). All cytokines were dissolved in 1ml of sterile saline, aliquoted into 100pg/μl volumes and stored at -20° C. L-arginine hydrochloride (Dorset, UK) and aminoguanidine from Aldrich (Dorset, UK) and tetrahydrobiopterin (25mg/vial) from ICN (Thame, UK). All drugs for infusion were prepared fresh, dissolved in physiological saline (0.9% sodium chloride; Baxter Healthcare Ltd) and passed through a 0.2μm bacterial filter, Acrodisc PF, Gelman Sciences (Michigan, USA) immediately prior to use.

Ethical considerations

Experiments in human volunteers are vital if we are to understand the mechanisms responsible for pathophysiology and to develop new therapies. Moreover, volunteers studies have become increasingly acceptable in the light of improvements in study design and technical developments which allow the use of very low doses of agents/drugs to be used. The Royal College of Physicians has established clear guidelines for the practice of research in human volunteers and patients which have been followed in all the studies undertaken in this thesis.

The studies described in this thesis were performed in healthy volunteers aged 19-40 who gave their written, informed consent to the procedures. The practical aspects of the study were specified in a detailed protocol conforming with the principles of good clinical practice. All subjects were given a written and verbal explanation of the study and were free to withdraw at any time during the experiment without explanation. Consent was based on an understanding of the nature and purpose of the study. Each subject was offered a small payment to cover expenses and inconveniences. Normal volunteers confirmed that they were healthy and taking no medication. At the start of all studies with agents that had not been previously used in humans, members of staff in the Clinical Pharmacology Unit were used. Moreover, an agreement was made with the Local Ethics Committee to report back to them at an early stage in all these studies to inform them on the outcome and progress and in the unlikely eventuality of any adverse reactions occurring.

All the studies described using the local infusion administration of drugs were done by the author in a specially designed and well-equipped Human Laboratory at St George's Hospital Medical School or University College London Medical School.

The technique of dorsal hand vein cannulation has been used for many years at St George's Hospital and causes only mild and transient discomfort to the subject. At the end of the study pressure is applied to the vein for 1-2 min to

prevent bleeding. The technique of isolation a segment of the dorsal hand vein for instillation of inflammatory agents was developed at St George's Hospital. Only transient discomfort is experienced whilst the wedges are applied to isolate the segment from the rest of the circulation. Over 350 of these studies have been performed by the author with no adverse effects having been recorded.

The doses of drugs used in the venous studies are 100-1000 times less than those required to have a systemic effect. The local infusion of drugs minimises any risk to the subject from the study in addition to allowing examination of the direct effects of drugs on veins. Many of the drugs used in the experiments described in this thesis have been widely used in man and are known to be associated with negligible risk to the subject. However, 3 types of the mediators used in this thesis deserve detailed examination.

Use of arachidonic acid, endotoxin and cytokines *in vivo*

Arachidonic acid

Arachidonic acid is a component of the membrane phospholipids of all mammalian cells and is released by the enzyme, phospholipase A_2 (Flower et al. 1976). It is then rapidly metabolised into oxygenated products, among them, the stable prostaglandins (Piper and Vane, 1969; Flower et al. 1976). Two important enzymes involved in arachidonic acid metabolism are lipoxygenase and cyclo-oxygenase (COX). Lipoxygenase catalyses the

formation of leukotrienes such as LTB_4 (Piper and Vane, 1969). The other enzyme is cyclo-oxygenase (COX-I) and results in the production of prostanoids (such as thromboxane (TXA_2 and prostacyclin, PGI_2). Metabolites from both the COX and the lipoxygenase family have now been used in human studies *in vivo* (Bisgaard and Kristensen, 1985; Camp et al. 1983; Bisgaard et al. 1982; Rosenkranz et al. 1980; Warrington et al. 1980; Leary et al. 1978). The pharmacology of the primary prostaglandins, especially prostacyclin (PGI_2) and thromboxane (TXA_2) has been studied intensively for many years and various physiological and pathological roles have been ascribed to them (Moncada and Vane, 1978; Moncada et al. 1977; Moncada et al. 1976). The significance of altered arachidonic acid metabolism has been the focus of investigation in disorders as diverse as myocardial infarction, unstable angina and rheumatoid arthritis (Holtzman, 1991).

At the time of undertaking the experiments in this thesis, very low doses of arachidonic acid had been infused into the brachial artery to assess biochemical generation of prostanoids (Nowak and Wennmalm, 1979) and arachidonic acid had been injected into the skin to assess generation of functionally active amounts of prostanoids in the skin microcirculation (Larkin et al. 1995); however, no studies involving an intravascular infusion of biologically active doses of arachidonic acid had been undertaken. Local infusion of arachidonic acid into the veins of healthy volunteers provides a direct method to study the physiological role of arachidonic acid metabolism in humans *in vivo*. It was hoped that the use of arachidonic acid, as a stimulant

of prostanoid synthesis, in healthy volunteers, would provide insights into the mechanisms controlling blood vessel tone, and might be useful to study the pathology underlying the vascular abnormalities that have been reported to occur in animal models of disease such as diabetes and hypertension (Lin and Nasjletti, 1992; Silberbauer et al. 1979).

In addressing ethical aspects concerning the use of this lipid, a careful and exhaustive literature search was undertaken to examine the evidence for the use of arachidonic acid in animal studies *in vivo* and *in vitro.* Aspects concerning, dose-dependency of the effects seen, recorded systemic adverse effects and the time course of action of the effects were all noted. A toxicological search on the known *in vivo* effects of the metabolites of arachidonic acid was also carried out. Together with information on the lowest dose known to produce a vascular effect in animals *in vivo* and the dose of radiolabelled arachidonic acid used in humans (Nowak and Wennmalm, 1979), the initial dose-range that was chosen for the experiments in this thesis were calculated to be at least 100-fold lower than the lowest systemically active dose. This dose was also based on *ex vivo* studies on platelet function and the minimum concentration required to induce platelet aggregation. In sum, therefore, the initial dose used was the lowest systemically active and least pro-aggregatory.

Arachidonic acid is the precursor of vasoconstrictor, prothrombotic prostanoids and bronchospastic leukotrienes and the theoretical concerns of

infusing this agent were the possibility of causing intravascular platelet aggregation, painful vessel wall inflammation during infusion and the risk of precipitating bronchospasm (no asthmatics were recruited). Care was taken with all studies to ensure that the risk of any possible harm to the subject was minimised, however, the nature of the investigations was such that there was no direct health benefit to the subject for taking part and all the subjects were aware that this was the case. Moreover, during the initial studies the dose of arachidonic acid was incrementally increased until a vascular response was seen. This dose was then repeatedly used to determine the local pharmacodynamics of arachidonic acid. After this had been established a dose range was calculated within these parameters to establish a dose response relationship of the effects seen. The dose that produced the maximal local effect (change in vessel tone) was chosen as the upper dose limit.

The studies in healthy volunteers were designed to extend what is known about the role of the prostanoid pathway in normal physiological responses that relate to the control of vascular tone in man. The studies have now been performed for over two years, and have caused no significant untoward effects and have contributed to our understanding of the human vascular endothelium as a modulator of vascular tone.

Endotoxin

Endotoxin is derived from the lipopolysaccharide fraction present in the cell wall of all Gram-negative bacteria. It is thought to be responsible for many of

the vascular effects seen during sepsis. Most animal studies *in vitro* and *in vivo* utilise this compound to initiate the endogenous inflammatory cascade thought to be associated with clinical sepsis. The dose of endotoxin used in animal models of septic shock often produce signs of severe vascular collapse and death. These studies have been designed to explore the pathophysiological changes that occur in severe sepsis and to test the use of pharmacological agents to attenuate or prevent this response. More recently models in which chronic low dose endotoxin are administered *in vivo* have been used to attempt to more closely reproduce the changes that may occur clinically (Rees et al. 1995; Rees et al. 1995). The use of endotoxin in humans was initially designed to explore the mechanisms of tolerance to pyrexia during administration of typhoid endotoxin and induction of endotoxin tolerance (Hornick and Griesman, 1978; Greisman et al. 1969). Prior to this Bradley and others were one of the first to note the haemodynamic changes that occurred in normotensive and hypertensive subjects following injection of endotoxin (Bradley et al. 1945). However, systemic administration of low endotoxin into healthy volunteers in order reproduce the early changes that occur in clinical sepsis was studied more systematically 1989 by Suffredini and others (Suffredini et al. 1989). Since then many studies using the same form and dose of endotoxin have been performed. These studies explored the changes in cardiovascular haemodynamics in addition to the changes that occurred in circulating inflammatory cells and mediators of inflammation (Suffredini et al. 1989). The experiments performed in this thesis that used endotoxin were based on the experience in these initial studies. The same endotoxin that had

been used by these investigators was administered. This endotoxin is prepared in the USA specifically for use in clinical studies (Hochstein et al, 1983).

Theoretical ethical concerns of using endotoxin include the possibility of systemic leakage into the circulation producing pyrexia, malaise, hypotension or an anaphylactic response. Moreover, the potential for local vessel thrombosis and infection was also considered. To overcome these concerns, the dose of endotoxin initially used was calculated to be 100-fold lower that that used by Suffredini and considerably lower than the minimum pyrogenic dose (Elin et al. 1981). Moreover, using the model of vein-isolation and occlusion described in this thesis, endotoxin was initially instilled for only 5-10 min and then withdrawn. Once it was established that no local or systemic adverse effects had occurred, the duration of incubation of the vein with endotoxin was gradually increased. At all time points a pharmacological evaluation of the vascular effects of endotoxin were made until a reproducible change was recorded with the lowest dose of endotoxin and the least exposure-time required to endotoxin. During the initial studies the pulse and blood pressure were recorded and all subjects were asked to report back the following day. Any subject with a history of recent infection, vaccination or drug anaphylaxis was excluded from participating in these studies.

The studies with endotoxin were used to gain further insight into the local mechanisms that may be responsible for the changes in vascular reactivity that occur following acute inflammation. More than 200 of these studies using

endotoxin have now been performed. At no stage during its use did subjects develop any local or systemic adverse effects.

Cytokines

Cytokines are endogenous peptides released during an inflammatory event. These biological modulators of inflammation are also released following systemic administration of endotoxin in humans (Kuhns et al. 1995; Michie et al. 1988).They are thought to be responsible for initiating the release of many of the chemical mediators of an acute inflammatory event. As a result, many of the pro-inflammatory cytokines have been used in animal models of septic shock (*in vitro* and *in vivo)* in order to initiate a vascular response. The 3 cytokines used in this thesis, TNFα, IL-1β and IL-6, are known to be released acutely following endotoxin administration (Kuhns et al. 1995). TNFα, IL-1β and IL-6 have all been used in clinical trials involving cancer patients (Chapman et al. 1987; Blick et al. 1987; Smith, 2d et al. 1992; Stouthard et al. 1996). The systemic effects of these agents are therefore well documented. However, only TNFα has been used in healthy volunteers as a means to explore the acute systemic changes in septic shock (Michie et al. 1988). Prior to the experiments in this thesis neither IL-1β nor IL-6 had been administered to healthy volunteers, either locally or systemically. Interestingly, Haefelli and others describe the effects on dorsal hand vein compliance of administering a systemically large bolus dose of IL-1β into patients with malignant melanoma (Haefeli et al. 1993). Following acute administration IL-1β induced a venoconstriction in the dorsal hand vein which was abolished by local

administration of phentolamine suggesting adrenergic activation possibly through the central nervous system. No studies either in patients or in healthy volunteers have looked at the local or systemic effects of combined cytokine administration.

The theoretical ethical considerations for the use of these agents were similar to those involving the use of endotoxin. The systemic effects of administering any of these cytokines resulted in fever, chills, malaise and hypotension in addition to their organ specific effects. The dose of TNFα chosen was based on a calculated dose 100-fold lower than that used by Michie and others in 1988. The doses of IL-1β and IL-6 were calculated to be 10-100-fold lower than those found in the systemic circulation following endotoxin administration in humans (Kuhns et al. 1995). As with the use of endotoxin, all cytokines were initially assessed individually using increasing doses until an effect on vascular tone was recorded. During the early studies pulse rate and temperature were recorded in all subjects and all volunteers were asked to report back to the Clinical Suite the following day for examination of the study vein.

At the time of writing this thesis more than 200 studies involving the use of these cytokines have been performed. On no occasion were any local or systemic adverse effect to instillation of these agents noted. Repeated use of these cytokines in the same subject did not appear to cause any local adverse effect and no local thrombosis or phlebitis occurred.

Chapter 3

Manipulation of the prostanoid pathway

Introduction

Continuous synthesis and release of prostacyclin is thought to contribute to the thromboresistant and vasodilator properties of the blood vessel wall and oppose the vasoconstrictor and prothrombotic effects of platelet-derived thromboxane (Moncada and Vane, 1978; Roth and Calverley, 1994). Aspirin, an irreversible inhibitor of cyclo-oxygenase, blocks the metabolism of arachidonic acid to prostanoids and this is its major mechanism of action (Roth and Calverley, 1994). When used to prevent vascular complications aspirin is given in low doses (Preston et al. 1981) and/or at long dose intervals (Heavey et al. 1985; Ritter et al. 1989) in an attempt to inhibit thromboxane production in platelets while sparing prostanoid production within endothelial cells. The rationale behind low-dose aspirin is that platelet cyclo-oxygenase is preferentially inhibited at low doses of aspirin given orally because platelets circulate and are exposed to a higher concentration of aspirin in the portal circulation before the drug is partially deactivated (Pedersen and FitzGerald, 1984) in the liver.

The rationale behind prolonged dose-intervals is that endothelial cell cyclo-oxygenase may be rapidly re-synthesised *de novo* to overcome the blockade, whereas once platelet cyclo-oxygenase is inhibited by aspirin, new platelets must be produced in order to overcome the blockade (Ritter et al. 1989).

In keeping with these observations, endothelial cells in culture regain the ability to synthesise prostacyclin within 35h of treatment with aspirin (Jaffe and Weksler, 1979) and aortic endothelium removed from rabbits 6h after intravenous injection of aspirin shows significant recovery of prostacyclin production (Frazer and Ritter, 1987). Indirect evidence for preferential inhibition of platelet cyclo-oxygenase after oral dosing is also provided by studies measuring stable metabolites of prostacyclin or thromboxane in blood or urine (Clarke et al. 1991; Hanley et al. 1981; Preston et al. 1981); in healthy volunteers bradykinin-stimulated prostacyclin production appears to return to baseline within 6h of a single 600mg dose of oral or intravenous aspirin whereas thromboxane production remains suppressed (Heavey et al. 1985; Ritter et al. 1989).

Despite these findings, it is not clear whether the amount of prostacyclin (or other prostanoids) produced by human blood vessels is sufficient to alter vascular tone or platelet function *in vivo*. Furthermore, it has become increasingly clear that, at least in animals, endothelial cells may also produce constrictor prostanoids (Pagano et al. 1991). Addition of arachidonic acid to human vessels or vascular cells *in vitro* increases prostanoid production (Meade et al. 1993; Miller and Vanhoutte, 1985; Schror and Seidel, 1988) and in the present study the effects of local infusions of arachidonic acid on the tone of single superficial dorsal hand veins *in vivo* were explored. COX inhibitors were used to determine the contribution of prostanoids to the

responses seen and developed the experimental system to study directly the dose-response and time-course of the vascular effects of aspirin.

Protocol

Studies were performed on male (10) and female (6) subjects aged 19 - 38. In this study (unless stated otherwise), after baseline recordings of stable vein size had been made, veins were preconstricted to between 30-50% of resting diameter by a continuous infusion of noradrenaline (20 - 160 pmol/min) for the remainder of the study. Study drugs were co-infused with noradrenaline. Measurement of vein size was made every 5min.

Effects of arachidonic acid: dose response, reproducibility and effects of local aspirin

In 3 subjects 3 doses of arachidonic acid (0.2nmol/min, 2 nmol/min, and 20 nmol/min, each dose for 5min) were infused into a preconstricted vein. Dose increments were made at 10min intervals. To determine the reproducibility of the response within a single experiment, 6 subjects were given 2 infusions of arachidonic acid (20 nmol/min for 5min) 30-45min apart and after a stable noradrenaline constriction was re-established. In a separate study in the same 6 volunteers, arachidonic acid (20 nmol/min for 5min) was infused into a pre-constricted vein before and after local infusion of aspirin (18μmol/min for 30min).

The effects of arachidonic acid (20 nmol/min for 5min) on unconstricted veins (n=3) and arachidonic acid vehicle on pre-constricted vessels (n=3) were also determined.

Effects, dose-response and time-course of oral aspirin and paracetamol

Subjects were given soluble aspirin (75 mg; n=3 or 1 g; n=3) or paracetamol (1g; n=3) and the response to arachidonic acid (20 nmol/min) determined 2h later. At the end of the study, glyceryl trinitrate (20 pmol/min) was infused for 5 min to determine whether the preconstricted vein would relax to an agent that does not utilise the prostanoid pathway. In a further study, 10 subjects were given high dose aspirin (1g). In 5 the response to arachidonic acid was determined at 0 (pre-dose) 2, 6 and 24h, and in the other 5 at 0 (pre-dose), 2 and 120h after aspirin. The response was measured as the maximal change in vein diameter within 15min of starting the infusion of arachidonic acid. The effects of aspirin on the venodilator drugs bradykinin and glyceryltrinitrate (GTN) were also studied. In 5 subjects dose response curves were constructed to bradykinin (2, 4, 8, pmol/min; each dose for 5 min) and GTN (1, 2, 4, pmol/min; each dose for 5 min) before and 2h after oral aspirin (1g).

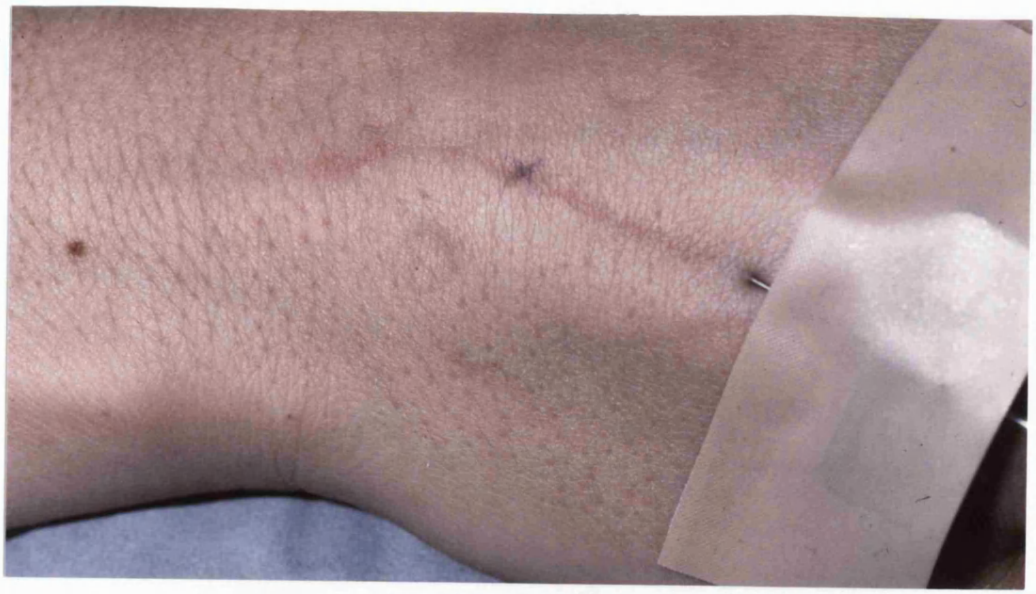
Platelet studies

Blood was drawn for platelet aggregometry in subjects taking part in the studies on oral aspirin (75mg, n=3; 1g, n=13) or paracetamol therapy (1g, n=3). Immediately prior to taking the drug, and 2h later 15ml of venous blood was drawn into a syringe containing 2.5ml of 3.15% (w/v) trisodium citrate. The maximal extent of aggregation was used for data analysis and results after drug administration were compared with control (pre-drug) values.

Results

Local infusion of arachidonic acid caused no adverse or systemic effects and subjects did not report any discomfort. In 7 subjects, after the infusion of arachidonic acid, a red line appeared on the skin overlying the vein and this persisted for up to 20 min (Figure 1).

Figure 1



A red line appeared on the skin overlying the vein within 5-10min infusion of arachidonic acid and persisted for up to 20min after the study.

The internal diameters of veins and the degree of pre-constriction for each part of the study are shown in Table 1.

TABLE 1

STUDY	BASAL VEIN SIZE (mm)	MEAN PRECONSTRICTION (%OF BASAL SIZE)	DOSE (NA) (pmol/min)
DOSE RESPONSE (n=3)	1.4±0.2	50±4	72±12
REPEATED INFUSION OF ARACHIDONIC ACID (n=6)	1.0±0.1	48±4	56±17
LOCAL ASPIRIN (n=6)	0.9±0.4	27±11 (Preaspirin) 55±9 (Post aspirin)*	78±13
ORAL ASPIRIN			
AA study: 1 g (n=13)	1.2±0.3	46±7	84±5
AA study: 75 mg (n=6)	0.7±0.4	50±4	60±15
BK and GTN study:1g (n=5)	1.4±0.3	52±9 (Preaspirin) 50±3 (Post aspirin)	45±5 45±5
PARACETAMOL (n=5)	1.1±0.3	55±11	77±12

The difference in preconstriction before and after infusion of aspirin was significant ($p< 0.05$). However, no such difference was observed in subjects before and after taking high dose (1 g) oral aspirin.

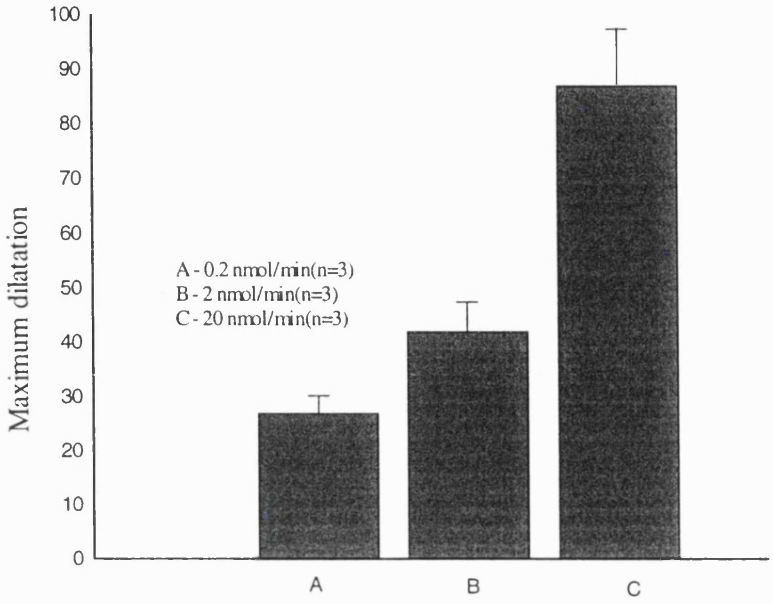
AA = arachidonic acid; BK = bradykinin; GTN = glyceryltrinitrate

Effects of arachidonic acid: dose response and reproducibility

Local infusion of arachidonic acid in 3 subjects produced a dose-dependent venodilatation such that 0.2pmol/min, 2 nmol/min and 20 nmol/min (each dose for 5min) resulted in dilatation of 27±3%, 42±3%, 87±10% respectively.

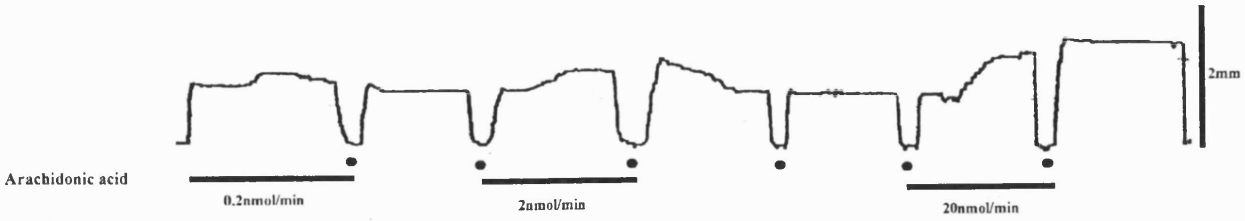
The lower two doses of arachidonic acid produced transient dilatation (lasting less than 10 min, whereas the duration of dilatation in response to the highest dose was in the order of 15 - 25min (Figures 2A, 2C, and 3). Arachidonic acid (20 nmol/min for 5 min) produced identical responses when infused twice in a single experiment (Figure 3) with no evidence of tachyphylaxis (n=6). The maximum dilatation after the first infusion was 62±11% and after the second was 56±7% (ns). 5 subjects participated in more than one part of the study and the response in these individuals gives an indication of the reproducibility of the response to arachidonic acid in a single subject on different days; each subject responded to arachidonic acid on every occasion (mean dilatation 77±4%; coefficient of variation ±20%; 5 subjects each studied on 3 occasions). The time to onset of dilatation to arachidonic acid and the time to reach maximal response varied between individuals and between days (maximal dilatation range 68-84±11%; time to reach maximal response range 5-15 min). Infusion of arachidonic acid vehicle (0.001% alcohol v/v) had no effect on a preconstricted vein. Infusion of arachidonic acid (20 nmol/min) for 5 min to a resting, unpreconstricted, vein produced no change in venous diameter.

Figure 2 A



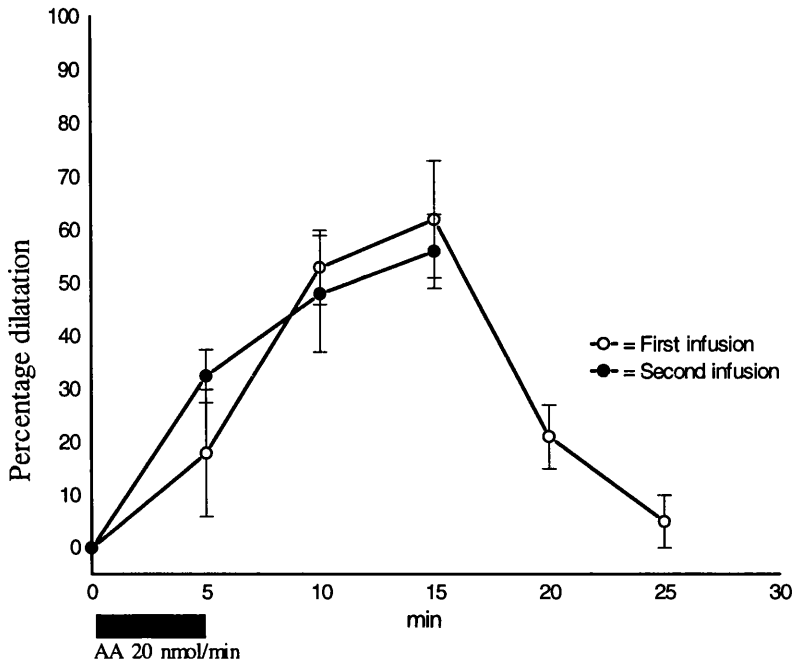
Dose response to arachidonic acid. Each subject received 3 doses of arachidonic acid (n=3). Each dose was given into a preconstricted vein for 5 min and the maximium dilatation recorded. Incremental doses were given at 10min intervals.

Figure 2B



Original trace shows arachidonic acid-induced dilatation. Vein size is recorded when the pressure in a congesting cuff placed around the upper arm is decreased from 40 to 0 mmHg (indicated by the symbol ●).

Figure 2C

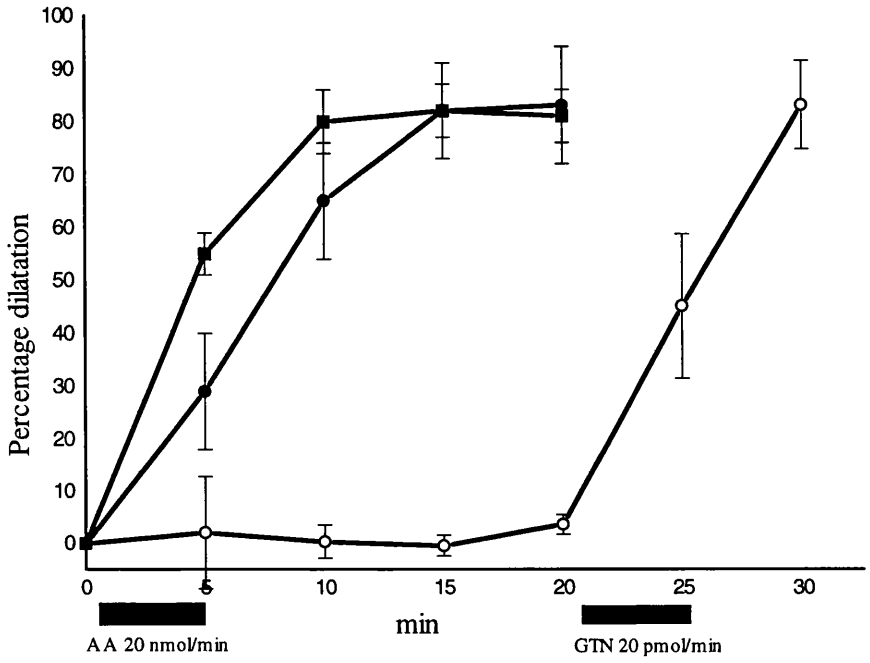


Response of arachidonic acid (AA) infused twice in a single study (n=6). Each 5 min infusion of AA was separated by an interval of at least 30min, and this dilatation persisted for 20min.

Effect of local aspirin

Aspirin (18μmol/min) infused directly into the study vein for 30 min abolished the dilator response to arachidonic acid (maximum dilatation before aspirin was 62±11% and after aspirin 0.5±20%; n=6; $p<0.05$). Assuming a flow in the vein of 0.5-1ml/min this dose of aspirin gives a local concentration in the order of 18-36mM.

Figure 3



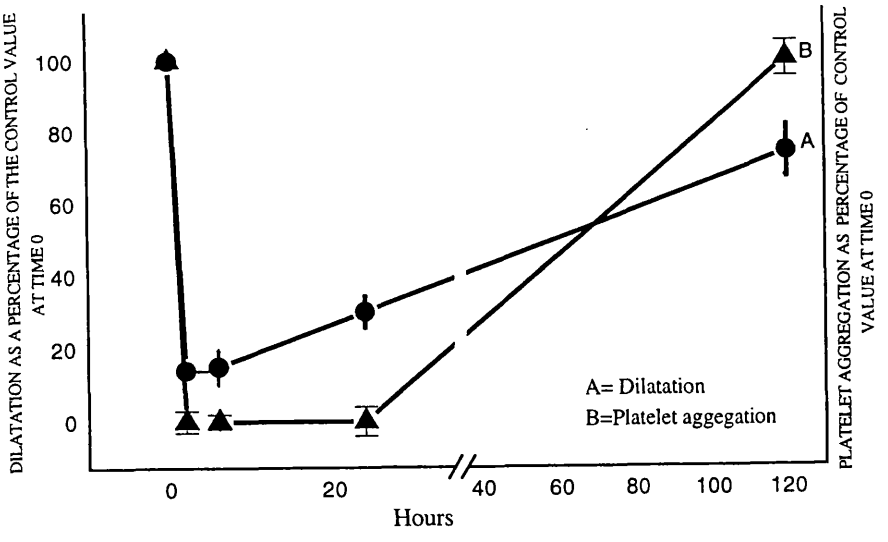
3 subjects received 1g of oral aspirin (O), 6 received 75mg (●), and 5 received 1g of paracetamol (■). The response to arachidonic acid (AA) was determined 2h later. In subjects receiving high dose aspirin a 5min infusion of glyceryl trinitrate (GTN) was given at the end of the experiment. Low dose aspirin and paracetamol did not affect the dilatation to arachidonic acid, whereas the response was abolished with 1g of oral aspirin.

Effects, dose-response and time-course of oral aspirin and paracetamol

Aspirin (75mg) or paracetamol (1g) had no effect on arachidonic acid induced venodilatation (72±20% and 84±14% dilatation respectively; Figure 3). In contrast, oral administration of aspirin (1g) taken 2h before the study inhibited arachidonic acid induced venodilatation (3.6±2% dilatation; n=3). In all

studies involving the use of high dose aspirin (n=13 subjects in total), the dilatation to arachidonic acid before aspirin was 80±11% (n=13) and 2 h after aspirin was 21±5% (n=13; $p<0.05$). Local glyceryltrinitrate (20 pmol/min for 5 min) still produced venodilatation after high dose aspirin (83±8% dilatation; n=3) indicating that the vein was still able to respond (Figure 3). The time-course of the inhibitory effects of oral aspirin on arachidonic acid-induced venodilatation is shown in Figure 4.

Figure 4



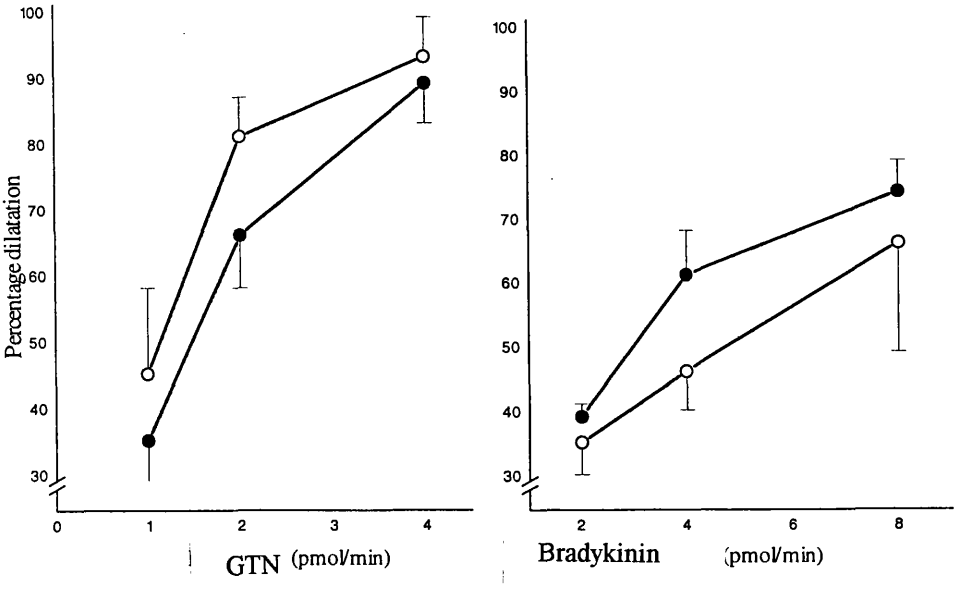
All subjects (n=13) receved 1g of oral aspirin. For studies of dilatation (●), all 13 subjects were studied at 2h, 5 were also studied at 6, 24, and 120h. At 6h and 24h, there was still a significant ($p<0.05$) degree of inhibition to arachidonic acid-induced venodilation, but by 120h the response had returned to 74±11% of the control value (ns). Platelet aggregation studies (■) showed a similar time course of recovery, reaching the control value at 120h.

Oral aspirin inhibited arachidonic acid induced venodilatation at 2, 6 and 24h (14±4, 15±3, 30±7% dilatation respectively). However, 5 days after aspirin, the dilator response to arachidonic acid had returned to 74±11% of the control dilatation (ns).

Effects of oral aspirin on the response to GTN and bradykinin

Oral aspirin (1g) did not alter the constrictor response to noradrenaline. Noradrenaline (45±5pmol/min) produced 55±9% and 40±3% (n=5) constriction for the GTN and bradykinin studies before aspirin and 41±5% and 40±3% 2h after aspirin. The response to bradykinin and GTN was unaltered by oral aspirin (Figure 5). The dilatation to bradykinin 2, 4, 8pmol/min before aspirin was 34±5%, 45±6%, 65±13%, and 2h after oral aspirin was 38±2%, 60±7%, 73±5% respectively. The dilatation to GTN 1, 2, 4 pmol/min was 44±13%, 80±6%, 92±6%, before aspirin and 2h after oral aspirin was 34±6%, 65±8%, 88±6% respectively.

Figure 5

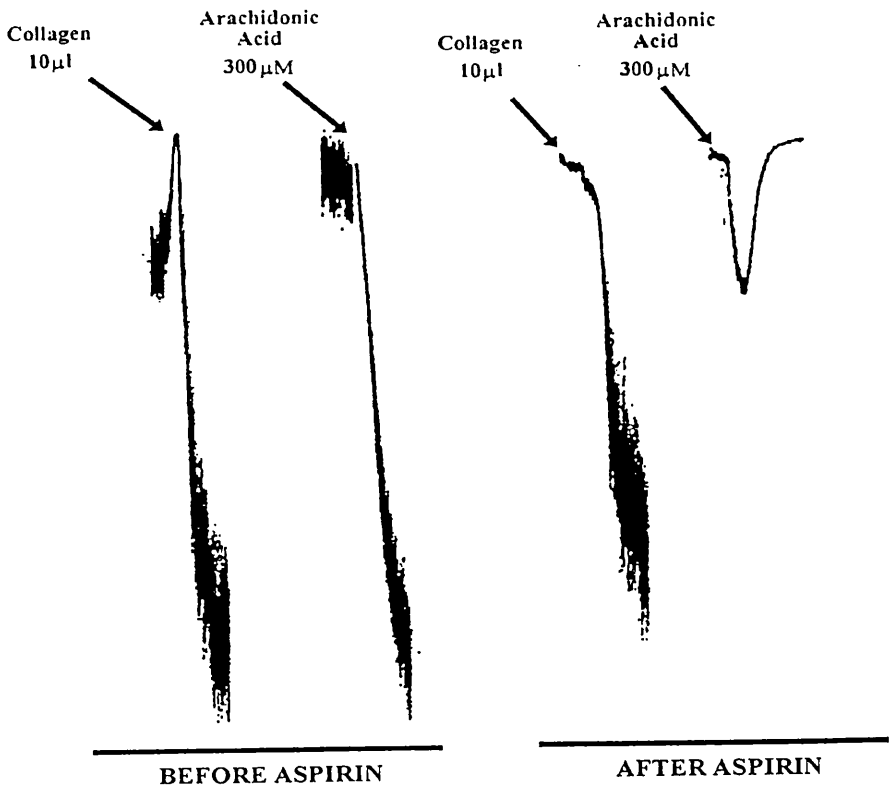


In 5 subjects dose response curves were constructed to bradykinin (left panel) and GTN (right panel) before (O) and 2h after (●) oral aspirin (1g). Oral aspirin did not affect the response to bradykinin or GTN.

Platelet studies

Arachidonic acid-induced platelet aggregation was inhibited by low (n=3) and high dose aspirin (n=13) but not by paracetamol (n=3) (Figure 6). The inhibitory effect of aspirin (1g) was still evident at 24h but not at 120h (Figure 6). The time-course of the effect of aspirin on arachidonic acid-induced platelet aggregation was similar to the time course of the effect of aspirin on arachidonic acid-induced venodilatation (Figure 6). Collagen induced platelet aggregation was not significantly affected by aspirin or paracetamol.

Figure 6



Tracings of platelet aggregation before and after 75mg aspirin: platelet response to 300μM arachidonic acid or 10μl collagen before and 2 hours after 75mg oral aspirin. Low dose aspirin completely abolished aggregation to arachidonic acid. Collagen-induced aggregation was not significantly affected.

Discussion

The results of this study demonstrate that arachidonic acid causes dilatation of human veins *in vivo*. The response was dose-dependent and inhibited by the COX inhibitor aspirin. Together, these findings suggest that provision of substrate is rate- limiting for prostanoid synthesis *in vivo* and that dilator prostanoids predominate, at least in these vessels. Using local infusions of arachidonic acid it was also possible to explore the effects and time-course of drugs on functional effects of endogenous prostanoids.

Arachidonic acid infused directly in a single blood-perfused dorsal hand vein caused dose dependent vasodilatation; there was no evidence of a constrictor response in preconstricted or unconstricted veins and the vehicle alone had no effect. Assuming a blood flow in the vein of 0.5-1ml/min, the dilatation occurred over the concentration range 200nM-20μM, with near maximal dilatation occurring in response to 20μM arachidonic acid. This concentration-range is an order of magnitude lower than that required to stimulate platelet aggregation (200-500μM) (Silver et al. 1973). The duration of the dilatation increased with dose and this might suggest that intracellular accumulation of arachidonic acid occurred during the 5min infusion period. Dose-dependent dilatation to arachidonic acid has also been reported in certain arteries including cerebral arteries of the dog (Toda et al. 1988) and cat (Kontos et al. 1984), coronary artery of the pig (Weintraub et al. 1994), and rat aorta (Dusting et al. 1981; Ezra et al. 1983). The effects occur in the concentration

range 0.1μM-600μM in studies in animal vessels *in vitro*. However, constriction of certain vessels has been reported; arachidonic acid contracts rabbit pulmonary artery (Salzman et al. 1980) and the aorta of hypertensive rats (Lukacsko et al. 1980). The reasons for the differences in response between vessels, between species, and between health and disease (Hill and Smith, 1994) are not known, but might be due to differences in distribution of specific prostaglandin synthetases (Dusting et al. 1978; Levy, 1980).

In the hand veins arachidonic acid-induced vasodilatation was inhibited by local or systemically administered aspirin, suggesting that prostanoids were responsible. Similar results have been found in some studies in animals (Cohen et al. 1973; Dusting et al. 1981; Ezra et al. 1983), but it is also clear that arachidonic acid can produce prostanoid-independent effects in certain vessels including pig coronary artery (Weintraub et al. 1994). It was not possible to determine which prostanoid is responsible for the dilatation of human hand veins but it has been shown previously that these vessels dilate in response to prostacyclin, PGA2, B1, E2 (Robinson et al. 1973), and using radiolabelled arachidonic acid, synthesis of PGD2, PGE2, 6-keto-PGF1 and 13,14-dihyro-15-keto-PGE2 has been demonstrated in the forearm vascular bed (Nowak and Wennmalm, 1979). Thus it seems likely that PGE2, PGD2 or prostacyclin account for the changes observed.

The demonstration of aspirin-inhibitable vasodilatation in humans *in vivo* provides direct evidence for a possible role for prostanoids in the regulation of

vascular tone. Indeed, the degree of dilatation seen was large, and if reproduced systemically would cause profound changes in haemodynamics. However, oral aspirin in doses sufficient to inhibit arachidonic acid induced vasodilatation, did not alter resting vascular tone or the constrictor response to noradrenaline and this argues against basal release of vasoactive amounts of prostanoids. The reason for the decrease in constrictor response to noradrenaline produced by local infusion of aspirin (Table 1) is not clear, but in the mM range aspirin acetylates various plasma proteins, enzymes and DNA (Pinckard et al. 1968) and this might produce non-specific effects on the response to constrictors.

The dilatation to arachidonic acid was used to explore the time-course and dose- dependent effects of aspirin. An anti-inflammatory dose of aspirin (1g) caused abolition of the dilatation whereas a cardioprotective dose (75mg) was without effect. Paracetamol, a drug reported to inhibit COX in the central nervous system but not in the periphery (Flower and Vane, 1972) was also without effect. Despite the different effects of the two doses of aspirin on vascular responses, arachidonic acid-induced platelet aggregation was blocked by aspirin 75mg and 1g. This pattern of responses is consistent with a selective anti-platelet effect of low dose aspirin (75mg) even within 2h of administration.

High dose aspirin (1g) produced a long lasting inhibition of venodilatation, and the time-course of this effect was similar to the inhibitory action of the

drug on platelet aggregation. This finding is in marked contrast to results of certain studies of the time-course of aspirin on biochemical measures of prostanoid production which indicate that circulating metabolites of prostacyclin re-appear in the circulation within 6h of oral dosing, whereas metabolites of thromboxane remain suppressed for 36h or longer (Heavey et al. 1985; Ritter et al. 1989). The reasons for this apparent discrepancy are not known, but might indicate that the dilatation seen was due to prostanoids other than prostacyclin. Alternatively, it may be that the biochemical studies differ because they detect prostacyclin synthesis from many different vessels and non-vascular sources. Consistent with the results of our functional study is the observation that prostanoid production is inhibited in human saphenous vein examined *ex vivo* (Preston et al. 1981) for up to 36h after oral aspirin therapy. Furthermore, smooth muscle COX remains inhibited for at least 24h after aspirin (Moncada et al. 1977), and it is possible that the venodilator prostanoid produced in response to arachidonic acid arises from the smooth muscle cells rather than endothelium.

Jaffe et al. (1979)

Whatever the mechanism, the results with acute administration of oral aspirin to healthy volunteers suggest that the selectivity of aspirin for platelets rather than blood vessels (at least in terms of dilatation) resides in the dose given rather than interval of dosing; low dose aspirin preferentially inhibits platelet thromboxane whilst sparing the synthesis of dilator prostanoids in the vessels wall. It will now be important to determine whether chronic oral dosing with low dose aspirin also spares vascular prostanoid synthesis. These results do

not preclude the possibility that anti-aggregatory prostanoids derived from the vascular endothelium are influenced more by timing of dose than dose itself, but dilator prostanoids are also anti-aggregatory (Moncada and Vane, 1978) and the ability of the vessel to dilate to arachidonic acid after low dose aspirin suggests that it would also retain certain anti-aggregatory properties.

The demonstration that arachidonic acid produces aspirin-inhibitable vasodilatation in humans provides direct evidence for a functional effect of locally generated prostanoids in the vessel wall. The relative simplicity and safety of the experimental system and the reproducibility of the response to arachidonic acid suggest that it may be possible to use this system to explore the role of locally generated prostanoids in disease states, such as hypertension (Lin and Nasjletti, 1992; Lin and Nasjletti, 1991), diabetes (Johnson et al. 1979; Silberbauer et al. 1979), and liver disease (Nanji et al. 1994) where endothelium-derived prostanoids have been implicated, or in inflammatory conditions when the inducible isoform of COX is expressed (Vane et al. 1990). In subsequent chapters the protocols developed here are used to probe the prostanoid pathway under conditions of inflammation.

Chapter 4

The effects of endotoxin on smooth muscle function

Introduction

Septic shock carries a high mortality (Parrillo, 1993). Characteristic haemodynamic changes include hypotension due to arterial and venous dilatation, and impaired cardiac contractility and there are also profound changes in metabolic, respiratory, haematological and host-defence functions (Guc et al. 1990; Parrillo, 1993). Decreased peripheral responsiveness to vasoactive agents contributes to the progressive decline of systemic blood pressure, which ultimately leads to tissue hypoperfusion and circulatory failure (Guc et al. 1990; Snell and Parrillo, 1991; Wright et al. 1992; Parrillo, 1993).

Systemic administration of endotoxin to healthy human volunteers produces cardiovascular changes similar to those seen in animal models; arterial (Suffredini et al. 1989; Gilbert RP, 1960) and venous (Bradley et al. 1945) dilatation and hypotension are seen within 60min and persist for up to 8h (Suffredini et al. 1989; Bradley et al. 1945) but it is difficult to dissect out the mechanisms in whole body studies. Human vessels *in vitro* show inconsistent responses to endotoxin (Thorin-Trescases et al. 1995) and it is possible that the vascular responses seen *in vivo* are dependent upon involvement of other cell types or tissues, increased concentrations of cytokines circulating in blood (Boujoukos et al. 1993), infiltration of inflammatory cells into the vessel wall (Cook et al. 1994) or neurohumoral effects of systemically administered endotoxin (Wang et al. 1991). One approach to gaining insight is to investigate

the local vascular effect of endotoxin, *in vivo*. This is what was attempted in the experiments in this section.

Protocol

Studies were performed on male (14) and female (18) subjects aged 19-38.

Assessment of venous response to noradrenaline

Cumulative dose response curves to noradrenaline (5-1280 pmol/min, each dose increment a doubling of the previous dose) were constructed. Each dose was infused for 5 min and doses were increased until a maximum response was achieved (no further constriction despite a doubling in dose). The response to noradrenaline varies between subjects but is consistent and reproducible within a single study in an individual (Collier et al. 1972). For repeat dose response curves in a single study in order to limit the duration of the study, 4 doses of noradrenaline were selected that produced 0, 20-40, 40-70, 70-100% constriction on the first occasion (these doses varied between individuals and for clarity were designated dose A, B, C and D). Maximum constriction refers to the response to dose D unless otherwise stated. Vein size was recorded in the same place as on the first occasion. Dose response curves to noradrenaline were constructed before and after local administration of endotoxin into an isolated portion of the hand vein.

Single deep breath as a venoconstrictor stimulus

Two adjacent dorsal hand veins were studied simultaneously. One was isolated and received endotoxin whilst the other was left unoccluded. Deep breath manoeuvres were performed before and 1h after endotoxin was instilled.

Instillation of endotoxin

Endotoxin (100 E.U. in 1ml saline) or saline (1ml) was injected into the isolated segment. This dose of endotoxin gives a calculated local concentration of about 20ng/ml, similar to that reported in the blood of patients with severe sepsis (Brandtzaeg et al. 1989). One hour later the contents of the segment were aspirated and the wedges removed. At this stage dose-response curves to noradrenaline were repeated.

Effects of endotoxin: time course (Study 1)

In 12 subjects the time course of the response to endotoxin was explored. Dose response curves were constructed to noradrenaline before, and at 1 and 2h after exposure to endotoxin or saline in 4 subjects, at 3h (after endotoxin only) in 4 subjects, and at 4h (after endotoxin only) in 4 subjects. In 3 further subjects, deep breath-induced venoconstriction was studied before and 1h after endotoxin. Two adjacent veins were compared. One was occluded and received endotoxin whilst the other was left unoccluded. Deep breath studies were performed in

both veins simultaneously, before and 1h after endotoxin. At the end of the study phentolamine (25nmol/min for 20 min) was infused into the control vein and the deep breath response repeated.

In all subsequent studies the response to endotoxin was assessed at 1h after endotoxin and, in order to study the response to drugs that might inhibit or reverse the effects of endotoxin, subjects were selected who in preliminary studies demonstrated a large response to endotoxin (arbitrarily defined as >40% suppression of constriction to noradrenaline).

Effects of endotoxin: local or systemic effect? (Study 2)

To determine whether endotoxin was producing a local rather than a systemic effect, dose response curves to noradrenaline were constructed simultaneously in 2 adjacent veins on the same hand (n=3). One vein was isolated and received endotoxin as before, while the other was left unoccluded. Dose response curves to noradrenaline were constructed in both veins simultaneously before, and 1h after, one vein was exposed to endotoxin.

Effects of repeated administration of endotoxin (Study 3)

In 5 subjects the effects of daily instillation of endotoxin for 3 days into a single vein were explored. On each day dose response curves to noradrenaline were constructed before and 1h after endotoxin. To determine whether the effects of

repeated exposure to endotoxin initiated a local or systemic response, on day 3 an adjacent vein on the same hand (ie a vein that had never previously been exposed to endotoxin) was exposed to endotoxin and a dose response curve to noradrenaline was constructed before and 1h after endotoxin.

Effects of inhibiting COX and/or NO synthase on the response to endotoxin (Study 4)

Five subjects were given soluble aspirin (1g), in a dose that inhibits the dilatation to arachidonic acid (Chapter 3) and the response to endotoxin was determined 2h later. Dose response curves were established to noradrenaline before and 1h after exposure to endotoxin. In a separate set of studies in 5 subjects, a dose response curve to noradrenaline was constructed before and 1h after exposure to endotoxin, and, at the end of the second dose response curve, N^G-monomethyl-L-arginine (L-NMMA; 100nmol/min for 10min) was co-infused with the same dose of noradrenaline that produced the maximum constriction prior to endotoxin. The combined effects of aspirin and L-NMMA were also studied. Five subjects were given oral aspirin (1g) 2h before the study and the response to noradrenaline determined before and at 1h after exposure to endotoxin. In addition, immediately after the second dose response curve to noradrenaline had been constructed, L-NMMA (100nmol/min) was coinfused with noradrenaline and the dose response curve repeated for a third time.

Effects of hydrocortisone on the response to endotoxin and to the development of endotoxin tolerance (Study 5 and 6)

Five subjects received hydrocortisone (100mg) 2h before the study. Dose response curves to noradrenaline were constructed before and 1h after exposure to endotoxin (Study 5).

To determine the effects of the glucocorticoid (Study 6) on repeated daily dosing of endotoxin (for 3 days, as above), 5 subjects were given hydrocortisone (100mg) 2h before the study on day 1 and day 2. On day 3 no steroid was given. Dose response curves to noradrenaline were constructed before and 1h after endotoxin.

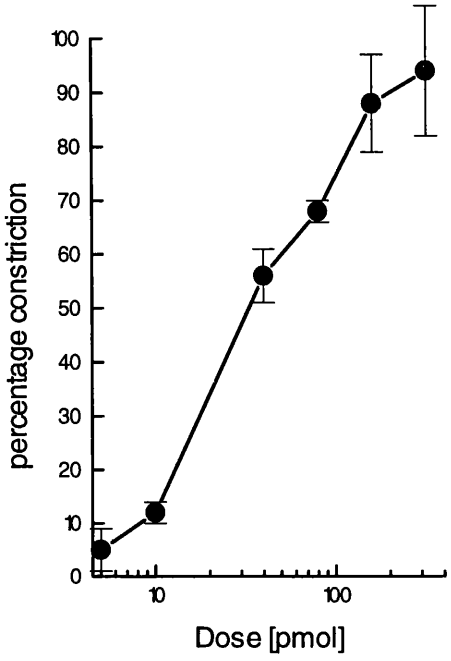
Results

Local instillation of endotoxin into the isolated vein had no effect on resting vein size and caused no local adverse or systemic effects. The average basal vein for each study is shown in Table 1. In each subject noradrenaline produced a dose-dependent venoconstriction (Figure 1) but as is recognised (Collier et al. 1972) there was variability in the response between individuals with the dose producing maximum constriction ranging from 40-1280 pmol/min.

Table 1

Study	Vein size
Study 1:	1.8±0.5mm
Study 2:	1.8±0.4mm
Study 3:	1.6±0.3mm
Study 4:	1.8±0.3mm
Study 5:	1.4±0.3mm
Study 6:	1.7±0.2mm

Figure 1

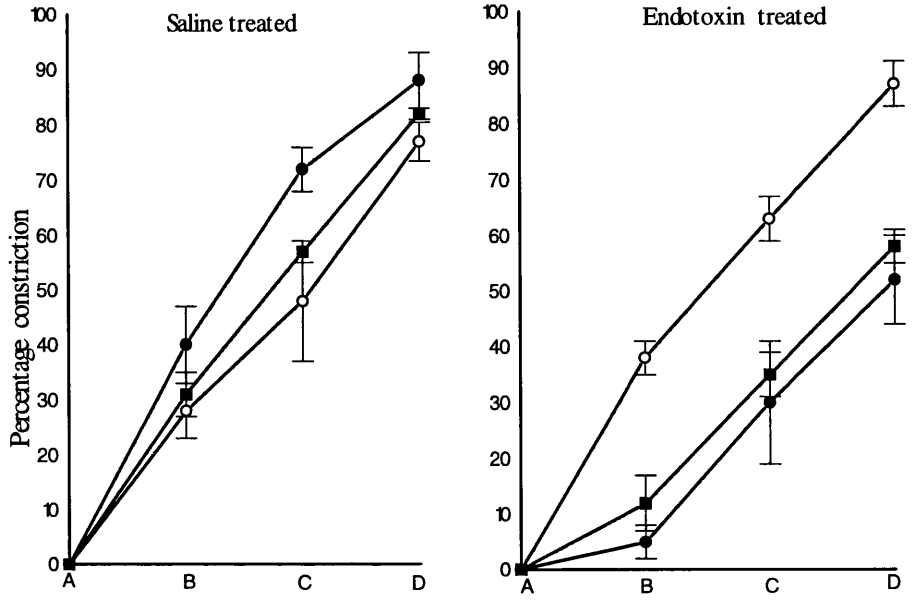


Dose response curve to noradrenaline (n=7)

Effects of endotoxin: response and time course (Study 1)

Local instillation of endotoxin caused a rightward shift in the dose response curve to noradrenaline (for example, the dose of noradrenaline producing a 40±3% constriction before endotoxin produced a 6±3% constriction 1h after endotoxin) and suppressed the maximum constriction achieved (Figure 2).

Figure 2



In 4 subjects dose response curves to noradrenaline (NA) were constructed before and at 1 and 2h after the vein was exposed to endotoxin (n=4) or saline (n=4). For the repeat dose response curves to NA at 1 and 2h after saline or endotoxin, 4 doses of NA were selected that produced 0, 20-40, 40-70, 70-100% constriction on the first occasion (designated A, B, C, D).

Left Pane : A = 23±19 pmol/min
B= 55±40 pmol/min
C= 208±148 pmol/min
D= 425± 292 pmol/min

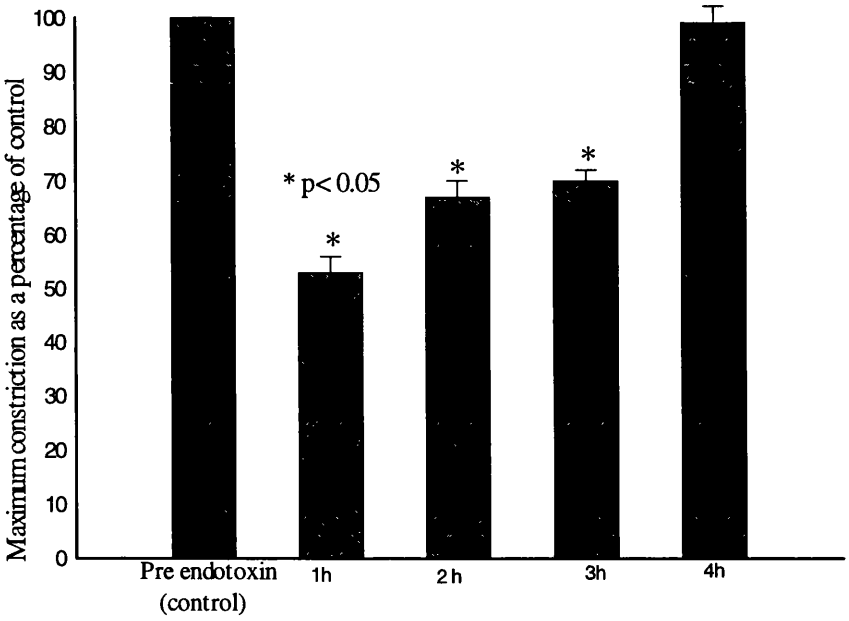
Right Panel: A= 6±2 pmol/min
B= 41±22 pmol/min
C= 93± 41 pmol/min
D= 220± 60 pmol/min

○ = Before saline or endotoxin
● = 1h after saline or endotoxin
■ = 2h after saline or endotoxin

In 7 subjects the dose of noradrenaline given after endotoxin was increased in an attempt to produce full constriction. In these subjects, maximum constriction (89±11%) was produced in response to 623±64pmol/min before endotoxin but 1h after endotoxin the constriction reached a plateau at 52±14% and even doses up to 2560pmol/min had no further effect. Attenuation of the noradrenaline response was seen at 1, 2 and 3h after endotoxin but by 4h the venoconstriction potency of noradrenaline was fully restored (Figure 3). The maximum constriction to noradrenaline (dose D) before endotoxin was 87±4% and 1h later was 52±8% ($p<0.05$). In contrast, the maximum constriction to noradrenaline 4h

after endotoxin was back to 99±0.2% of the control value (ns; Figure 3). The effect was specific for endotoxin since there was no change in the dose response curve to noradrenaline at 1 or 2h after instillation of saline (maximum constriction before saline: 77±4%, 1h later, 88±5% and 2h later, 82±2%; Figure 2).

Figure 3

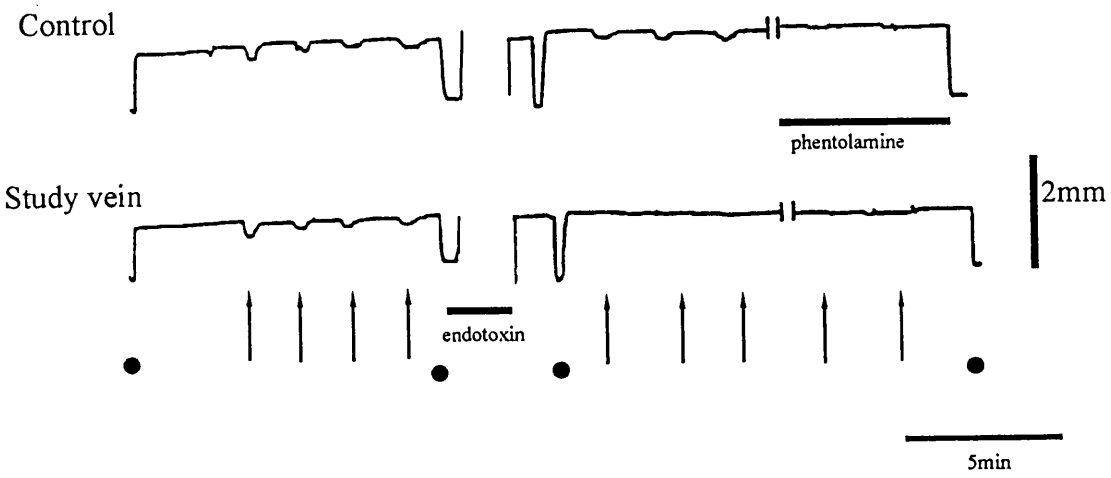


In 12 subjects the time course of the response to endotoxin was explored. Dose response curves were constructed to noradrenaline (NA) before and at 1 (n=4), 2 (n=4), 3 (n=4), and 4 (n=4) hours after exposure to endotoxin. Results are expressed as maximum percentage constriction to NA relative to the initial control value for that study.

The potential physiological significance of attenuation of the constrictor response to noradrenaline is illustrated in the 3 subjects who took part in the study of deep breath responses. Deep breath produced simultaneous transient venoconstriction in both veins (13±4% constriction. Figure 4). After endotoxin,

the constrictor deep breath response was abolished in the treated (0% constriction) but not in the control vein (11±3% constriction). Infusion of phentolamine (25nmol/min for 20 min) into the control vein abolished the deep breath response in this vessel (Figure 4), confirming that the constriction produced by this manoeuvre was due to sympathetic nervous system activation and noradrenaline release.

Figure 4

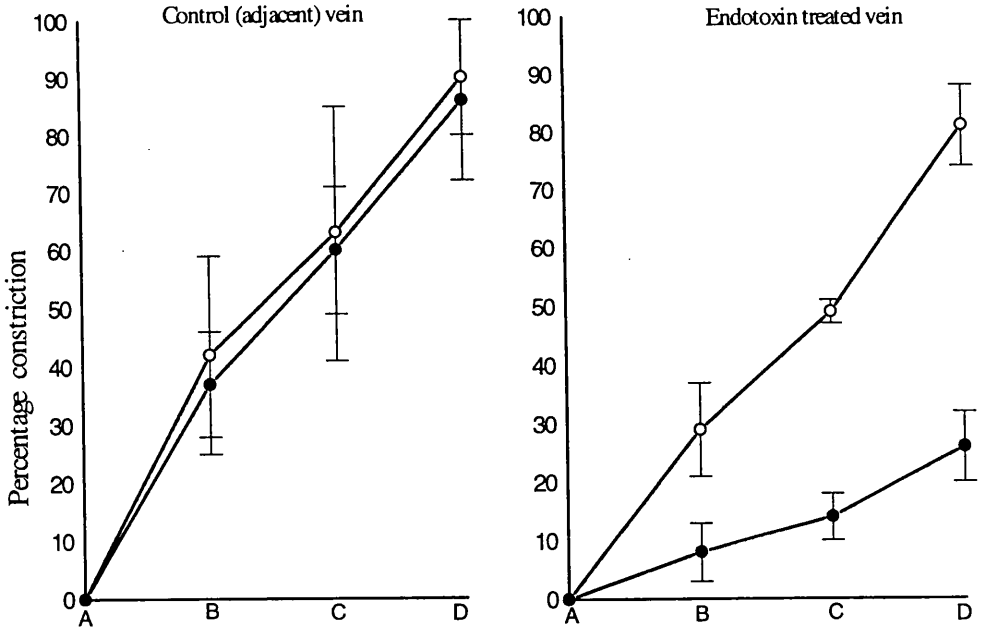


In 3 subjects, sympathetically mediated venoconstriction responses were assessed simultaneously in 2 adjacent veins. The control vein (top panel) was left unoccluded while the other vein was isolated and received endotoxin (lower panel). Deep breath (↑) venoconstrictor responses were performed simultaneously in both veins before and 1h after instillation of endotoxin. A deep breath produced simultaneous transient venoconstriction in both veins. After endotoxin, the constrictor deep breath response was abolished (lower panel). Finally, in the control vein, phentolamine (25nmol/min for 20 min) was infused, and the deep breath response was assessed again (top panel).

Effects of endotoxin: local or systemic effect? (Study 2)

In experiments comparing two adjacent veins on the same hand, there was a rightward shift of the noradrenaline dose response curve in the vein receiving endotoxin and suppression of the maximum constriction at 1h (maximum constriction before endotoxin, 81±7% and after endotoxin 26±6%; $p<0.05$). In contrast, in the control vein there was no change in the response to noradrenaline at the same time points (maximum constriction at time 0, 86±13% and at 1h after the instillation of endotoxin into the adjacent vein, 90±10%; Figure 5 and 6).

Figure 5



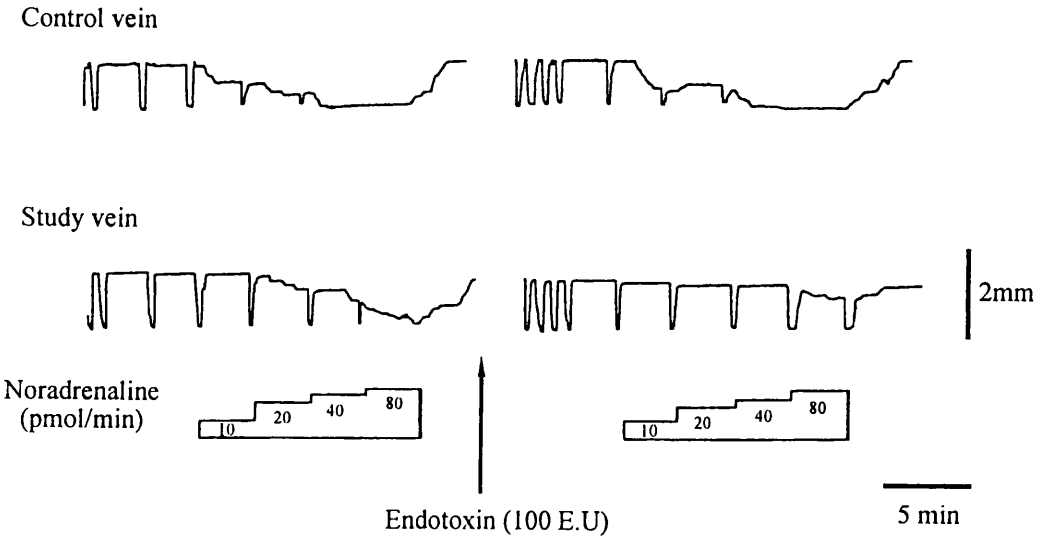
In 3 subjects dose response curves were constructed simultaneously in 2 adjacent veins. The control vein was left unoccluded while the other vein was isolated and received endotoxin. Dose response curves to NA were constructed in both veins before and 1h after one vein was exposed to endotoxin. For the repeat dose response curves to NA at 1 and 2h after saline or endotoxin, 4 doses of noradrenaline were selected that produced 0, 20-40, 40-70, 70-100% constriction on the first occasion (designated A, B, C, D).

Left Panel: A= 4±1 pmol/min
B= 10±0 pmol/min
C= 40± 0 pmol/min
D= 107± 27 pmol/min

Right Panel: A= 7±2 pmol/min
B= 30±10 pmol/min
C= 67± 14 pmol/min
D= 160± 0 pmol/min

O= Before endotoxin
●= 1h after endotoxin

Figure 6



A typical trace of the results shown in Figure 5.

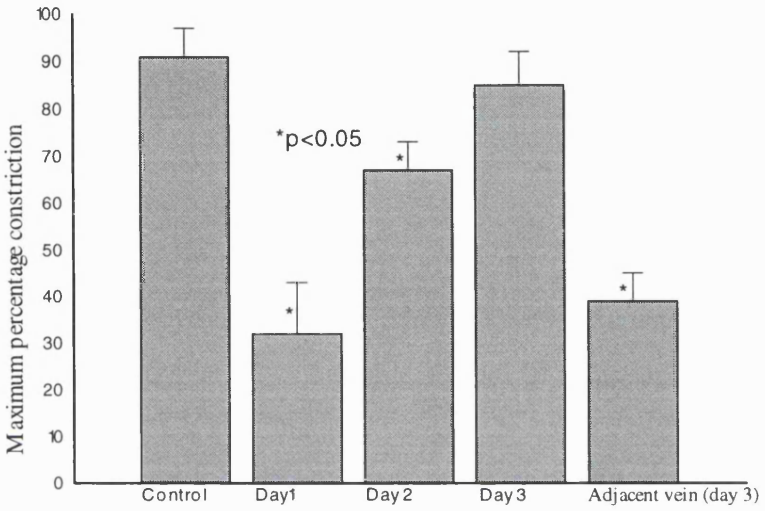
Two adjacent veins were studied. One was left unoccluded (top panel) while the other vein was occluded and received endotoxin (lower panel). Dose response curves to noradrenaline were constructed in both veins simultaneously before and 1h after endotoxin.

Effects of repeated administration endotoxin (Study 3)

Instillation of endotoxin into the same vein on 3 consecutive days resulted in the development of tolerance to the effects of endotoxin such that by day 3 there was no shift in the dose response curve to noradrenaline after endotoxin (maximum constriction before endotoxin: day 1, 91±6%; day 2, 98±2%; day 3, 94±4%. 1h after instillation of endotoxin: day 1, 32±11%, $p<0.001$; day 2, 67±6%, $p<0.05$; day 3, 85±7%, ns. Figure 7). In contrast, on day 3, the adjacent

vein which had not been exposed to noradrenaline previously, still demonstrated sensitivity to the effects of endotoxin; maximum constriction before endotoxin, 94±4% and 1h after endotoxin, 39±6%, Figure 7).

Figure 7



In 5 subjects the effects of repeated instillation of endotoxin for 3 days into a single vein were explored. On each day dose response curves to NA were constructed before and 1h after endotoxin. To determine whether the effects of repeated exposure to endotoxin initiated a local or systemic response, on the 3rd day an adjacent vein on the same hand was exposed to endotoxin and a dose response to NA was constructed before and 1h after endotoxin.

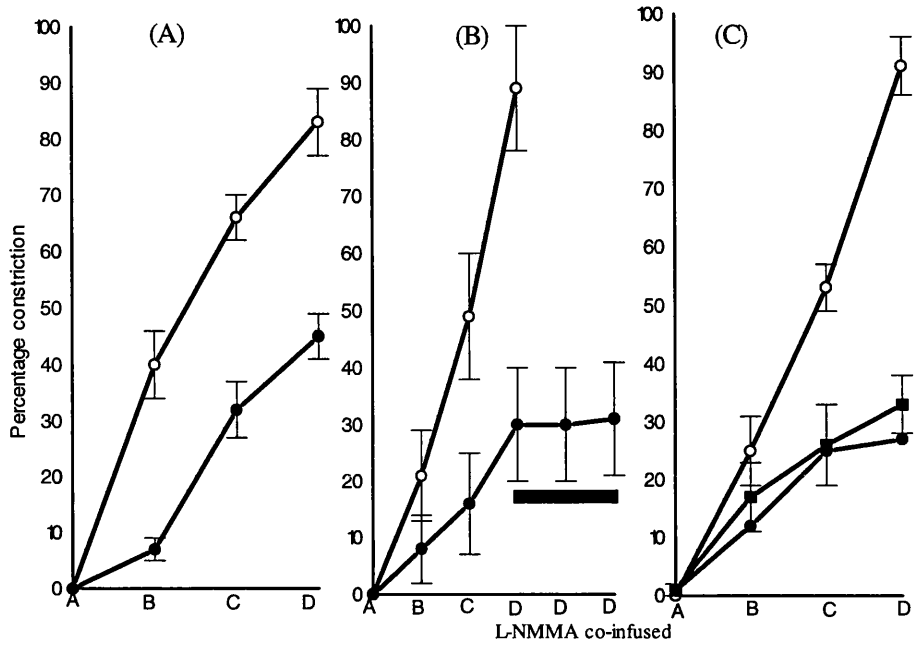
Effects of COX and/or NO synthase inhibitors on the response to endotoxin (Study 4)

Neither aspirin nor L-NMMA modified the shift in the noradrenaline response induced by endotoxin (Figure 8).

L-NMMA co-infused for 10min with a dose of noradrenaline (123±66pmol/min) that produced maximal constriction before endotoxin did not alter vein size (maximum constriction before endotoxin, 88±11%, after endotoxin, 30±10%, and after endotoxin and in the presence of L-NMMA for 10min; 31±10% $p<0.05$; Figure 7).

Similarly, the combination of aspirin (1g) taken 2h before the study plus L-NMMA co-infused with noradrenaline did not affect the endotoxin-induced dose response shift to noradrenaline (maximum constriction before endotoxin 90±5%, 1h after endotoxin 33±5% and 1h after endotoxin and in the presence of aspirin and L-NMMA: 27±5% n=5; $p<0.05$; Figure 8).

Figure 8



(A) 5 subjects were given soluble aspirin (1g) and the response to endotoxin determined 2h later. Dose response curves were established to NA before and 1h after exposure to endotoxin.
A= 7±2 pmol/min B= 40±11 pmol/min C= 224± 113 pmol/min D= 464± 220 pmol/min

(B) In 5 subjects a dose response curve to NA was constructed before and 1h after endotoxin. At the end of the 2nd dose response curve L-NMMA (100nmol/min for 10 min) was co-infused with the same dose of NA that produced the maximum constriction prior to endotoxin.
A= 7±2 pmol/min B= 23±7 pmol/min C= 41± 9 pmol/min D= 124± 66 pmol/min

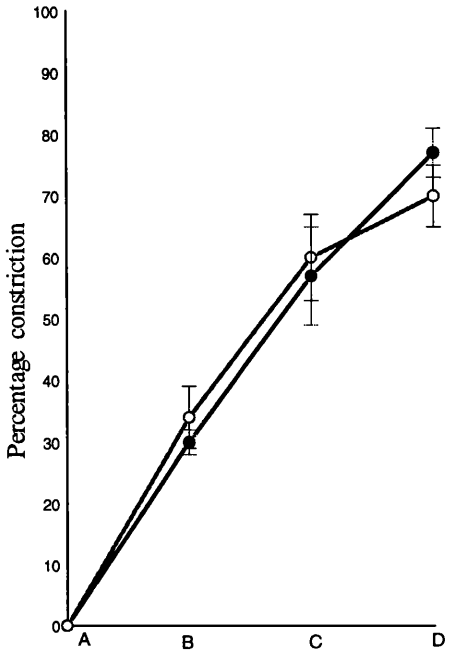
(C) The combined effects of L-NMMA and aspirin were studied in 5 subjects. All subjects received oral aspirin (1g) 2h before the study and the response to noradrenaline was determined before and 1h after exposure to endotoxin. In addition, immediately after the 2nd dose response curve to NA had been constructed, L-NMMA (100nmol/min) was co-infused with NA and the dose response curve repeated for a 3rd time.
A= 8±1 pmol/min B= 32±5 pmol/min C= 80± 18 pmol/min D= 173± 32 pmol/min

O = Before endotoxin ● = 1h after endotoxin ■ = 1h after endotoxin with L-NMMA co-infused

Effects of hydrocortisone on the response to endotoxin and development of tolerance (Study 5 and 6)

Oral hydrocortisone (100mg) taken 2h before the study abolished the endotoxin-induced shift in the response to noradrenaline at 1h (maximum constriction before endotoxin, 77±4% and 1h after endotoxin, 70±5%; Figure 9).

Figure 9



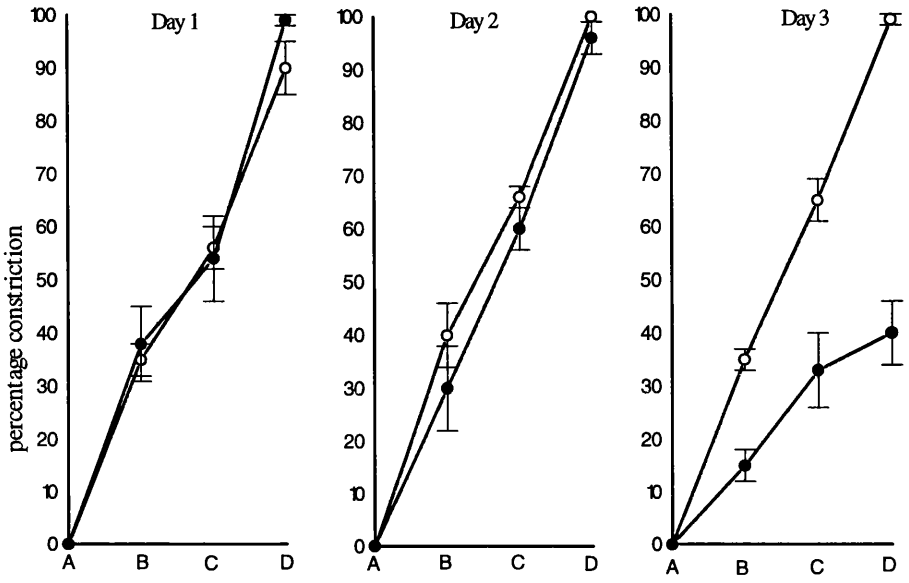
In 5 subjects oral hydrocortisone (100mg) was taken 2h before the study and the response to endotoxin determined 2h later. Dose response curves were established to NA before and 1h after exposure to endotoxin.

A= 6±1 pmol/min B= 28±11 pmol/min C= 57± 22 pmol/min D= 133± 39 pmol/min

O = Before endotoxin ● = 1h after endotoxin

Hydrocortisone, taken 2h before each study, prevented the development of hyporesponsiveness to noradrenaline on day 1 and day 2. On day 3 (in the absence of steroid) endotoxin caused a rightward shift in the dose response curve to noradrenaline (maximum constriction before endotoxin: day 1, 89±5%, day 2, 99±1%, day 3, 100±0%; and 1h after endotoxin: day 1, 99±1%, day 2, 97± 3%, day 3, 40±6%; $p<0.05$, Figure 10).

Figure 10



On 2 consecutive days 5 subjects were given oral hydrocortisone (100mg) 2h before the study. Dose response curves to NA were constructed before and 1h after endotoxin. On day 3 no steroid was given and the dose response curve to NA established before and 1h after endotoxin.

Day 1: A = 7±1 pmol/min B = 18±2 pmol/min C = 36±4 pmol/min D = 88±20 pmol/min
Day 2: A = 9±1 pmol/min B = 36±11 pmol/min C = 72±23 pmol/min D = 144±46 pmol/min
Day 3: A = 8±1 pmol/min B = 28±5 pmol/min C = 56±10 pmol/min D = 112±20 pmol/min

O = Before endotoxin ● = 1h after endotoxin

Discussion

Injection of endotoxin into animals or humans causes systemic arterial (Suffredini et al. 1989) and venous (Bradley et al. 1945) dilatation and a fall in blood pressure, similar to the changes seen in patients with septic shock. The results of the present study provide direct evidence for a local vascular action of endotoxin in human veins *in vivo* and demonstrate that this effect is suppressed by a glucocorticoid. Using a system in which drugs are given into a single

superficial vein in very low doses sufficient only to produce changes in the study vessel, endotoxin attenuated the constrictor response to noradrenaline, causing a shift in the dose response curve and suppressing the maximum constriction achieved. This effect was greatest 1h after exposure to the endotoxin and had waned by 4h, by which time the noradrenaline response had fully returned. Pre-treatment with oral hydrocortisone abolished the endotoxin-induced hyporesponsiveness to noradrenaline; however, the local vasoactive mediators nitric oxide and prostaglandins appeared not to contribute to the changes seen. Repeated exposure to endotoxin induced tolerance.

Noradrenaline released from sympathetic nerves is the major determinant of venous tone (Lewis and Landis, 1935) and these studies suggest that systemic administration of endotoxin causes venodilatation by inhibiting constriction induced by neuronally-derived noradrenaline. This suggestion is supported directly by the observation that the venoconstriction produced by activation of the nervous system by deep breath was abolished by treatment with endotoxin.

Hypotensive and vasodilator effects of endotoxin in healthy volunteers have been reported previously (Suffredini et al. 1989), but it has not been determined whether these effects are due to a systemic reaction to endotoxin or direct effects on the vessels themselves. Indeed, following intravenous injection of endotoxin a series of systemic effects occurs including pyrexia, altered white cell count, increased concentration of cytokines circulating in blood and activation of coagulation factors (Mechanic R et al. 1962; Suffredini et al. 1989; Hosford et

al. 1989; Boujoukos et al. 1993). In the present study a segment of the vein was isolated from the circulation and the endotoxin was instilled locally for 1h and then withdrawn, at which time the vessel was opened again to the circulation. The hyporesponsiveness to noradrenaline produced by this manoeuvre was due to the endotoxin since instillation of sterile saline had no effect. Several observations support the conclusion that the effect of endotoxin was local rather than systemic: the total dose of endotoxin instilled and then removed was less than the minimum pyrogenic dose (Hochstein et al. 1983); subjects experienced no systemic symptoms; and, most conclusively, reactivity of an adjacent, untreated, vein on the same hand was unaltered.

Hyporesponsiveness to noradrenaline was evident 1h after endotoxin and persisted for at least 3h. This rapid effect is consistent with the acute effects of endotoxin seen in certain animal models (Gilbert RP, 1960) and with the time course of the haemodynamic changes that occur in healthy volunteers after systemic administration of endotoxin (Suffredini et al. 1989). The effects of endotoxin persisted for more than 2h after the endotoxin had been removed, at a time when the vessel was no longer exposed to this stimulus. This suggests that exposure to endotoxin triggered longer lasting pharmacological and biochemical changes in the vessel wall.

The effects of endotoxin could be abolished by prior administration of oral hydrocortisone indicating that whatever mediates the response to endotoxin is suppressed by an anti-inflammatory dose of glucocorticoid. Studies in animals

and *in vitro* have demonstrated that exposure to endotoxin leads to the delayed expression of iNOS and COX-II, a process that is inhibited by glucocorticoids (Thiemermann and Vane, 1990; Paya et al. 1993). Furthermore, blocking activity of iNOS or COX-II (Hom et al. 1995) reverses many of the vascular effects of endotoxin indicating that the products of these enzymes, nitric oxide and prostanoids, contribute to the changes seen. However, in the hand veins, although glucocorticoids were effective, neither the nitric oxide synthase inhibitor L-NMMA nor the COX inhibitor aspirin had any effect on the hyporesponsiveness to noradrenaline. Thus nitric oxide or prostanoids are unlikely to contribute to the response to endotoxin that was detected in these veins. This finding is consistent with certain previous studies of the acute response to endotoxin in animals (Paya et al. 1993) and a study of the delayed effects of endotoxin on human saphenous veins *in vitro* (Thorin-Trescases et al. 1995). It is unlikely that an insufficient dose of L-NMMA was used since this dose has been demonstrated to attenuate the response to bradykinin [Chapter 6 and 7] (an agent whose actions in the hand vein are known to be mediated by nitric oxide(Vallance et al. 1989)). The studies performed in the previous chapter demonstrated that the dose of aspirin used was sufficient to inhibit the dilatation to arachidonic acid. Thus the mechanism of the change observed in the veins remains uncertain but possibilities include direct stimulation of guanylate cyclase (Beasley and McGuiggin, 1994), local generation of platelet activating factor (PAF), leukotrienes, cytokines, isoprostanes or other glucocorticoid suppressible inflammatory mediators (Hosford et al. 1989;

Michie et al. 1988; Boujoukos et al. 1993; Larkin et al. 1995). Further studies with specific antagonists of these mediators are now needed.

By 4h the effects of endotoxin had disappeared. This contrasts with the effects seen when endotoxin is administered systemically, when the hypotension persists for 8h or longer (Suffredini et al. 1989). The reasons for this are not known, but might include persistence of endotoxaemia after systemic administration, differences between arteries and veins or between vascular beds, or that systemic rather than direct local effects of endotoxin contribute to the delayed and more long lasting response to endotoxin. In animals studies, the chronic vascular response to endotoxin seems to be largely due to expression of iNOS (Wright et al. 1992; Cook et al. 1994; Hom et al. 1995) in the vessel wall, a process that takes longer than 2-3h to develop fully and might in part be due to a systemic response due to cytokines or infiltration into the vessel wall of activated circulating macrophages (Cook et al. 1994). Such a systemic response would have been absent in our study and it should now be possible to dissect out which mechanisms are generated locally and which are systemic in origin.

Repeated daily exposure to endotoxin for 1h for 3 consecutive days induced tolerance to the effects of endotoxin by the third day. Again this effect appeared to be a local phenomenon since at a time when the treated vein was tolerant to endotoxin, an adjacent vein on the same hand was not. Interestingly, tolerance to endotoxin on day 3 did not occur when the biological response (hyporesponsiveness to noradrenaline) on day 1 and 2 had been blocked by prior

administration of hydrocortisone. These results suggest that tolerance occurs as a result of a down-regulation or depletion of pathways mediating the response to endotoxin rather than down-regulation of the endotoxin receptors. This is consistent with studies performed *in vitro* on macrophage and monocyte cell-lines demonstrating tolerance occurring as a result of decreased endotoxin-induced expression of mRNA for TNF and decreased G protein function (Larsen and Sullivan, 1984), with no alteration of endotoxin receptors (Fahmi and Chaby, 1993). These results do not support the suggestion that **systemic** elevation of endogenous glucocorticoids is the mechanism of vascular tolerance to endotoxin (Szabo et al. 1994). There is interest in the possibility of utilising endotoxin tolerance therapeutically in high risk patients (Astiz et al. 1995). The results of the present study suggest that tolerance to vascular effects of endotoxin is achievable but indicate that it may not be possible to induce tolerance without first eliciting the biological response.

The finding that endotoxin produces an acute venous hyporesponsiveness to the constrictor effects of noradrenaline and to activation of the sympathetic nervous system, might account for the changes in cardiac filling pressure that occur in septic shock (Snell and Parrillo, 1991; Parrillo, 1993). This condition is associated with profound venodilatation despite high circulating levels of catecholamines and activation of the sympathetic nervous system (the major determinant of venous tone) (Chernow et al. 1982). The observation that the hyporesponsiveness to noradrenaline is inhibited acutely by glucocorticoids but appears not to involve prostanoids or nitric oxide suggests that other

unidentified mediators contribute to the overall vascular response to endotoxin. This suggestion is supported by the finding that animals lacking the gene encoding for inducible nitric oxide synthase (iNOS) still show a degree of hypotension to endotoxin (MacMicking et al. 1995) and that the acute hypotensive effect of endotoxin remains unaltered in iNOS knockout mice (D. Rees; personal communication).

Chapter 5

The effects of cytokines on smooth muscle function

Introduction

In the previous Chapter experiments were described which explored the effects of endotoxin on the venous response to noradrenaline. Endotoxin produced a transient hyporesponsiveness to noradrenaline and the effect was not mediated by NO or prostanoids. However, when systemic endotoxaemia occurs several cytokines are generated and it is possible that these cytokines are responsible for the delayed hypotensive response to endotoxin. In this Chapter experiments are described that were designed to investigate the effects of IL-1β, TNFα and IL-6 on the venoconstrictor response to noradrenaline. Pharmacological and molecular studies were undertaken to identify the mechanisms of the changes seen and specifically to determine the role of NO synthase isoforms.

Protocols

Dose response curves to noradrenaline were constructed before and after exposure of the vessel to cytokines. Cytokines were instilled using the same technique as described in Chapter 4.

In all studies with cytokines the doses used were:
TNFα: 1ng
IL-1β: 1ng
IL-6: 100pg

Constrictor stimuli

(A) *Dose response curves to noradrenaline*

In 35 subjects constrictor dose response curves to noradrenaline (10-640pmol/min) were constructed before and at 1, 6, 24h, after instillation of either TNFα alone (n=5), IL-1β alone (n=5), IL-6 alone (n=5), TNFα and IL-1β together (n=5), or TNFα, IL-1β and IL-6 together (n=5) [a 48h dose response curve was also constructed when all 3 cytokines were added].

(B) *Sympathetic nervous system activation*

Deep breath venoconstrictor response was assessed in two adjacent dorsal hand veins studied simultaneously. One was isolated and received IL-1β whilst the other was left unoccluded. Six hours after instillation of IL-1β into the study vein, a local infusion of L-NMMA (1μmol/min) was administered into both veins simultaneously and the deep breath response repeated.

Effects of NO synthase inhibitors and glucocorticoids on the response to cytokines

L-NMMA and aminoguanidine

In 10 subjects constrictor dose response curves to noradrenaline were constructed before and 1, 6 and 24h after instillation of a combination of TNFα, IL-1β and IL-6. At the 6h time point, L-NMMA (1μmol/min; n=5) or

aminoguanidine (1μmol/min; n=5) was co-infused with noradrenaline and the dose response curve to noradrenaline repeated. L-arginine (1μmol/min) then was co-infused with noradrenaline and a repeat dose response curve constructed.

Hydrocortisone

Subjects were given oral hydrocortisone (100mg) 2h before the study and constrictor dose response curves were constructed before and 1h after instillation of TNFα, IL-1β and IL-6 (n=5).

eNOS activation

To determine the effects of L-NMMA and aminoguanidine on eNOS activity, a dilator dose response curve to bradykinin (2, 4, and 8 pmol/min; each dose for 5 min) was constructed in 5 subjects. The dose response curves were then repeated in the presence of L-NMMA (1μmol/min; n=5) or aminoguanidine (1μmol/min; n=5).

Effects of tetrahydrobiopterin (BH_4) infusion

In 6 subjects BH_4 (250μmol/min for 20min) was administered twice in a single study. The 1st infusion was given once a stable preconstriction had been achieved. At the end of this infusion the vein was reconstricted and when a stable constriction was re-established the second infusion of BH_4 was administered. In the same subjects (on a different occasion), during the 2nd

infusion, aminoguanidine (1μmol/min) was co-infused with BH_4 for the last 10 min.

Vein biopsy

In 4 subjects, a combination of TNFα, IL-1β and IL-6 was instilled for 1h. Three hours later the portion of the vein that was occluded and had received the cytokines was surgically removed under local anaesthesia (1% lignocaine). In 5 control subjects that did not receive cytokines, a portion of a single dorsal hand vein was removed under the same conditions. All samples were handled minimally and immediately frozen to -80^0C following dissection.

Detection of nitrite *in vitro*

Human umbilical vein endothelial cells (HUVECs) were incubated with TNFα (1ng), IL-1β (1ng), and IL-6 (100pg) for 1, 3, 6 and 24h. In a separate series of studies the same protocol was repeated but in the presence of L-NMMA (1mM)). The supernatant was then analysed for nitrite production by chemiluminescence (Palmer RMJ et al 1987). 100μl of the cell free supernatant was injected into a reaction vessel containing 90ml of glacial acetic acid and 6% sodium iodine: 3:1. NO formed from nitrite in the refluxing mixture was reduced in a steam of N_2, mixed with ozone and the chemiluminescent product measured with a photomultiplier and converted to

pmol of NO by reference to a nitrite standard curve. Studies were performed in triplicate.

Detection of eNOS protein in HUVECs by fluorescence-activated cell sorter (FACS)

HUVECs were incubated for 24 h in the presence of IL-1β (1ng), TNFα (1ng) and IL-6 (100pg). The cells were then trypsinised with 0.025% trypsin/0.01%EDTA. Membrane permeabilisation to immunostain cytosolic eNOS was carried out by resuspending the cell pellet with 5mg/ml octyl β-n glucopyranoside for 10 min. For the immunostaining, 10^6 HUVECs/ml were kept in FACS' buffer (PBS+5% foetal calf serum + 0.02% azide) at 4°C and divided into 3 groups for incubation with the first antibody:

1. Control group: Isotype-matched unrelated mouse IgG antibody.
2. Second group: Monoclonal antibody against a 20.4 kDa protein from the amino acid fragment 1030-1209 of human eNOS (Transduction Laboratories).
3. Third group: Monoclonal antibody against eNOS pre-absorbed with 8μg/ml of the neutralising eNOS protein extract.

After 30 min incubation at 4°C with the first antibody, cells were washed with cold FACS' buffer, centrifuged at 200g for 5 min and all cell groups were incubated with an FITC-conjugated sheep anti-mouse IgG F(ab')2 antibody for another 30 min at 4°C. The eNOS positive cells were analysed by the FACScan. Rabbit polyclonal antibody (Insight Biotechnology) against the

amino acids 1135-1153 mapping at the carboxy terminus of iNOS was used to detect the presence of iNOS protein.

Detection of mRNA

Exraction from veins

Following surgical excision of the study vein. Poly-A mRNA was extracted from the tissue using the Invitrogen MicroFastrack kit according to the manufacturer's instructions and stored as a suspension in ethanol/sodium acetate at -70°C.

Exraction from HUVECs

Following treatment with 0.025% trypsin and resuspension in PBS, the cells were centrifuged to produce a cell pellet. Poly-A mRNA was extracted using the Invitrogen MicroFastrack kit according to the manufacturer's instructions and stored as a suspension in ethanol/sodium acetate at -70°C.

Reverse transcription and PCR

First strand cDNA was synthesised in a volume of 20µl using random primers and a recombinant reverse transcriptase (Superscript II, Gibco BRL) according to the manufacturer's protocol. 1µl of the reaction mixture was used as a template for PCR using gene specific primers for human eNOS (5'-AGTGTCCAACATGCTGGAAATCAAATTG-3'[sense] and 5'-

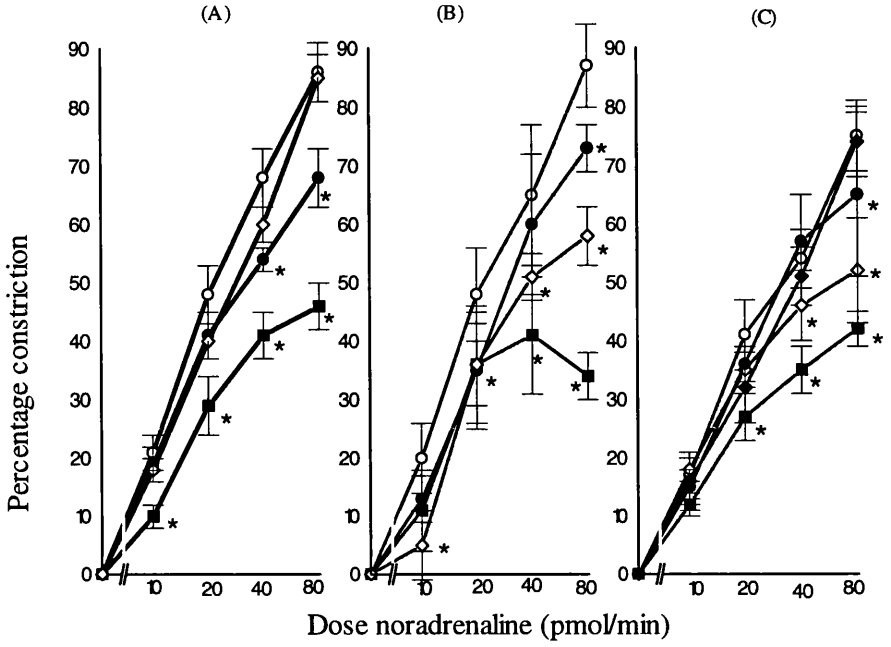
TAAAGGTCTTCTTCTGGTGATGCC-3'[antisense]), human iNOS (5'-CAGTACGTTTGGCAATGGAGACTGC-3' and 5'-GGTCACATTGGAGGTGTAGAGCTTG-3' [antisense]) and human GTP cyclohydrolase-I (5'-TTGGTTATCTTCCTAACAAG-3' [sense] and 5'-GTGCTGGTCACAGTTTTGCT-3' [antisense]). PCR amplification was performed in a volume of 25μl (containing 200μM dNTPs, 1.5mM MgCl2, 15pmol of specific forward and reverse primers and 1U of Taq polymerase). All reactions were conducted for 35 cycles. PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide and visualised by UV-induced fluorescence.

Results

Effects of cytokines on exogenous and endogenous constrictor responses

Instillation of IL-1β into a single superficial blood vessel caused a rightward shift in the dose response curve to noradrenaline and suppressed the maximal constriction achieved (Figure 1A). This effect was present at 1 and 6h after exposure of the vessel to IL-1 β, but by 24h the potency of noradrenaline was fully restored. Instillation of either TNFα (1ng in 1ml of saline), or IL-6 (100pg in 1ml of saline) alone produced no significant change in the response to noradrenaline, but co-instillation of these cytokines with IL-1β for 1h caused a prolonged (>24 h) attenuation of the constrictor response to noradrenaline (Figure 1B and C).

Figure 1



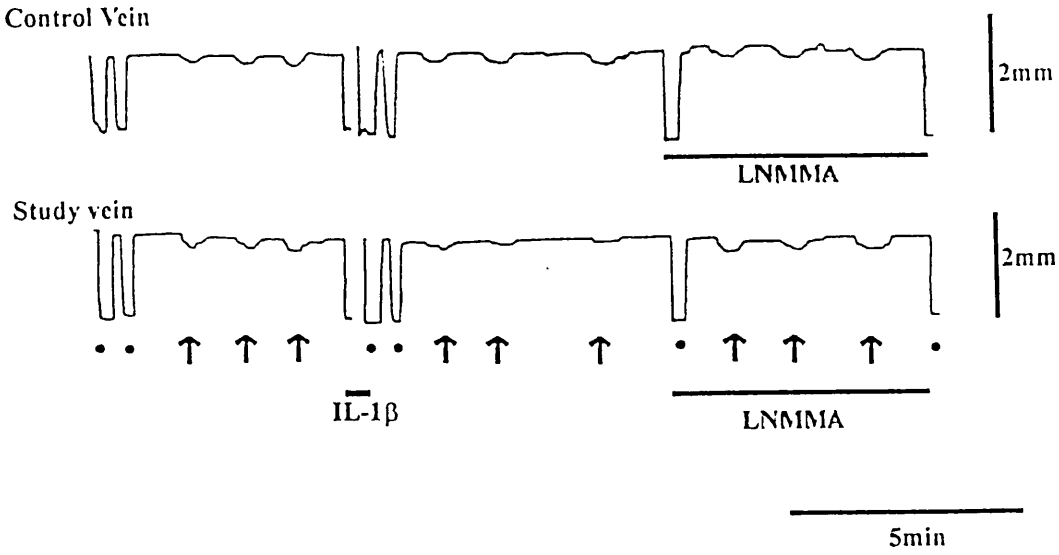
(A) Dose response curves were constructed to noradrenaline before (o) and 1(•), 6 (■), and 24h (◊) after instillation of IL-1β (1ng).

(B) Dose response curves were constructed to noradrenaline before and 1, 6, and 24h after instillation of IL-1β and TNFα (1ng).

(C) Dose response curves were constructed to noradrenaline before and 1, 6, 24 and 48h (◆) after instillation of IL-1β (1ng), TNFα (1ng) and IL-6 (100pg). *indicates $p<0.05$

The potential physiological significance of the vascular effects of the cytokines is illustrated in Figure 2. Instillation of cytokines virtually abolished endogenous venoconstriction due to activation of the sympathetic nervous system. Activation of the sympathetic nervous system produced simultaneous transient venoconstriction in two adjacent superficial veins (15±6% constriction). After IL-1β, the constrictor response to deep breath was abolished in the treated (3±5%) but not in the control vein (17±7%).

Figure 2



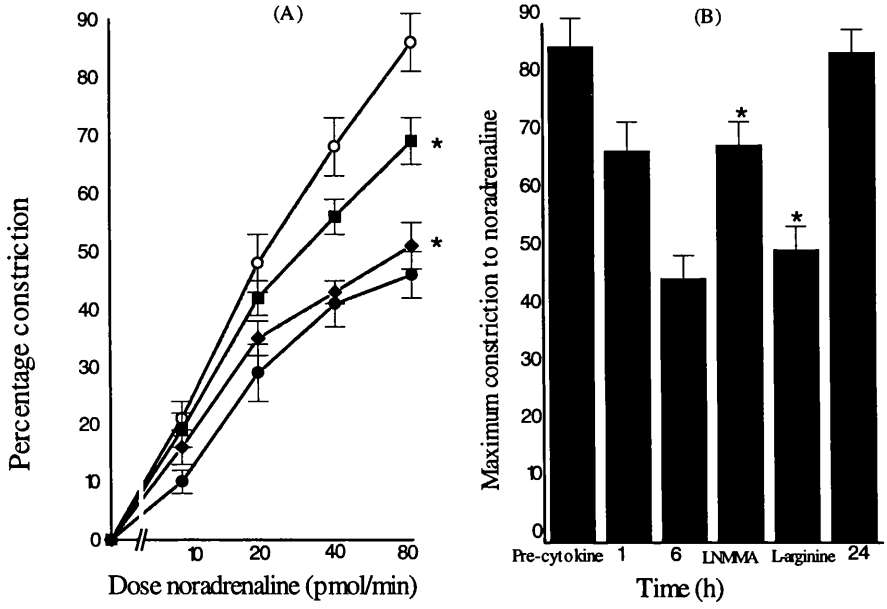
In 3 subjects, sympathetically-mediated venoconstriction responses were assessed simultaneously in two adjacent veins. The control vein (top) was left unoccluded while the other vein was isolated and received IL-1β (bottom). Deep-breath venoconstrictor responses (↑) were performed simultaneously in both veins before and 6h after instillation of IL-1β. Deep breath produced simultaneous transient venoconstriction in both veins. After IL-1β, the constrictor deep-breath response was abolished (bottom). Finally, in the control (untreated) vein and IL-1β-treated vein, L-NMMA (1μmol/min) was infused 15 min before and throughout a repeat deep-breath response. • indicates deflation of the cuff.

Inhibition of NOS

In order to determine whether increased generation of NO contributed to the hyporesponsiveness to the constrictor stimuli, the effects of L-NMMA (a standard NOS inhibitor (Rees et al. 1989)) , and aminoguanidine (a compound that shows some selectivity for inhibition of iNOS (MacAllister et al. 1994)) were tested. Both L-NMMA and aminoguanidine caused significant reversal of the IL-1β-induced attenuation in the dose response curve to noradrenaline (Figure 3 and 4). Inhibition of NOS also restored the constrictor response to

sympathetic nerve stimulation (Figure 2). Infusion of L-arginine, reversed the effect of L-NMMA or aminoguanidine (Figure 3 and 4). In Chapter 4 the studies showed that that L-NMMA does not have any direct constrictor action in the dorsal hand vein, or alter the response to noradrenaline in veins not exposed to cytokines.

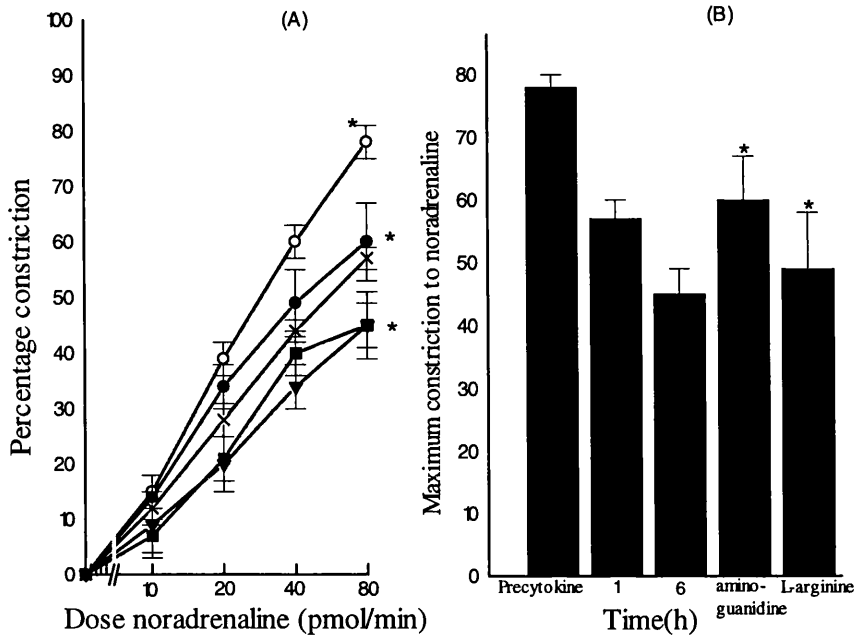
Figure 3



(A) Dose response curves were constructed to noradrenaline before (o) and 6h (•) after instillation of IL-1β. Six hours after instillation of IL-1β a repeat dose response curve was constructed with L-NMMA (1μmol/min; ■) co-infused with noradrenaline. After a stable degree of preconstriction had been re-established (noradrenaline only), a repeat dose response curve to noradrenaline was constructed with L-arginine (1μmol/min; ◆).

(B) Panel B shows the maximum constrictor response to noradrenaline before and 1, 6 and 24h after IL-1β and the effects of L-NMMA and arginine co-infused 6h after IL-1β. *indicates $p<0.05$

Figure 4



(A) Dose response curves were constructed to noradrenaline before (o) and 1 (•) and 6h (■) after instillation of IL-1β. Six hours after instillation of IL-1β a repeat dose-response curve was constructed with aminoguanidine (1μmol/min; x) co-infused with noradrenaline. After a stable degree of preconstriction had been re-established (noradrenaline only), a repeat dose response curve was constructed with L-arginine (1μmol/min; ▼) co-infused with noradrenaline.

(B) Panel B shows the maximum constrictor response to noradrenaline before and 1, 6 and 24h after IL-1β and the effects of aminoguanidine and L-arginine co-infused 6h after IL-1β. *indicates $p<0.05$

Effects of hydrocortisone

Oral hydrocortisone (100mg) taken 2h before the administration of the cytokine mix prevented the development of hyporesponsiveness to noradrenaline. In subjects pre-treated with hydrocortisone, the constriction to the 4 doses of noradrenaline used were: 25±5%, 53±7%, 71±11%, 89±12% before cytokine and 27±6%, 48±5%, 68±4% and 85±10% 1h after instillation of cytokine.

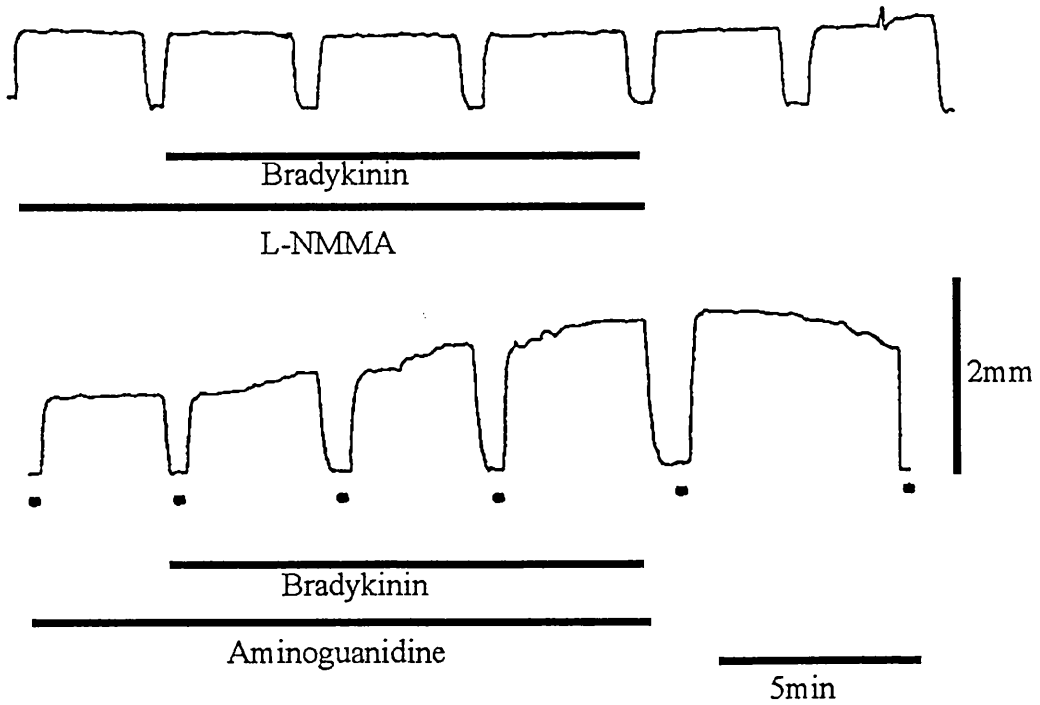
Effects of aminoguanidine on eNOS

To explore the selectivity of aminoguanidine for inhibition of iNOS, the response to agents that stimulate eNOS was examined. L-NMMA inhibited the maximum dilatation to bradykinin by 88% whereas aminoguanidine had no effect (Figure 5).

Effects of tetrahydrobiopterin infusion

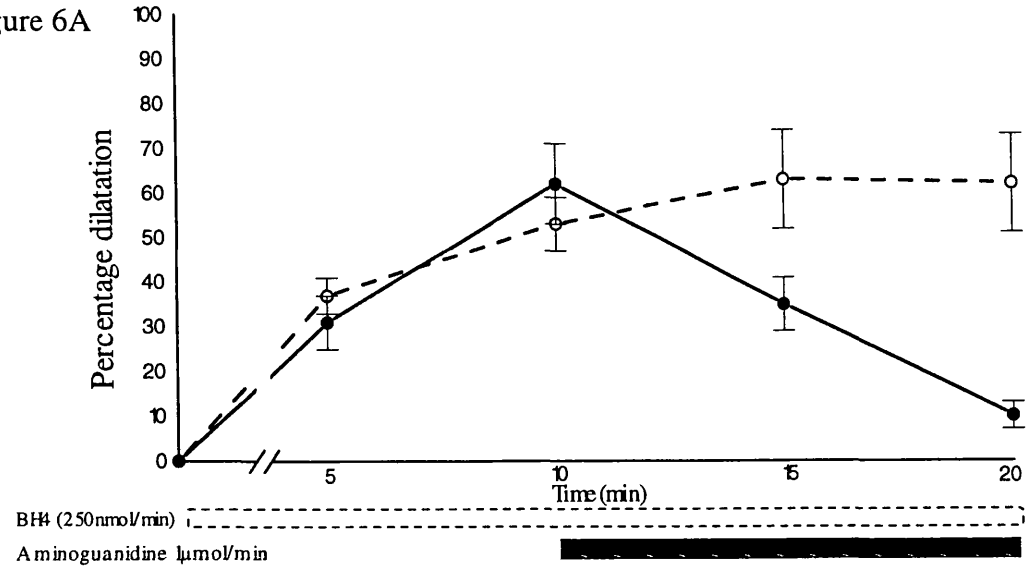
Infusion of tetrahydrobiopterin resulted in venodilatation (maximum dilatation: 60±7%; n=6) which was inhibited by aminoguanidine (Figure 6)

Figure 5



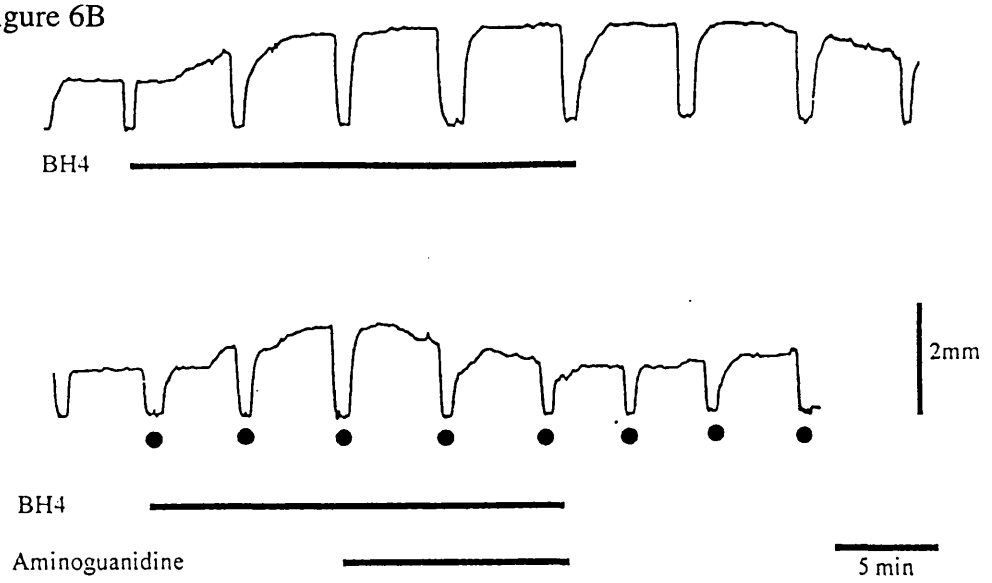
In 6 subjects a continuous infusion of L-NMMA (1μmol/min) and bradykinin (8pmol/min) was given (upper panel) into a preconstricted vein. In the same subjects on a different occasion, a continuous infusion of aminoguanidine (1μmol/min) and bradykinin (8pmol/min) was given into a preconstricted vein (lower panel). The figure shows a typical trace of the results seen. • indicates deflation of the cuff.

Figure 6A



In 6 subjects a repeated infusion of tetrahydrobiopterin (BH4) was administered into a preconsticted vein. The second infusion (●) was then given after a 20min saline washout. During the last 10min of the 2nd infusion, aminoguanidine (1μmol/min) was administered for 10min. A representative trace of Figure 6A is seen in Figure 6B.

Figure 6B

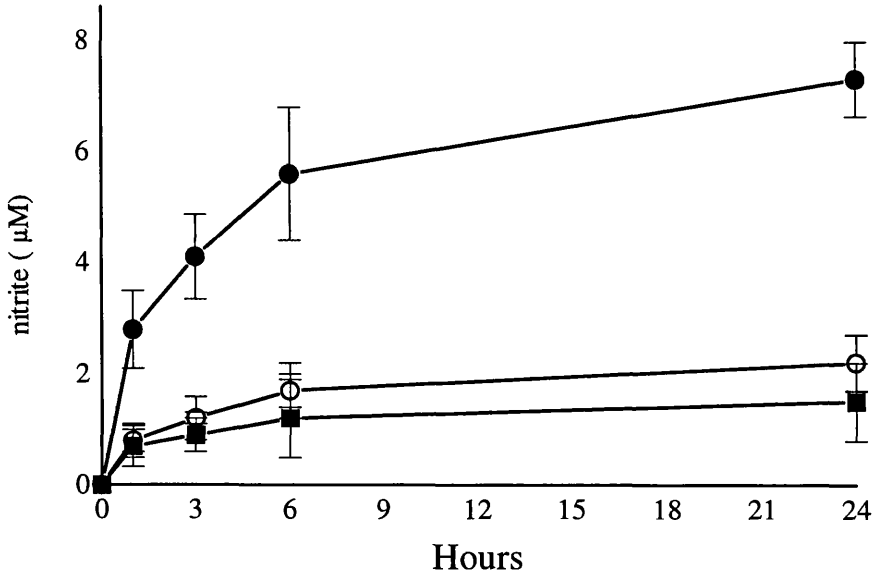


The top panel shows a typical trace during an infusion of tetrahydrobiopterin into a preconstricted vein. The lower panel shows a repeat trace but with an aminoguanidine (1μmo/min) coinfusion during the last 10min of the study. • indicates deflation of the cuff.

Nitrite studies *in vitro*

Nitrite levels increased in a time-dependent-manner following incubation of HUVECs with the 3 cytokines (Figure 7). All the nitrite and FACS studies were performed by Dr Miriam Palacios (Cruciform Project, University College London).

Figure 7

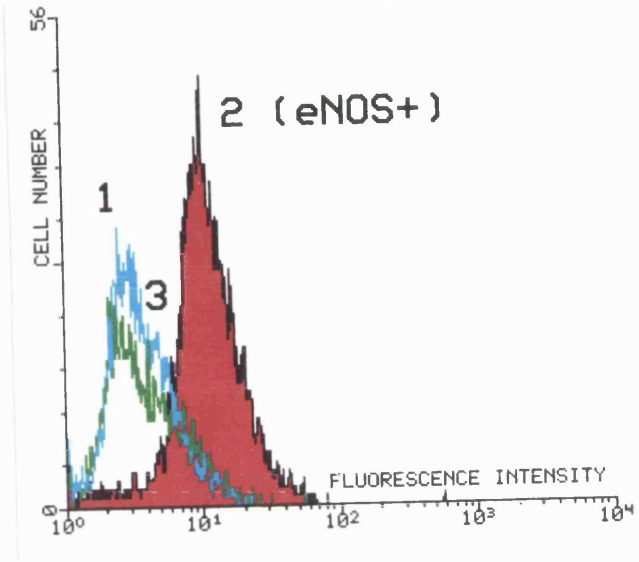


HUVECs were incubated with IL-1β (1ng), TNFα (1ng) and IL-6 (100pg) for 1, 3, 6h, and 24h. Nitrite measurements were performed at each time point. O = control cells ● = cytokine treated cells ■ = cytokine-treated cells incubated in the presence of L-NMMA (1mM).

Detection of eNOS and iNOS protein by FACS

Protein for eNOS was detected in all HUVECs (control and cytokine treated) but no protein for iNOS was detected. (Figure 8).

Figure 8



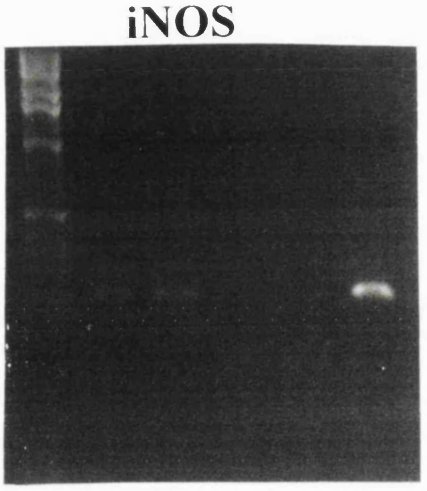
HUVECs were incubated with IL-1β (1ng), IL-6 (100pg) and TNFα (1ng) for 24h. Expression of eNOS was detected using specific monoclonal antibody and FITC-conjugated sheep anti-mouse IgG F(ab')2 antibody (group 2). Percentage of eNOS positive stained cells (shaded in red) is compared with the control cells (blue, group 1). The green curve (group 3) represents cells incubated with eNOS antibody preabsorbed with neutralising eNOS protein extract and shows reversal of the rightward shift (red curve) at the specific immunofluorescence in the group 2 cells.

Molecular studies

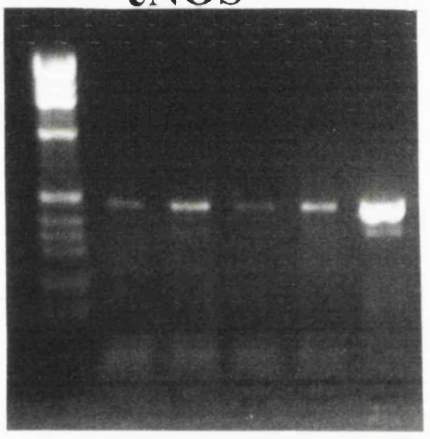
In 9 subjects a portion of the hand vein was excised to explore the molecular basis for the functional induction of NOS. Three hours after the instillation of IL-1β, IL-6 and TNFα mRNA for eNOS was detected in all of the samples, but there was no consistent induction of mRNA for iNOS. However, mRNA for GTP-cyclohydrolase-I was present in veins exposed to the 3 cytokines but not in the control samples (Figure 9). All the molecular studies were performed by Drs Aroon Hingorani and Ian Charles, Cruciform Project, University College London).

Figure 9

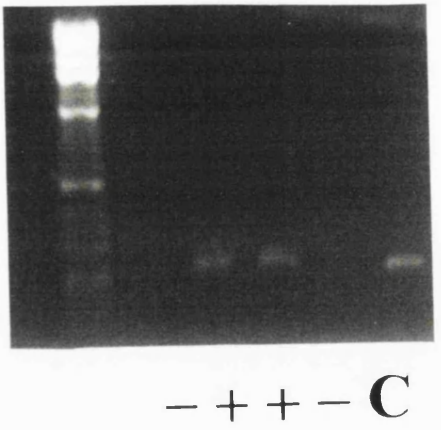
Panel A



Panel B



Panel C



Key

M=molecular weight marker
C=no template
1=no cytokine exposure
2=cytokine exposure for 1h, then cells harvested
3=cytokine exposure for 1h, cells harvested after 3h
4=cytokine exposure for 3h, then cells harvested
5=cytokine exposure for 1h, cells harvested after 6h
6=cytokine exposure for 6h, then cells harvested
+=positive control template for eNOS, iNOS or GTPCyclohydrolase-I (GTPCH 1) as appropriate

Detection of messenger RNA for iNOS (Panel A), eNOS (Panel B) and GTP Cyclohydrolase-I (Panel C) in dorsal vein tissue using RT-PCR.

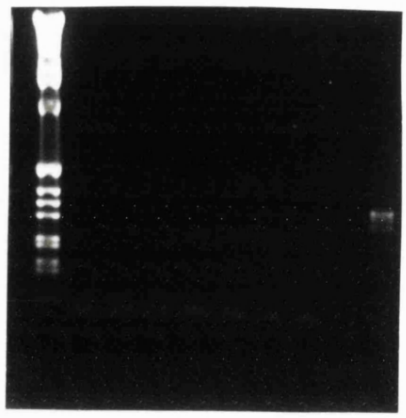
Following surgical excision of the study vein, Poly-A mRNA was extracted using the Invitrogen MicroFastrack kit and reverse transcribed to synthesise first strand cDNA. PCR was conducted in a volume of 25µl (containing 200µM dNTPs, 1.5mM MgCl2, 15pmol of specific forward and reverse primers and 1U of Taq polymerase) for 35 cycles on a Biometra-TRIO thermocycler with denaturing at 95°C for 1min, annealing at 56°C for 1min and extension at 72°C for 2min. 4 µl of each PCR product was electrophoresed at constant voltage in a 1.5% agarose gel and detected by ethidium bromide staining.

Detection of message for eNOS and iNOS in HUVECs following incubation with cytokines

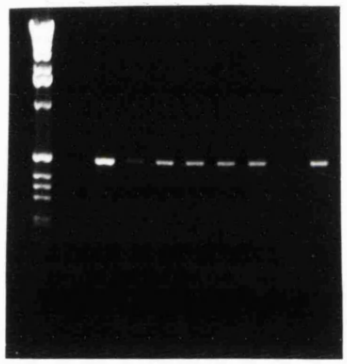
Message for eNOS was present in both the control and cytokine exposed cells. However, no iNOS message was found before or 1h, 3h, 6h, and 24h after incubation of HUVECs with the 3 cytokines. In addition, mRNA for GTP-cyclohydrolase-I was present in HUVECs exposed to the 3 cytokines but not in the control samples (Figure 10).

Figure10

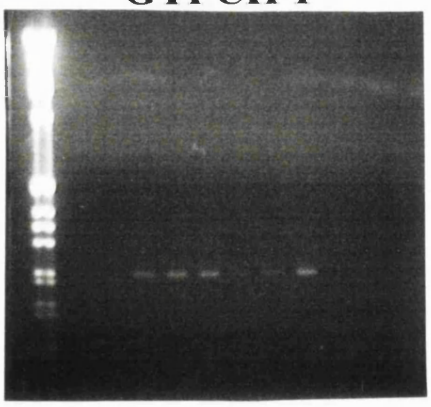
Panel A



Panel B



Panel C



Key

M=molecular weight marker
C=no template
1=no cytokine exposure
2=cytokine exposure for 1h, then cells harvested
3=cytokine exposure for 1h, cells harvested after 3h
4=cytokine exposure for 3h, then cells harvested
5=cytokine exposure for 1h, cells harvested after 6h
6=cytokine exposure for 6h, then cells harvested
+=positive control template for eNOS, iNOS or GTPCyclohydrolase-I (GTPCH 1) as appropriate

Detection of messenger RNA for iNOS (Panel A), eNOS (Panel B) and GTP Cyclohydrolase-I (Panel C) in cultured HUVECs using RT-PCR.

Passage 4 HUVECs were seeded into 6 well flasks and grown to subconfluence. Cells were exposed to vehicle or a combination of IL-1β (1ng), TNF-α (1ng) and IL-6 (10pg) for periods of 1h, 3h, or 6h. Poly-A mRNA was extracted using the Invitrogen MicroFastrack kit and reverse transcribed to synthesise first strand cDNA. PCR was conducted in a volume of 25μl (containing 200μM dNTPs, 1.5mM MgCl2, 15pmol of specific forward and reverse primers and 1U of Taq polymerase) for 35 cycles on a Biometra-TRIO thermocycler with denaturing at 95°C for 1min, annealing at 56°C for 1min and extension at 72°C for 2min. 4 μl of each PCR product was electrophoresed at constant voltage in a 1.5% agarose gel and detected by ethidium bromide staining.

Discussion

Venodilatation is a prominent and an important component of the cardiovascular response to acute systemic inflammation, and contributes to the hypotension and changes in cardiac output in septic shock (Bradley et al. 1945; Snell and Parrillo, 1991; Parrillo, 1993). The studies in this Chapter demonstrate that a brief (1h) exposure to IL-1β induces a significant and long-lasting attenuation of venoconstriction in human veins *in vivo*, and that this effect is mediated by increased generation of NO. The most likely mechanism for the increased generation of NO is stimulation of basal eNOS activity following induction of GTP cyclohydrolase-I and enhanced synthesis of tetrahydrobiopterin. The functional significance of this increased NO generation is to blunt the ability of the sympathetic nervous system to alter venous tone. These findings have implications for the design of drugs to treat septic hypotension and inflammatory vasodilatation

IL-1β is the key cytokine

The effects of 3 cytokines that are generated in response to infection or systemic inflammation were studied. These studies show that IL-1β (in concentrations similar to that which may occur clinically (Basaran et al. 1993; Guillen et al. 1995; Miyao et al. 1993; Neumann et al. 1995; Tashiro et al. 1995)) attenuated the response to noradrenaline and abolished sympathetically-mediated venoconstriction. The effects were maximal 6h after the vein was

treated with the cytokine and disappeared by 24h. In contrast, TNFα or IL-6 when given alone did not affect the responses of the vein, although when co-instilled with IL-1β they prolonged the hyporesponsiveness. The advantage of the model used here is that it is possible to expose the vessel *in situ* to each cytokine separately, whereas following systemic administration, a cytokine cascade is initiated, making interpretation of responses to individual cytokines more complex.

The venous hypo-responsiveness to constrictors is due to nitric oxide

The vessels studied do not generate functionally active amounts of NO basally (Vallance et al. 1989), and in healthy veins L-NMMA does not alter resting vessel tone (Vallance et al. 1989), affect the response to noradrenaline or alter sympathetically-mediated venoconstriction (Figure 2). However, following exposure to any cytokine mix that included IL-1β, L-NMMA enhanced the constrictor response to noradrenaline, evoked a constrictor response when infused into a vessel pre-constricted with noradrenaline, and restored the venoconstriction elicited by sympathetic activation. Together these results indicate that following exposure to IL-1β the veins generated NO "basally" in amounts sufficient to suppress contractions to exogenous or endogenous noradrenaline. Similar findings have been obtained previously in the rabbit jugular vein studied *in vitro* (Vallance et al. 1992).

Sympathetic nervous system activity is a major determinant of venous tone in humans and sympathetic blockade causes profound venodilatation and postural hypotension (Lewis and Landis, 1935). Our findings indicate that the profound venodilatation that accompanies sepsis or a systemic inflammatory response is likely to be mediated by an induction of basal generation of NO in the venous system, and that inhibition of NOS would lead to venoconstriction. This explains the observation that systemic administration of L-NMMA does not alter venous pressure in healthy volunteers (Haynes et al. 1993; Stamler et al. 1994) but increases it in patients with septic shock (Petros et al. 1994).

Origin of the nitric oxide

The cytokine-induced hypo-responsiveness developed slowly and was prevented by prior administration of a glucocorticoid. Furthermore, aminoguanidine (a NOS inhibitor with some selectivity for iNOS), given in a dose which did not affect the dilator response to bradykinin, reversed the hypo-responsiveness to noradrenaline. This pattern of results is usually considered as indicative of induction of iNOS (Auphan et al. 1991; MacAllister et al. 1994). However, in the biopsies of veins exposed to cytokines, eNOS mRNA was detected in all samples but no consistent induction of mRNA encoding iNOS was detected. It is unlikely that iNOS message was not detected for technical reasons, since the biopsies were taken at a time when the functional changes were already occurring, PCR was used with multiple cycles of amplification and a positive control was included.

In HUVECs, cytokines (particularly IL-1β) increase NO generation by increasing levels of the co-factor tetrahydrobiopterin (Rosenkranz Weiss et al. 1994). Tetrahydrobiopterin is synthesised by GTP cyclohydrolase-I. Similar to iNOS, this enzyme is inducible (Hattori and Gross, 1993; Ichinose et al. 1995; Werner Felmayer et al. 1993), is expressed in response to cytokines or endotoxin (Schoedon et al. 1993; Gross et al. 1993; Hattori and Gross, 1993; Werner et al. 1993), and the induction can be prevented by glucocorticoids (Schoedon et al. 1993). In the hand vein studies the experiments showed evidence for induction of mRNA for GTP-cyclohydrolase suggesting that this is the mechanism for increasing NO generation *in vivo.*

Intriguingly, aminoguanidine reversed the dilatation to tetrahydrobiopterin, indicating that this compound can act as an inhibitor of eNOS in the presence of high levels of tetrahydrobiopterin. This finding is consistent with recent observations made using purified enzyme preparations (Wolff and Lubeskie, 1995) and supports the suggestion that tetrahydrobiopterin might affect the allosteric properties of the arginine binding site of NOS enzymes (Werner et al. 1993; Wolff and Lubeskie, 1995). Together these results demonstrate that the pharmacological profile of increased eNOS activity due to induction of GTP cyclohydrolase-I is similar to the induction of iNOS.

The studies in this Chapter identify IL-1β as being the key mediator causing venodilatation in humans by increasing NO generation and indicate that the source of the NO is most likely to be eNOS rather than iNOS. The NO

generated was sufficient to abolish sympathetic-mediated vasoconstriction. It remains to be determined whether this also occurs in the arterial system and how much the effects have seen contribute to the overall haemodynamic changes that occur in sepsis. However, neopterin levels are increased in the plasma of patients with sepsis (Delogu et al. 1995; Waydhas et al. 1992; Strohmaier et al. 1992) and, with the known species differences in iNOS genes, the difficulty in inducing active iNOS in human cells (Schneemann et al. 1993; Yan et al. 1994; Cameron et al. 1990), and the relatively low levels of nitrate seen in human sepsis (Evans et al. 1993), it is possible that the results from the studies in this Chapter may be more generally relevant to the response of the human vascular system to infection and inflammation.

Chapter 6

Effects of endotoxin on endothelial function

Introduction

Previous chapters explored the effects of inflammatory mediators on constrictor responses. In this and the subsequent chapter the experiments that are described were designed to explore whether endothelial dilator function is also affected by inflammation. An imbalance between endothelium-derived relaxing and contracting factors, between anti- and pro-coagulant mediators or growth-inhibiting and growth-promoting factors could lead to changes in the normal state of the cardiovascular system from one that prevents atheroma, vasospasm and thrombosis to one that supports it. Indeed, endothelial dysfunction is now recognised as a major mechanism underlying acute and chronic cardiovascular disease (Luscher et al. 1991). In experimental models, infection and inflammation have been shown to cause endothelial dysfunction (Luscher et al. 1991). Furthermore, chronic infection has been implicated as a possible causative agent in atherogenesis (Ross, 1986), and acute systemic infection has been suggested as a factor predisposing to acute myocardial infarction in the general population and post-operatively (Mamode et al. 1995). In large quantities endotoxin is known to produce cardiovascular changes similar to those seen in septic shock (Suffredini et al. 1989). However, experiments *in vitro,* suggest that it might also induce more subtle changes in the vessel wall including endothelial damage and/or dysfunction (Koshi et al. 1993; Richardson and Parbtani, 1987).

In this chapter the effects of a brief exposure to endotoxin on the reactivity of blood vessels in humans both *in vivo* and *in vitro* was examined. Endothelium-dependent relaxation to bradykinin and the dilator response to arachidonic acid were used to explore the L-arginine-NO pathway and prostanoid production respectively.

Protocol

Studies were performed on male (10) and female (14) subjects aged 19 - 38.

Instillation of endotoxin

Endotoxin or control solution (saline) was instilled into an isolated dorsal hand vein as described in Chapter 4. The endotoxin was left *in situ* for 1h after which the contents of the vessel were aspirated and the vessel re-connected with the rest of the circulation.

Dilator dose-response curves

Vessels were preconstricted to approximately 50% of resting diameter by a continuous infusion of noradrenaline (5-1280pmol/min, doses selected as above). Then bradykinin (2, 4, 8pmol/min, each dose for 5 min), arachidonic acid (0.2, 2, and 20nmol/min, each dose for 5 min) or GTN (1, 2, 4pmol/min,

each dose for 5 min) were co-infused with the noradrenaline and relaxation was recorded.

Study 1: In 5 subjects a dose response curve was constructed to bradykinin and arachidonic acid before and 1h after instillation of endotoxin (n=5) or saline (n=5).

Study 2: In a separate study dose response curves were constructed to bradykinin and GTN before and 1h after instillation of endotoxin (n=5).

Study 3: In a further 5 subjects dose response curves to bradykinin and arachidonic acid were constructed before and at 1h, 24h, 48h and 7 days after endotoxin.

In all studies a 10-15 min washout period (infusion of saline and noradrenaline) separated the dose response to different agonists.

Effects of hydrocortisone

Study 4: In 5 subjects the dose response curve to noradrenaline was constructed and oral hydrocortisone (100mg) given. Endotoxin was instilled 2h later. Dose response curves to bradykinin and arachidonic acid were constructed before and 1h after instillation.

Measurement of vascular tone *in vitro*

In preconstricted, endothelium-intact saphenous vein, concentration response curves were constructed to bradykinin (1nM-1μM) and GTN(1nM-1μM). After the first concentration response-curve, endotoxin (100 Endotoxin Units, LOT EC-5) or an equal volume of physiological saline was added to the organ bath and allowed to incubate with the saphenous vein rings for 1h. Concentration response curves to bradykinin and GTN were then repeated. Studies with arachidonic acid were not undertaken since patients undergoing by-pass surgery would have been taking aspirin which would interfere with the relaxant response to arachidonic acid.

Electron microscopy

Saphenous tissue was fixed and prepared for electron microscopy by Mr Ray Moss, Department of Anatomy, St George's Hospital Medical School.

All specimens for electron microscopy were assessed by a microscopist blinded to the sample code. Saphenous vein rings were added directly to fixative or incubated for 1h in either 10ml of Krebs' solution, or 10ml of Krebs' with endotoxin (100EU) prior to fixing in 3% gluteraldehyde in 0.1M cacodylate buffer pH 7.2 for 2h. Rings were divided longitudinally, rinsed in buffer, post-fixed in 1% osmium tetroxide, rinsed again in buffer and dehydrated through ascending grades of ethanol (30-100%). One half was critical point dried with

liquid CO_2, mounted on aluminium stubs, coated with gold and examined by scanning electron microscopy (Higman et al. 1994). The other half was embedded in Spurr's resin, polymerised, ultramicrotomed and sections stained with uranyl acetate and Sato's lead. These were examined using a transmission electron microscope.

Results

Effects of endotoxin on noradrenaline responses

As reported in Chapter 4, instillation of endotoxin decreased the contractile response to noradrenaline such that the dose of noradrenaline producing 48±7% (n=15) constriction before endotoxin produced only a 12±3% constriction 1h after endotoxin ($p<0.05$), and the maximal constriction achieved was reduced from 87±6% to 53±3%. Consistent with the studies in Chapter 4 the hyporesponsiveness to noradrenaline disappeared by 4h and the response to noradrenaline was normal at 24h, 48h and at 7 days after endotoxin. The degree of preconstriction and dose of noradrenaline used for each part of the dilator study is shown in Table 1.

Table 1

Dose of noradrenaline used and percentage preconstriction of the vein

Study	#Time(h)	Dose of noradrenaline (pmol/min)	Percentage preconstriction
Study 1	0	84±22	43±4
ETX/Bradykinin	1	296±100*	44±8
Study 1	0	84±22	42±6
ETX/Arachidonic acid	1	296±100*	40±3
Study 1	0	160±120	48±3
Saline/Bradykinin	1	152±121	44±6
Study 1	0	160±120	49±4
Saline/Arachidonic acid	1	152±121	47±5
Study 2	0	52±11	52±3
ETX/Bradykinin	1	384±227*	45±6
Study 2	0	52±11	45±4
ETX/GTN	1	384±227*	47±4
Study 3	0	108±53	48±5
ETX/Bradykinin	1	320±85	52±6
	24	76±19	51±4
	48	72±23	54±3
	7 days	68±24	50±8
Study 3	0	108±53	50±5
ETX/Arachidonic acid	1	320±85	54±3
	24	76±23	50±3
	48	68±12	51±8
	7 days	68±24	47±7
Study 4	0	40±10	48±2
ETX/Bradykinin/ Steroid	1	56±28	52±5
Study 4	0	40±10	43±5
ETX/Arachidonic acid/ Steroid	1	56±28	50±3

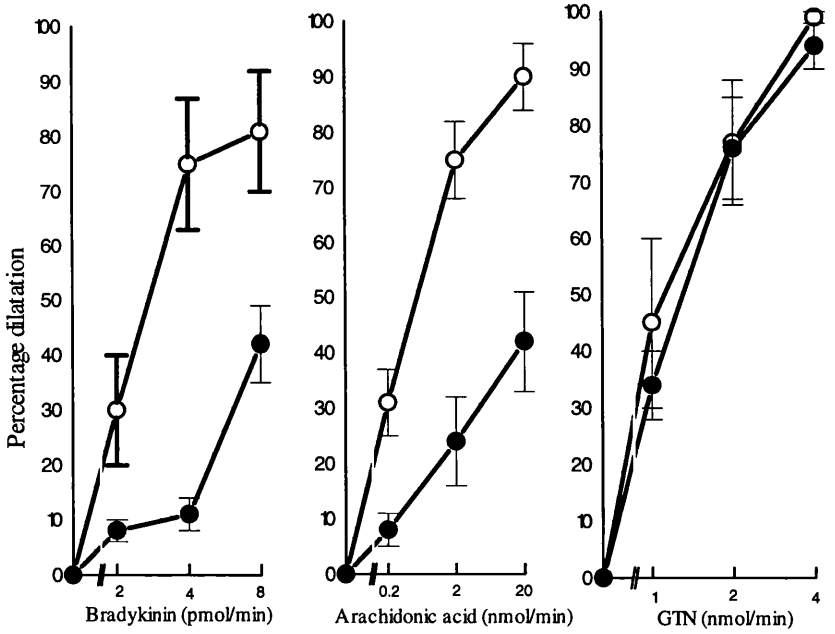
Time 0, 1, 6, 24, and 48h and 7 days represent the time before (0) and the designated time after instillation of endotoxin or saline. * $p<0.05$ - p values compared with time 0

Effects of endotoxin on endothelium-dependent relaxation

Exposure to endotoxin attenuated the dilator response to bradykinin and arachidonic acid whereas the dilator response to GTN was unaffected (Figure 1). In contrast, after instillation of saline there was no significant change in the dose response curves to any dilator (Figure 1). In subjects receiving endotoxin, dilatation to the highest dose of each drug before endotoxin (time 0) was: bradykinin: 81±6% (n=15); arachidonic acid: 90±6% (n=10) and GTN: 99±2% (n=5); 1h after endotoxin, dilatation to the highest dose of each drug was:

bradykinin: 40±4% (n=10; p<0.05), arachidonic acid: 42±9% (n=10; p<0.05); and GTN: 94±4% (n=5). In control subjects (saline instillation), dilatation to the same doses before instillation of saline were: bradykinin 90±3% (n=5), and arachidonic acid 87±4% (n=5), and 1h after instillation of saline were: bradykinin 90±6% (ns), and arachidonic acid 91±6% (ns).

Figure 1

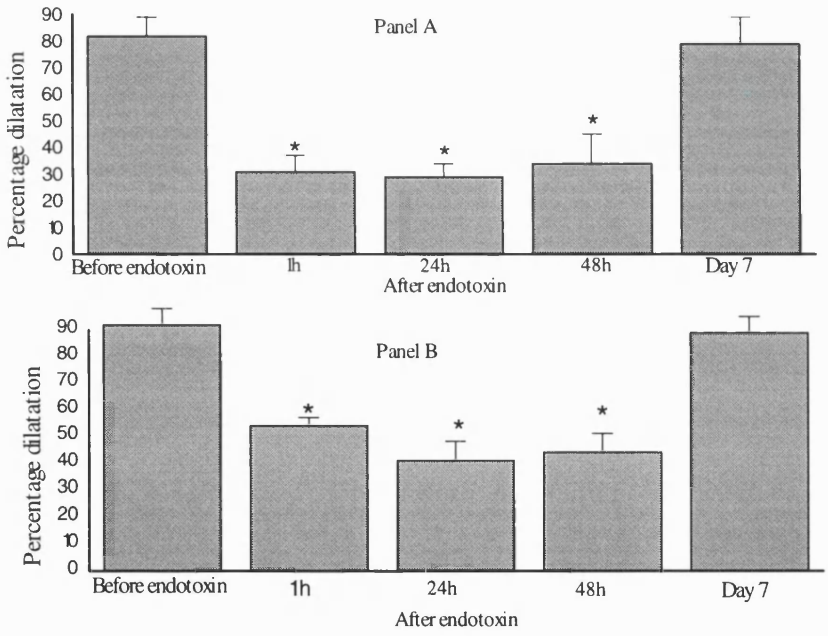


Vessels were preconstricted to approximately 50% of resting diameter by a continuous infusion of noradrenaline (5-1280pmol/min). Then bradykinin (2, 4, 8pmol/min, each dose for 5 min), arachidonic acid (0.2, 2, 20nmol/min, each dose for 5 min) or GTN (1, 2, 4pmol/min, each dose for 5 min) were coinfused with the noradrenaline and relaxation recorded. Studies were undertaken before (O) and 1h after endotoxin (●) was instilled.

The endotoxin-induced attenuation in the response to bradykinin and arachidonic acid (Figure 2) persisted for at least 48h and returned to pre-

endotoxin values by 7 days. Before endotoxin, dilatation to the highest dose of drug was: bradykinin 82±7% (n=5), arachidonic acid 92±6% (n=5). 1h after endotoxin dilatation to the same dose of drug was: bradykinin 31±6% (n=5; $p<0.05$), arachidonic acid 54±3% (n=5; $p<0.05$); 24h later: bradykinin 29±5% (n=5; $p<0.05$), arachidonic acid 41±7% (n=5; $p<0.05$); 48h later: bradykinin 34±11%(n=5; $p<0.05$), arachidonic acid 44±7%(n=5; $p<0.05$); and 7 days later: bradykinin 79±10%(n=5; ns), arachidonic acid 89±6% (n=5; ns).

Figure 2

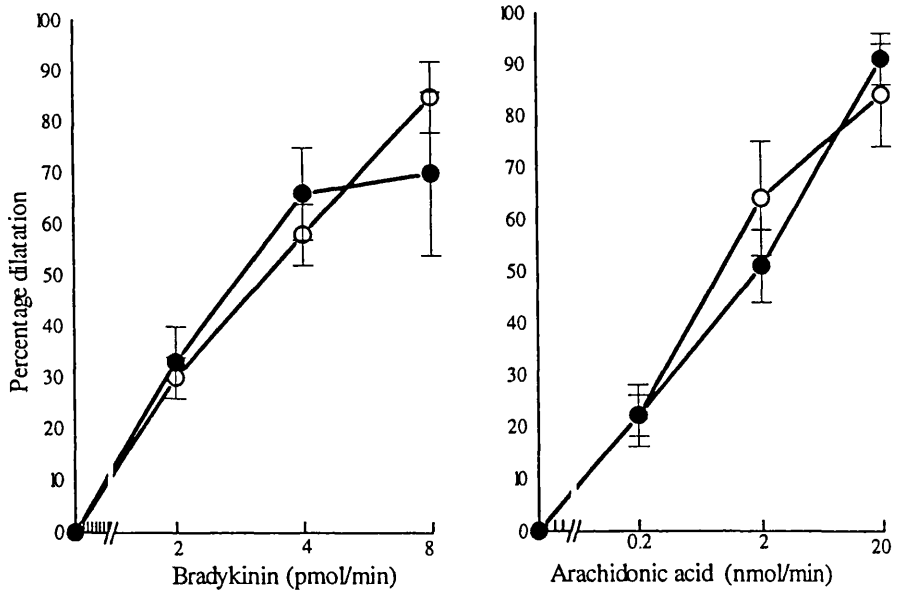


In 5 subjects dose response curves to bradykinin and arachidonic acid were constructed before and at 1h, 24h, 48h and 7 days after endotoxin. At each time point the maximum dilatation achieved to bradykinin (Panel A) and arachidonic acid (Panel B) is shown. * $p<0.05$

Effects of hydrocortisone

Hydrocortisone (100mg) given 2h before the study inhibited the effects of endotoxin. Before endotoxin, dilatation to the highest dose of each drug used was: bradykinin 85±6% (n=5), arachidonic acid 84±10% (n=5). Following hydrocortisone treatment, 1h after endotoxin the dilatation to the same dose of bradykinin was: 70±16% (ns), arachidonic acid 91±5% (ns) (Figure 3).

Figure 3



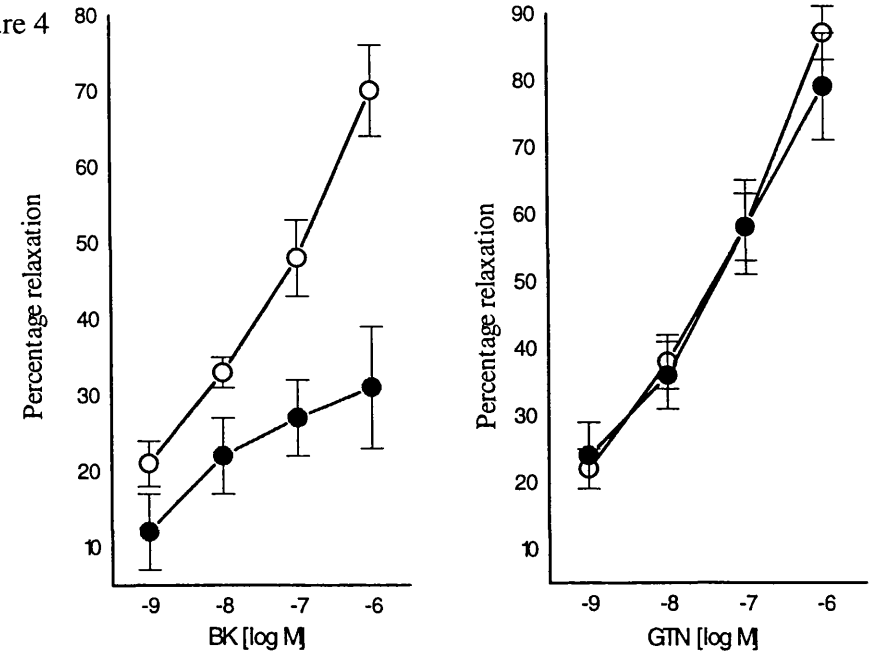
In 5 subjects oral hydrocortisone (100mg) was given. Endotoxin was instilled into the vein 2h later. Dose response curves to bradykinin and arachidonic acid were constructed before (○) and 1h after instillation (●).

Vascular relaxation *in vitro*

Bradykinin and GTN caused concentration-dependent relaxation of preconstricted endothelium-intact saphenous vein. Incubation with endotoxin for 1h did not affect the contractile response to phenylephrine but resulted in a rightward shift in the concentration response curve to bradykinin. The concentration response curve to GTN was unaffected by endotoxin (Figure 4).

Maximum dilatation before endotoxin was bradykinin (1μM) 70±5% (n=5); GTN (1μM) 87±4% (n=5). 1h after endotoxin maximum dilatation to bradykinin was 31±4% ($p<0.05$) and to GTN was 79±8% (ns). In the control rings, not exposed to endotoxin maximum dilatation to bradykinin (1μM) was 67±9% and to GTN (1μM) was 77±7%; 1h later maximum dilatation to bradykinin was 72±11% (ns) and to GTN was 67±4% (ns).

Figure 4



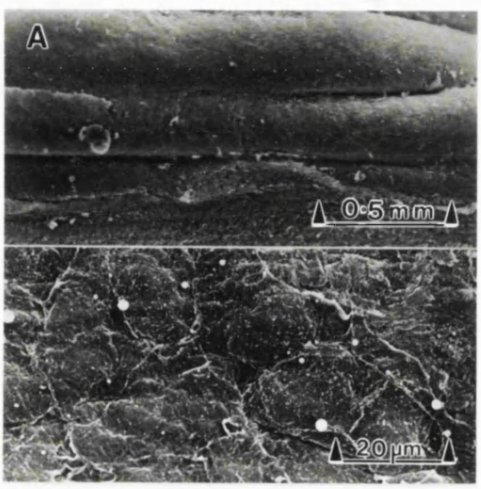
In preconstricted saphenous vein, concentration response curves were constructed to bradykinin (1nM - 1μM) and GTN (1nM - 1μM). After the 1st concentration response curve (o), endotoxin (●) (left panel) or saline (●) (right panel) was added to the organ bath and allowed to incubate with the saphenous vein rings for 1h. Concentration response curves to bradykinin and GTN were then repeated.

Electron microscopy

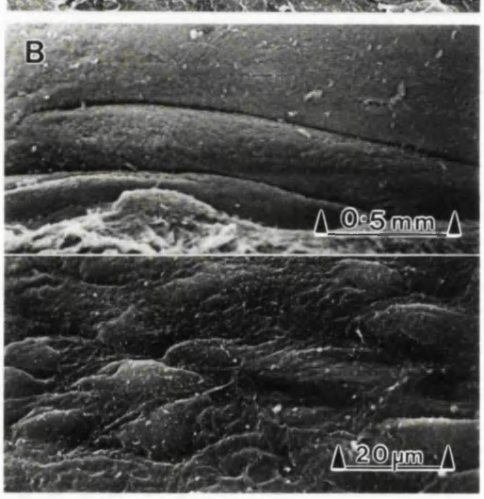
Electron microscopy revealed no clear differences between the endotoxin-treated vein (n=5), the vein that was allowed to remain in Krebs' for 1h before adding fixative (n=5) and the vein that had been added immediately to fixative (n=5), Figure 5.

Figure 5

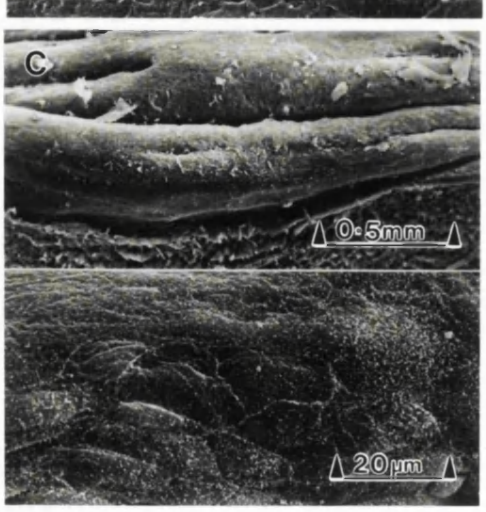
For each of the groups the micrographs show in the upper portion a survey of the lumen and in the lower part a higher magnification detailing the endothelial cells.



Saphenous vein rings were incubated for 1h 10ml of Krebs' solution with endotoxin [100 EU; (A)] shows the vein lumen lined by endothelial cells and at higher magnification their microvillus surface and ridged edges.



Saphenous vein rings were incubated for 1h in Krebs' solution alone. This shows the vein lumen lined by endothelial cells, higher magnification shows a reduced amount of microvilli on their surface and less distinct edges.



Saphenous vein rings added directly to a glutaraldehyde fixative immediately after harvesting. This shows the vein lumen lined by endothelial cells with some plasma protein and red cells on the surface. Higher magnification shows endothelial cells with distinct ridged edges and microvillus surface

Discussion

The results of this study suggest that a brief (1h) exposure to bacterial endotoxin impairs endothelium-dependent relaxation for several days. This effect was termed endothelial stunning since studies *in vitro* suggested that the endothelium was not altered morphologically. Stunning was prevented by prior treatment with a glucocorticoid, indicating that locally generated inflammatory mediators contribute to the effect. If the endothelium of other vessels can also be stunned by a brief exposure to endotoxin this would have implications for vascular function and cardiovascular pathophysiology during infection or post-operatively when endotoxin levels increase transiently (Casey et al. 1992; Mamode et al. 1995; Nieminen et al. 1993).

Endotoxin (in concentrations similar to those that may occur clinically (Brandtzaeg et al. 1989)) attenuated the dilator response to bradykinin and arachidonic. The response to GTN, an endothelium-independent dilator, was unaffected. The attenuation of endothelium-dependent dilatation was greatest 1h after exposure to endotoxin but was still significant 48h later. By 7 days the response had returned to normal. Studies *in vitro* using saphenous vein demonstrated a similar shift in the concentration-response curves to bradykinin after endotoxin whilst the concentration response curves to GTN again was unaltered. This functional change in endothelial-dependent relaxation was not associated with endothelial abnormalities on electron microscopy. These results

suggest that the effects seen were due to endothelial dysfunction rather than de-endothelialisation of the vessel wall.

These studies may provide an insight into the cardiovascular changes that might occur in a wide variety of patients who have transient endotoxaemia (Casey et al. 1992; Shenep et al. 1988) without developing any clinically obvious sepsis. The findings in Chapter 4 suggest that the vascular smooth muscle recovers rapidly from the effects of endotoxin whereas the endothelium does not.

Possible explanations for the reduced relaxant response observed after endotoxin include structural damage to endothelium or smooth muscle, functional antagonism due to differing degrees of preconstriction in different parts of the study, or induction of biochemical and pharmacological changes in the vessel wall. The studies with saphenous vein *in vitro* suggest that endothelial denudation is unlikely to account for the changes seen. Damage to the smooth muscle is also unlikely since the relaxant response to the endothelium-independent dilator GTN was unaltered by endotoxin. Similarly functional antagonism would not explain these findings since the effect was specific for bradykinin and arachidonic acid. Despite the difference in sensitivity to noradrenaline 1h after endotoxin treatment, the degree of preconstriction achieved was similar for each part of the study, and at 24h, when the sensitivity to noradrenaline had returned to normal, the relaxant response to bradykinin and arachidonic acid remained attenuated. Thus the most likely explanation is that a brief exposure to endotoxin induces endothelial dysfunction by stimulating

biochemical and pharmacological changes within the endothelium. This interpretation is strengthened by the finding that hydrocortisone, a drug that inhibits the production of a wide variety of inflammatory mediators and cytokines (Snijdewint et al. 1995; Marx, 1995), prevented the endotoxin-induced abnormality of endothelium-dependent relaxation.

Mechanisms underlying the pharmacological changes induced by endotoxin or cytokines might include an effect on the stability of mRNA for the enzymes nitric oxide synthase and COX (Feng et al. 1995; Yoshizumi et al. 1993), changes in the coupling of receptor stimulation to mediator production (for example G protein related pathways (Daniel-Issakani et al. 1989)) or alterations in the stability or function of the enzymes themselves. Decreased endothelium-dependent relaxation has also been observed in some (Cook et al. 1994; Szabo C et al. 1995) studies in vessels exposed to endotoxin *in vitro* or in animals. Indeed, even in conditions of gross experimental endotoxic shock, when iNOS is expressed (Baxter, 1995; Guc et al. 1990) and generates nitric oxide in amounts sufficient to produce profound vasodilatation and hypotension, decreased 'physiological' endothelium-dependent dilatation has been reported (Parker et al. 1994). These changes observed in animals might be due to an effect of nitric oxide generated from iNOS on the expression or function of the normal constitutive, eNOS. However, functionally active iNOS is not expressed in the veins in response to endotoxin (Chapter 4), yet endothelium-dependent dilatation is markedly diminished. Furthermore, these results in humans clearly indicate that the endotoxin-induced abnormality is not confined to the L-

arginine:nitric oxide pathway but extends to production of dilator prostanoid and persists long after the smooth muscle changes have returned to normal. The effect appears to be generated locally within the vessel wall and the finding that it was reproducible *in vitro* indicates that it is not dependent upon the presence of circulating inflammatory cells. It remains to be determined whether other bacterial toxins (for example staphylococcal toxin) or inflammatory cytokines that are also elevated post-operatively (Baigrie et al. 1993; Syrjanen, 1993) or during the process of unstable angina and myocardial infarction (Guillen et al. 1995) also stun the endothelium. Glucocorticoids (or possibly anti-endotoxin or anti-cytokine antibodies) should prevent the development of endothelial stunning if administered before the inflammatory process has started and it would now be important to identify agents that might reverse established stunning. Arterial endothelium differs from venous endothelium and it would be important to extend these studies to determine whether arterial endothelium is also affected by exposure to endotoxin. It would be important to determine whether other endothelial functions are also deranged during the period of stunning.

There is a largely unexplained association between infection or inflammation and the subsequent development of vascular pathology including myocardial infarction and stroke (Syrjanen, 1993). A preceding febrile respiratory infection is a major risk factor for stroke in young and middle-aged adults (Syrjanen et al. 1988) and transient endotoxaemia often occurs post-operatively, a time when the incidence of myocardial infarction and stroke rises (Baigrie et al. 1993; Casey et

al. 1992; Engstrom et al. 1992; Mamode et al. 1995; Nieminen et al. 1993; Syrjanen et al. 1988). If other vessels behave similarly to the hand veins (particularly, clinically relevant arteries), it is possible that sufficient bacterial toxins or inflammatory cytokines may be present in a variety of conditions to stun the endothelium for several days or longer. Loss of physiological nitric oxide and prostanoid-mediated effects in coronary arteries and other important vessels is associated with vasospasm and a predisposition to thrombosis and occlusion (Luscher and Noll, 1994).

Many studies exploring predisposition to cardiovascular disease have investigated chronic 'stable' factors and their effects on endothelial function. These studies in healthy volunteers indicate that endothelial function may alter on a day-to-day basis and provide a mechanism to link infection or inflammation to increased risk of an acute cardiovascular event. These studies suggest the existence of a novel variable and potentially modifiable cardiovascular risk factor, that of transient 'endothelial stunning'.

Chapter 7

The effects of inflammatory cytokines on endothelium-dependent dilatation

Introduction

In the previous chapter a brief exposure to bacterial endotoxin was shown to impair endothelium-dependent relaxation for many days and this suggests that transient "stunning" of endothelial function might provide a mechanism linking infection to increased risk of infarction.

Cytokines mediate many of the biological effects of endotoxin (Martich et al. 1993) and local concentrations of certain pro-inflammatory cytokines are significantly elevated in patients with unstable angina and myocardial infarction (Basaran et al. 1993; Guillen et al. 1995; Miyao et al. 1993). Indeed, it has been suggested that an inflammatory response might trigger the transition from stable to unstable atheroma (Liuzzo et al. 1994). In this chapter the effects TNFα, IL-1β and IL-6 on endothelial function in healthy volunteers was assessed.

Protocol

Studies were performed in (8) male and (14) females subjects aged between 18-36.

TNFα (1ng in 1ml of saline), IL-1β (1ng in 1ml of saline) and IL-6 (100pg in 1ml of saline) were instilled for 1h, either individually or together. The

calculated concentration of cytokine was in the order of 300-1000pg/ml (TNFα and IL-1β) and 30-100pg/ml (IL-6).

Dilator dose-response curves

Vessels were preconstricted to approximately 50% of resting diameter by a continuous infusion of noradrenaline (5-640pmol/min). Then bradykinin (2, 4, 8pmol/min, each dose for 5 min), arachidonic acid (0.2, 2, and 20nmol/min, each dose for 5 min) or glyceryl trinitrate [GTN] (1, 2, 4pmol/min, each dose for 5 min) was co-infused with the noradrenaline and relaxation recorded. In all studies a 10-15 min washout period (infusion of noradrenaline alone) separated the dose response to different agonists.

Effects of cytokines

In 40 subjects dilator dose response curves were constructed before and at 1, 6, 24, and 48h after instillation of either TNFα alone, IL-1β alone, IL-6 alone, TNFα and IL-1β together, or TNFα, IL-1β and IL-6 together.

Effects of antiinflammatory drugs on the response to cytokines

Hydrocortisone

Subjects were given oral hydrocortisone (100mg) 2h before the study and dilator dose response curves were constructed before and 1h after instillation

of TNFα alone (n=5), IL-1β alone (n=5) or a combination of TNFα, IL-1β and IL-6 (n=5).

Aspirin

Subjects were given oral aspirin (75mg or 1g) 2h before the study and dilator dose response curves were constructed before and 1h after instillation of TNFα alone (n=5; highest dose of aspirin only) or a combination of TNFα, IL-1β and IL-6 (n=5; each subject studied twice, once with high dose and once with low dose aspirin).

Results

Neither TNFα alone nor IL-6 alone affected the constrictor response to noradrenaline. However, as described in Chapter 5, IL-1β alone and combinations of IL-1β with the other cytokines decreased the constrictor responses, and in order to maintain the same degree of preconstriction the noradrenaline dose was increased as necessary (Table 1).

Table 1 **Dose of noradrenaline used and percentage preconstriction of the vein**

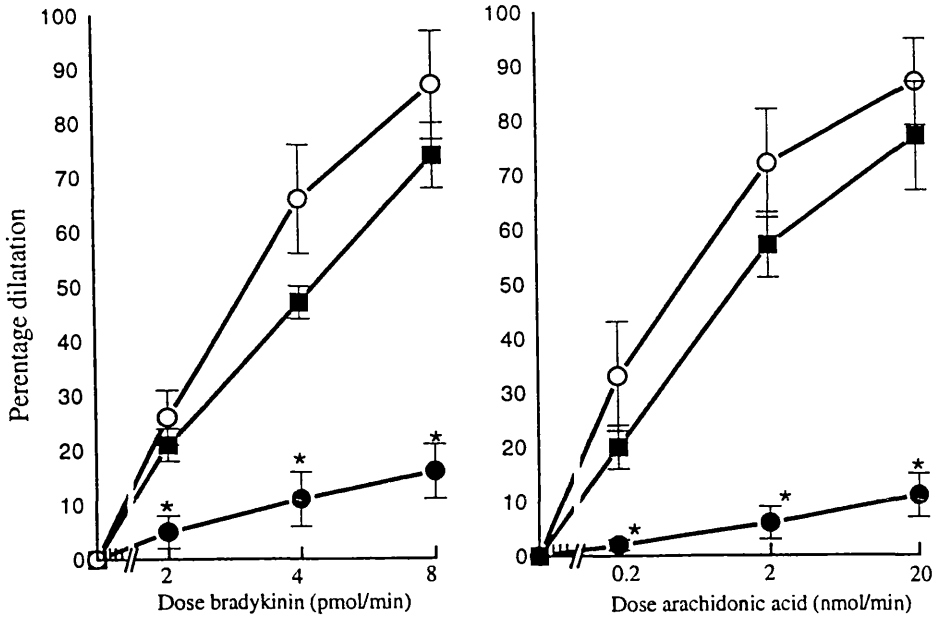
Study	# Time(h)	Dose of noradrenaline (pmol/min)	Percentage preconstriction
TNFα	0	34±12	52±4
	1	36±11	45±2
	6	40±11	52±6
IL-1β	0	27±5	48±3
	1	73±21	51±4
	6	93±15*	55±3
	0	42±16	48±8
IL-6	1	38±6	52±6
	6	45±9	43±7
TNFα and IL-1β	0	24±4	48±3
	1	36±4	50±2
	6	72±8*	52±2
	24	40±4	50±2
TNFα, IL-1β and IL-6	0	40±11	44±4
	1	72±23	44±2
	6	160±44*	48±2
	24	96±56	49±3
	48	36±4	52±5
1g aspirin and TNFα	0	40±11	49±2
	1	53±6	47±2
	6	48±8	46±3
75mg aspirin + TNF α, IL-1β and IL-6	0	36±4	44±5
	1	64±10	48±2
1g aspirin + TNF α, IL-1β and IL-6	0	50±9	52±2
	1	96±16	47±2
Hydrocortisone and TNFα	0	24±4	52±6
	1	28±5	47±2
Hydrocortisone + TNFα, IL-1β and IL-6	0	56±10	44±2
	1	36±4	54±2

Time 0, 1, 6, 24, and 48h represent the time before (0) and the designated time after instillation of cytokine. * $p<0.05$ - p values compared with time 0

Effects of cytokines on dilator-response

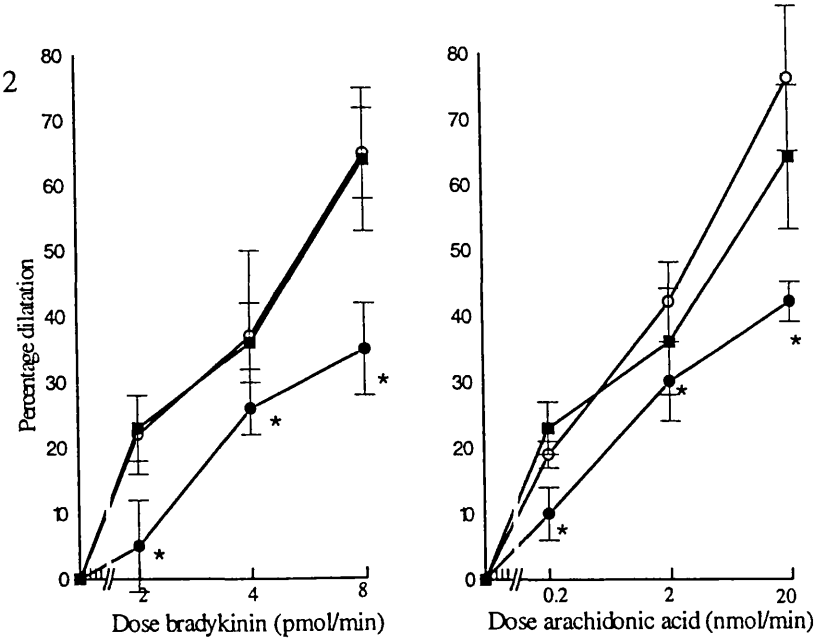
TNFα and IL-1β attenuated the dilatation to bradykinin and arachidonic acid (Figure 1 and 2). IL-6 was without effect.

Figure 1



Vessels were preconstricted to approximately 50% of their resting diameter by a continuous infusion of noradrenaline (10-1280pmol/min). Then bradykinin (2, 4, 8pmol/min, each dose for 5min - left panel) and arachidonic acid (0.2, 2, 20nmol/min, each dose for 5 min - right panel) were coinfused with the noradrenaline and relaxation recorded. Studies were undertaken before (o), 1h (●) and 6h (■) after TNFα was instilled (n=5). * indicates $p<0.05$

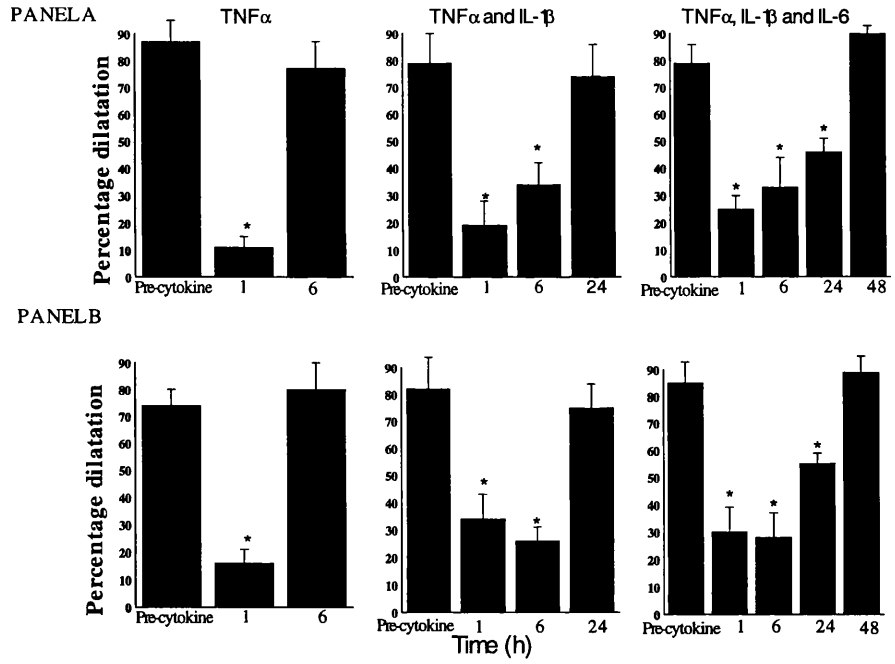
Figure 2



Vessels were preconstricted to approximately 50% of their resting diameter by a continuous infusion of noradrenaline (10-1280pmol/min). Then bradykinin (2, 4, 8pmol/min, each dose for 5min - left panel) and arachidonic acid (0.2, 2, 20nmol/min, each dose for 5 min - right panel) were coinfused with the noradrenaline and relaxation recorded. Studies were undertaken before (o), 1h (●) and 6h (■) after IL-1β was instilled (n=5). * indicates $p<0.05$

The duration of the effect of TNFα alone or IL-1β alone was short-lived and the dilatation to bradykinin and arachidonic acid returned to normal by 6h. Combining TNFα, IL-1β and IL-6 did not increase the magnitude of the effect on endothelium-dependent dilatation but increased the duration (Figure 3). When all 3 cytokines were instilled together for 1h the impairment of endothelium-dependent dilatation persisted for at least 24h (Figure 3).

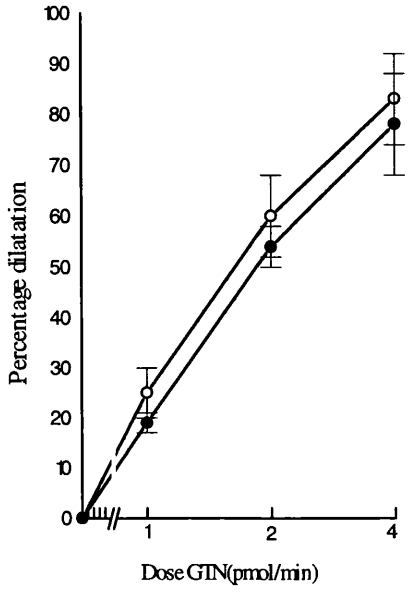
Figure 3



Dose response curves to bradykinin (Panel A) and arachidonic acid (Panel B) were constructed before and at varying intervals after TNFα or IL-1β or IL-6 alone or in combination. At each time point the maximal dilatation achieved to bradykinin and arachidonic acid is shown (n=5). * $p<0.05$

In contrast to the effects of cytokines on endothelium-dependent dilatation, the response to GTN was unaltered by instillation of cytokines (Figure 4).

Figure 4



Dose response curves were constructed before (o) and 1h after TNFα, IL-1β and IL-6 (●) were instilled in combination.

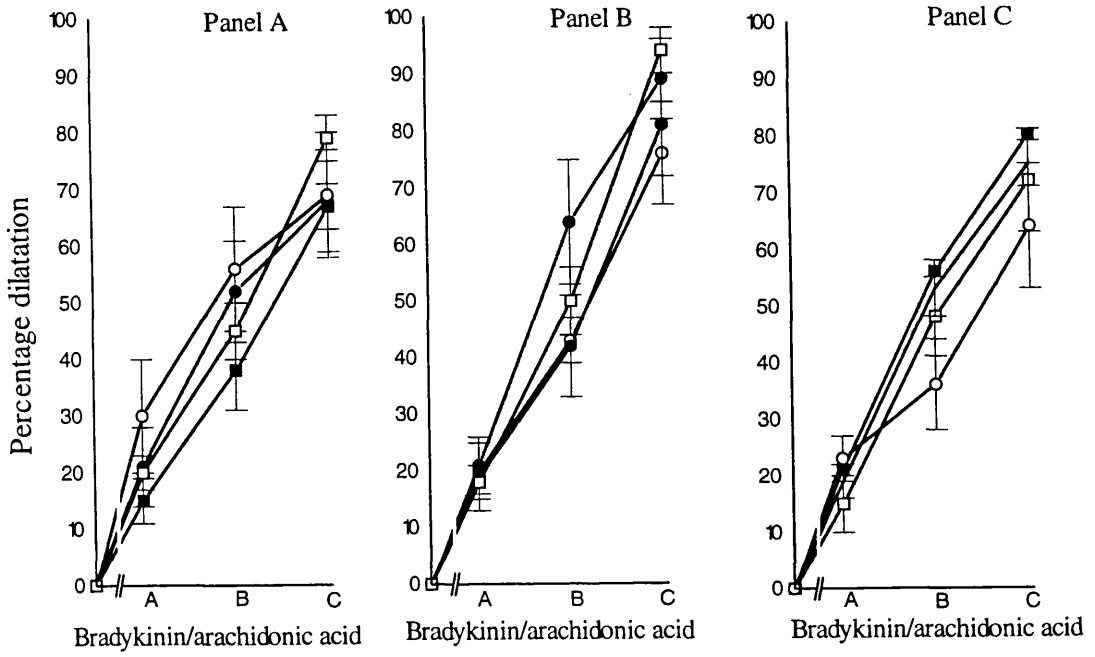
Effects of antiinflammatory drugs on the response to cytokines

Hydrocortisone

Prior administration of oral hydrocortisone prevented the effects of cytokines on dilatation to bradykinin and arachidonic acid (Figure 5). For example, in the hydrocortisone pre-treatment group the dilatation to bradykinin (2, 4, and 8pmol/min) was 19±5%, 43±4%, and 81±9% before, and 21±5%, 64±11% and 89±7% 1h after instillation of TNFα, IL-1β and IL-6. Similarly, the dilatation to arachidonic acid before instillation of the cytokines was: 19±6%,

42±10% and 81±9% and 1h after instillation of TNFα, IL-1β and IL-6 was 81 ±4%, 50±6% and 94±4%.

Figure 5

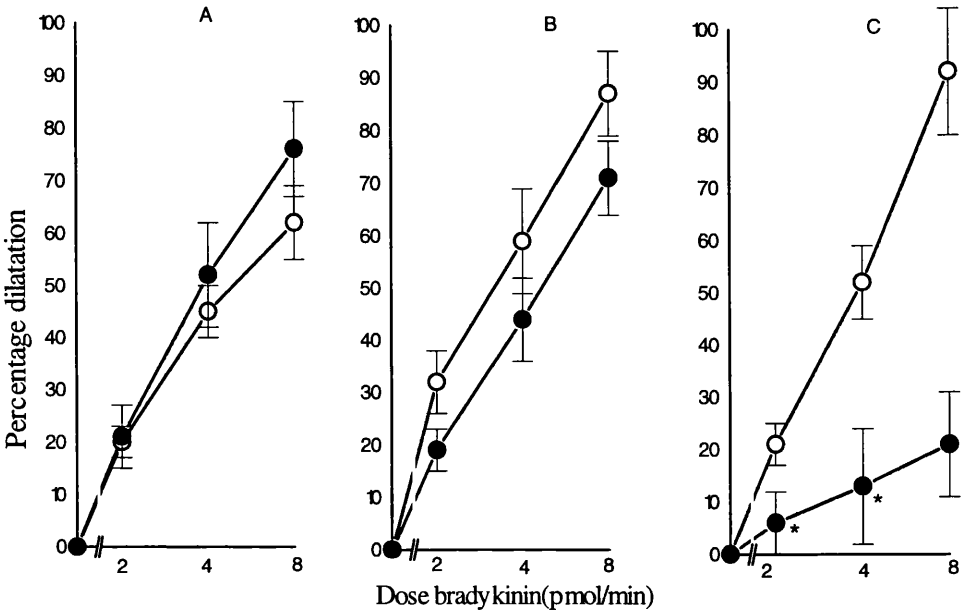


Subjects were given oral hydrocortisone (100mg). Two hours later TNFα (panel A), IL-1β (Panel B) or a combination of TNFα, IL-1β, and IL-6 (Panel C) was instilled into the vein. Dose response curves to bradykinin (O) and arachidonic acid (□) were constructed before (open symbols) and 1h after (closed symbols) instillation. Doses of arachidonic acid: A = 0.2nmol/min B = 2nmol/min C = 20nmol/min
Doses of bradykinin: A = 2pmol/min B = 4pmol/min C = 8pmol/min

Aspirin

Prior administration of high dose aspirin (1g) significantly attenuated ($p<0.05$) the inhibitory effects of either TNFα alone or the combination of TNFα, IL-1β and IL-6 (Figure 6 A and B). However, pre-treatment with low dose aspirin was without effect (Figure 6 C).

Figure 6



(A) In 5 subjects high dose oral aspirin (1g) was given. TNFα was instilled into the vein 2h later. Dose response curves to bradykinin were constructed before (O) and 1h after instillation (●).

(B) In 5 subjects high dose aspirin (1g) was given. TNFα, IL-1β and IL-6 was instilled into the vein 2h later. Dose response curves to bradykinin was constructed before (O) and 1h after instillation (●).

(C) In 5 subjects low dose oral aspirin (75mg) was given. TNFα was instilled into the vein 2h later. Dose response curves to bradykinin were constructed before (O) and 1h after instillation (●).

* indicates $p<0.05$

Discussion

The results of this study in healthy volunteers demonstrate that a brief (1h) exposure to certain pro-inflammatory cytokines results in prolonged endothelial dysfunction. The studies in Chapter 6 demonstrated that endotoxin

causes endothelial dysfunction in this model and the term "endothelial stunning" was used to describe the phenomenon. The present study demonstrates that doses of cytokines sufficient to produce a local concentration similar to those that occur during infective and non-infective inflammatory states (Basaran et al. 1993; Cannon et al. 1990; Guillen et al. 1995; Hesse et al. 1988; Miyao et al. 1993; Neumann et al. 1995; Tashiro et al. 1995), also results in prolonged and profound endothelial stunning and indicates that cyclooxygenase activity may contribute to the genesis of the dysfunction. This is the first demonstration that cytokines impair endothelium-dependent dilatation in humans and suggests a novel cardioprotective effect of aspirin.

Experiments described in the previous chapter demonstrated that a brief exposure to endotoxin impairs endothelium-dependent relaxation for several days. This effect was also seen when human saphenous vein was incubated with endotoxin *in vitro* and was not due to structural damage to the endothelial layer. Similarly it has been reported that acute exposure to endotoxin impairs the production of nitric oxide in response to bradykinin in bovine endothelial cells in culture (Myers et al. 1994). In the present study, the effects of 3 cytokines that have been implicated in mediating the inflammatory response and which are found in elevated local concentrations in the plasma of patients with acute myocardial infarction or unstable angina (Basaran et al. 1993; Guillen et al. 1995; Miyao et al. 1993; Neumann et al. 1995; Tashiro et al. 1995) were explored. IL-1β and TNFα impaired the relaxant responses to

bradykinin (a mediator that acts through stimulation of nitric oxide production in these vessels (Vallance et al. 1995; Vallance et al. 1989)) and to arachidonic acid (the precursor of prostanoid synthesis (Moncada and Vane, 1978)). The effect was specific for endothelium-dependent dilators since the response to the nitric oxide donor GTN was unaltered. IL-6 had no effect on its own, but the combination of all 3 cytokines produced the most long-lasting impairment of endothelium-dependent relaxation, with recovery only occurring by 48h. The manoeuvre of isolating and wedging the vein for 1h in the absence of cytokines does not cause endothelial dysfunction (Chapter 6).

Care was taken to ensure that the degree of preconstriction was identical for each study (Table 1). IL-1β induces hyporesponsiveness to noradrenaline as described in Chapter 5 and more noradrenaline was used to induce the same degree of constriction after instillation of IL-1β. However, it is unlikely that the impaired relaxation observed was due to differences in the amount of noradrenaline required to preconstrict the vessels for the different parts of the study, since TNFα alone did not alter the constrictor response to noradrenaline but was the most effective cytokine to selectively impair endothelium-dependent relaxation. Furthermore, high dose aspirin abolished the endothelial dysfunction induced by the combination of TNFα, IL-1β and IL-6 without affecting the hyporesponsiveness to noradrenaline.

Prior administration of hydrocortisone prevented the inhibitory actions of the cytokines on endothelial dilator function suggesting that expression of

inflammatory enzymes and generation of local inflammatory mediators were responsible for the effects seen. An involvement of COX is suggested by the observation that an anti-inflammatory dose (1g) of aspirin significantly reduced the effects of cytokines on endothelial function, whereas a low cardioprotective dose (75mg) of aspirin had no effect. The experiments performed in Chapter 3 demonstrated that this antiinflammatory dose of aspirin (1g) abolished vascular prostanoid synthesis whereas a low cardioprotective dose of aspirin (75mg) abolished arachidonic acid-induced platelet aggregation without affecting arachidonic acid-induced venodilatation. Thus the endothelial dysfunction induced by cytokines appears to depend on prostanoid synthesis within the vessel wall. One possibility is that inducible COX-11 was expressed in response to cytokines and that the activity of this enzyme contributes to the endothelial dysfunction. It seems unlikely that COX-1 activity in the endothelium was responsible, since the usual dilatation to arachidonic acid was abolished. Consistent with this possibility, mRNA encoding for COX-11 activity was present in the tissue samples, although due to small quantities obtained, it was not possible to undertake quantitative studies to determine whether significant induction of COX-11 occurred.

Administration of TNFα depresses endothelium-dependent relaxation *in vivo* (Wang et al. 1994), and *in vitro* TNFα reduces the half-life of mRNA coding for nitric oxide synthase (Yoshizumi et al. 1993). In addition, in patients with heart failure, significantly elevated levels of TNFα have been documented (Levine et al. 1990) and, in experimental heart failure, reduced gene

expression of endothelial NO synthase and COX-1 activity has been reported (Smith et al. 1996). It is not known whether COX-II activity contributes to these effects of TNFα, however generation of free radicals as a by-product of COX activity (Darely-Usmar and Halliwell, 1996) might affect endothelial function, and in studies in animals, the endothelial dilator dysfunction that occurs during endotoxaemia is significantly restored in the presence of free radical scavengers (Siegfried et al. 1992). In vessels from the Wistar Kyoto rat, incubation of vascular rings with IL-2 induces a change in the vessel wall such that arachidonic acid becomes a constrictor agent (Casey et al. 1993). Together with this study these earlier reports support a role for COX in mediating endothelial dysfunction. It is unlikely that increased synthesis of nitric oxide due to expression of the iNOS contributed to the effects observed, since TNFα does not induce iNOS expression in this model (Chapter 5). Further studies will be required to explore these mechanisms and to understand why such a brief (1h) exposure to cytokines causes such prolonged endothelial dysfunction (lasting at least 24h).

The cytokines were instilled in doses sufficient to produce a local concentration similar to those found in patients with certain infections (Casey et al. 1993), acute myocardial infarction and unstable angina (Basaran et al. 1993; Guillen et al. 1995; Miyao et al. 1993; Neumann et al. 1995; Tashiro et al. 1995). The effects are generated locally within the blood vessel and previous studies (Chapter 4) have indicated that even an adjacent vessel on the same hand is unaffected by agents instilled into the isolated segment. Of

course it remains to be determined whether the observations made in the superficial hand vein are relevant to what might happen in the coronary or carotid artery or other clinically important vessels. However, the pharmacology and physiology of the hand veins is similar to that of the saphenous vein (Aellig, 1981; MacAllister et al. 1995; Vallance et al. 1989; Aellig, 1994), a vessel widely used for by-pass grafting (Luscher et al. 1988), and the vessels were studied *in situ* in the usual physiological environment.

What are the potential clinical implications of this study? The incidence of acute myocardial events or stroke appears to rise significantly after a febrile or bacteraemic illness (Pesonen and Siitonen, 1981; Spodick et al. 1984; Syrjanen et al. 1988). The experiments in Chapter 6 demonstrated that exposure of the vessel to bacterial endotoxin impaired endothelial function for several days. These studies show that certain cytokines that might be generated in a much wider range of infectious or inflammatory conditions, and which have been implicated in the pathogenesis of acute cardiovascular events (Basaran et al. 1993; Guillen et al. 1995; Miyao et al. 1993; Neumann et al. 1995; Tashiro et al. 1995), also impair endothelium-dependent dilatation in healthy volunteers *in vivo*. TNFα appears to be the critical in this process. The impairment is not confined to the L-arginine: NO pathway but also affects dilator prostanoid production. If other human vessels are affected by these inflammatory cytokines in the same way as the hand veins are, these results demonstrate a mechanism by which an acute inflammatory response (for example, due to infection or surgery) might be linked to transiently increased risk of acute cardiovascular events including deep vein thrombosis. It remains

to be determined whether the protective effects of aspirin observed contribute to the efficacy of this drug in unstable angina or acute myocardial infarction.

Chapter 8

Summary and conclusions

The experiments in this thesis have attempted to explore the change in vessel wall tone that occurs following exposure to known inflammatory mediators. The studies have provided strong evidence to suggest that the vascular wall is equipped with most of the necessary receptor and mediator-response functions to orchestrate a local dilator response to inflammatory signals - without the need to elicit a systemic response.

Studies in different species have shown a variable response to arachidonic acid and its metabolites both *in vitro* and *in vivo* (Dusting et al. 1981; Kontos et al. 1984; Salzman et al. 1980; Toda et al. 1988; Weintraub et al. 1994; Redl et al. 1996). Indeed, intradermal injection of arachidonic acid in humans causes microvessel constriction rather than dilatation. Prostanoid-receptor populations vary within the different vascular beds and this may account for the variable functional response. Nevertheless, the results of the studies in Chapter 3 suggest that, although there does not appear to be any basal prostanoid production, the vessel wall is capable of producing vasoactive amounts of prostanoid that can effect major changes in vascular tone.

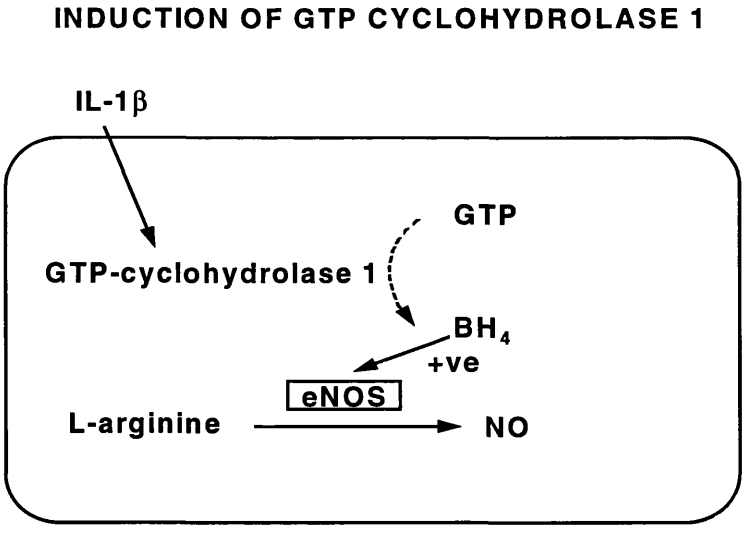
From the studies performed in this thesis, it is not clear whether the prostanoids derive from the endothelium or smooth muscle. Both components of the vessel wall are capable of releasing prostacyclin and other vasodilator prostanoids and an important experiment to determine the origin of prostanoid release *in vivo* would be infusion of arachidonic acid into a de-endothelialised dorsal hand vein.

The studies in Chapter 4 explored the effect of an exogenous inflammatory mediator, endotoxin on vascular tone. Prior to these studies other investigators examined the effects of a systemically injected bolus doses of endotoxin into healthy volunteers in order to examine the inflammatory response. Injection of endotoxin in these experiments produced a characteristic and reproducible biphasic drop in systemic blood pressure. The initial loss of vascular reactivity is also seen in many animal models of endotoxaemia and several studies have suggested a role for eNOS or COX-I in contributing to the immediate response to endotoxin. The studies in this thesis do not support these observations - neither the NO nor the prostanoid pathway appear to be involved. The possibility remains that the acute effects of endotoxin may be due to the production of metabolites generated by reactive oxygen species (such as isoprostanes) (Wolin, 1996) or changes in α-adrenergic receptors. It would be important to explore the effects of endotoxin on the response to other vasoconstrictor agents such as angiotensin II and constrictor prostanoids, to determine whether the hyporesponsiveness observed was specific or generalised. It would also be important to determine whether in isolated blood vessels exposed to endotoxin addition of CD14 would allow these results to be repeated and the mechanisms explored *in vitro.*

Following systemic injection of endotoxin several key proinflammatory cytokines are released into the circulation. Many *in vitro* and *in vivo* animal studies have highlighted the importance of TNFα and IL-1β in inducing

changes in vessel wall reactivity (predominantly as a result of excess NO production) that occur during endotoxaemia. Many animal studies suggest TNFα to be the important systemically circulating cytokine responsible for this increased production of NO as a result of the induction of iNOS. In contrast to these animal experiments the studies in Chapter 5 demonstrated that IL-1β (and not TNFα) induced the greatest local vascular hyporesponsiveness. Moreover, and again in contrast to the animal studies, the increased NO production was not due to the induction of iNOS. The experiments showed that IL-1β appeared to regulate the expression and activity of eNOS through the induction of GTP-cyclohydrolase-I, resulting in greater cofactor production (tetrahydrobiopterin) for increased NO generation (Figure 1).

Figure 1



IL-1β might regulate the activity of eNOS through the induction of GTP cyclohydrolase-I, resulting in greater cofactor production (tetrahydrobiopterin - BH4) for increased NO generation.

The results in the hand veins do not preclude the possibility that iNOS is expressed in other vessels or in disease states but indicate a novel route for the induction of NO generation. Increased eNOS activity, rather than induction of iNOS would be consistent with many of the observations made by other researchers and might help explain certain anomalies. It is known that the iNOS gene is evolving rapidly and differs markedly between mouse and man and that the levels of nitrate in patients with sepsis are elevated by only about 50% on average compared with the 1000% elevation seen in rodent models of shock (Evans et al. 1993; Tracey et al. 1995). Indeed, the nitrate levels in human sepsis are very similar to the levels seen in animals rendered hypotensive through maximal stimulation of **eNOS** by infusion of agonists (for example, Substance P and bradykinin). It would be important to extend the observations in this thesis by examining vascular tissue from septic and other inflammatory diseases to determine whether there is induction of GTP-cyclohydrolase-I with absent iNOS. It would also be interesting to determine whether there are arteriovenous differences and whether the basal generation of NO in arteries is linked to basal expression of GTP-cyclohydrolase-I.

The studies in Chapters 6 and 7 explored the effect of endotoxin and cytokines on endothelial dilator function. This area has been less extensively researched since inflammation has been thought of as a vasodilatory stimulus. In contrast to the findings in Chapter 5, TNFα (rather than IL-1β) appeared to be the more important cytokine responsible for the endothelial dysfunction seen.

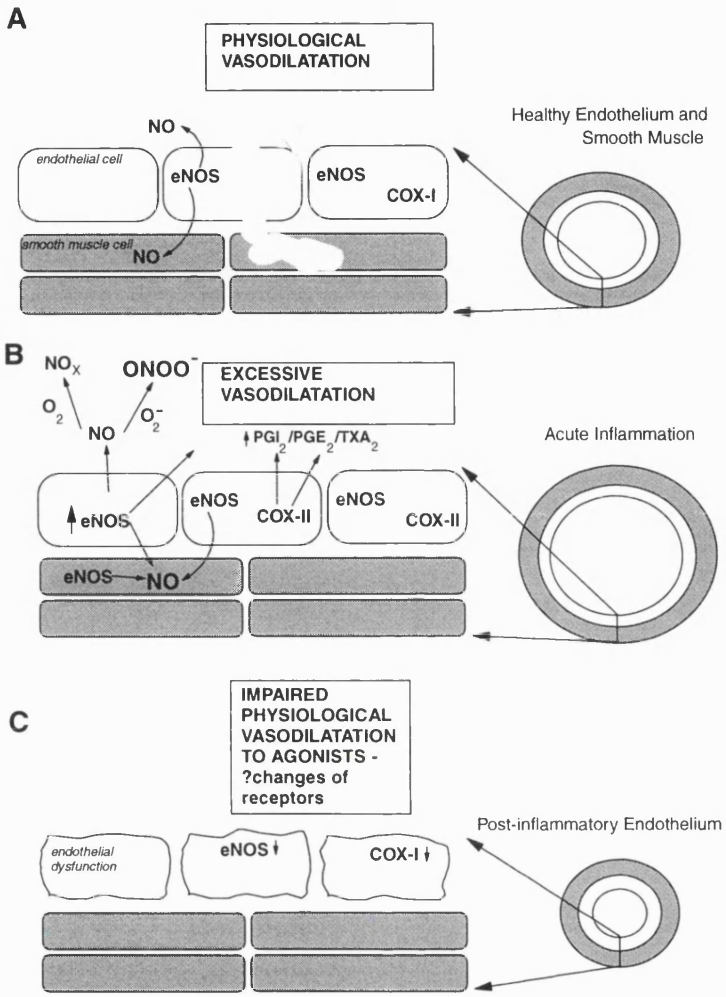
Moreover, the dysfunction appeared to affect both the agonist-stimulated NO and the prostanoid pathway and persisted for considerably longer than the effects of cytokines on smooth muscle tone. Many animal studies have also confirmed the importance of TNFα in the genesis of endothelial dysfunction (Myers et al. 1994; Wang et al. 1994).

The clinical relevance of the loss of endothelium-dependent relaxation in inflamed vessels is not known. However, if infection (and transient endotoxaemia - resulting in a transient cytokinaemia) predisposes to an increased risk of cardiovascular events, it is possible that endothelial dysfunction might provide a link. From case control studies it has been estimated that about 4% of bacteraemic patients will develop an acute myocardial infarction within one month of the onset of an infection and that up to 10% of all strokes may be associated with preceding bacteraemic infections (Valtonen et al. 1993). Abdominal surgery is often associated with transient bacteraemia or leakage of endotoxin into the circulation, and is accompanied by a systemic inflammatory response with cytokine production (Baigrie et al. 1993). Again, there is an increase in the risk of cardiovascular disease, with the incidence of acute myocardial infarction remaining elevated for several weeks after surgery (Mamode et al. 1995).

The normal metabolic activity of vascular endothelium exerts a basal thrombo-resistant and vasodilator influence upon the cardiovascular system. Infection of endothelial cells with respiratory or other viruses, or exposure to

certain pro-inflammatory cytokines leads to expression of tissue factor, cell surface adhesion molecules and induction of procoagulant activity (Vane et al. 1990; Brody et al. 1992; Paleolog et al. 1994; Vallance, 1995; Visseren et al. 1996). The studies in Chapter 5, 6 and 7 suggest that perhaps initially the infection or inflammation may lead to vasodilatation due to increased generation of NO or products of the arachidonic acid cascade. After the acute vasodilator stage of the illness has resolved the residual endothelial changes may tip the balance of mediators produced in favour of thrombosis and vasospasm (Figure 2), and this might be important in the pathogenesis of arterial and venous thromboembolic disease (Valtonen et al. 1993). To explore the clinical relevance of this hypothesis further it will be necessary to undertake studies in defined patient groups, to identify the mechanisms of any changes seen, and to explore the effects of additional endothelial dysfunction on the already abnormal endothelium that overlies atheromatous plaques.

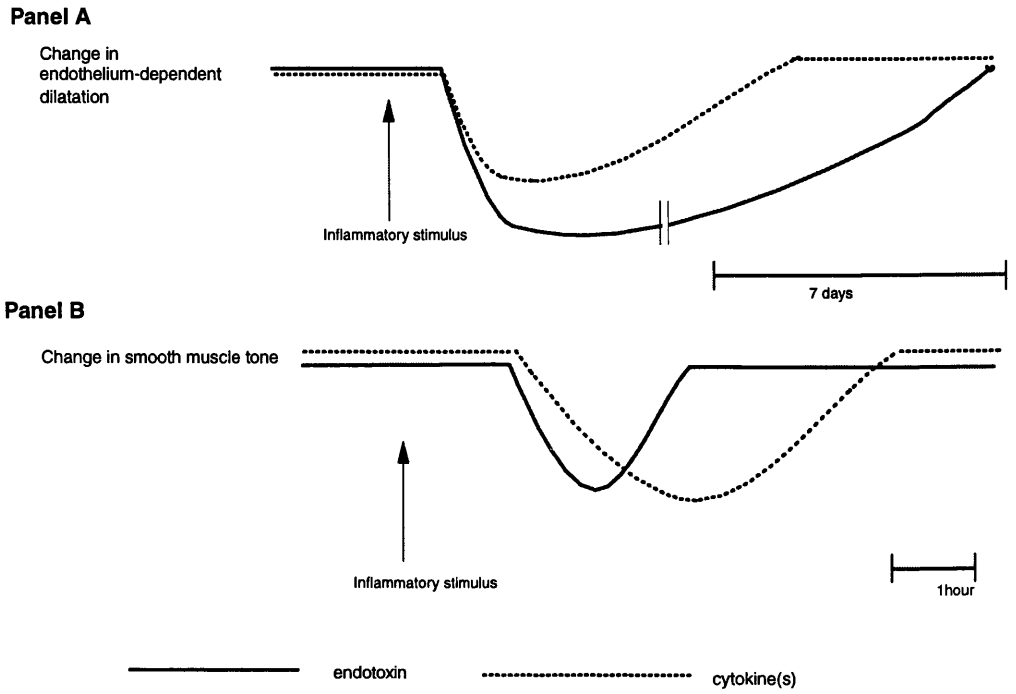
Figure 2



A: Healthy endothelium produces anticlotting, antiaggregatory, antiadhesive and vasodilator mediators. B: In infective or inflammatory states increased NO and prostanoid production results in excessive vasodilatation. C: After recovery of the acute insult the endothelium may remain dysfunctional for a long time before fully recovering its ability to contribute towards homeostatic functions. This loss may result in a predisposition towards vasospasm and thrombosis.

Endotoxin and cytokines produced a prolonged state of hyporesponsiveness to the endothelium-dependent vasodilator agents used (Figure 3, Panel A). In contrast, the response of the smooth muscle was much shorter-lived both to endotoxin and cytokines (Figure 3, Panel B). Paradoxically, there appeared to be a greater production of endogenous basal NO generated in response to IL-1β in spite of an impaired responses to 'endothelium-dependent' agonists.

Figure 3



How does one resolve the differences seen, with TNFα function producing a reduced response to bradykinin and to IL-1β increasing production of NO? A possible explanation is that TNFα exerts an acute effect on endothelial function resulting in a bradykinin and prostanoid receptor down-regulation (or a change in the receptor sub-types) and that IL-1β is responsible for the more delayed up-regulation of eNOS through induction of GTP-cyclohydrolase-I. The excess NO production results in vascular hyporesponsiveness to noradrenaline but the ability to stimulate eNOS using bradykinin is attenuated due to a change in receptor type or affinity. It would be important to extend the observations in this thesis by exploring the effects of agents that by-pass the receptors involved in mediating NO/prostanoid release in order to probe

further the possible sites of endotoxin/cytokine induced endothelial dysfunction. An example would be the use of the calcium ionophore A23187, an agent that activates eNOS by increasing intracellular calcium concentration. An absence of any change in the dilatation seen to this agent before and after exposure to endotoxin or cytokine would lend support to the receptor-change theory alluded to above. The results highlight the importance of using probes for specific parts of pathways when exploring and interpreting pharmacological changes *in vivo*.

The limitation of these studies is that they were performed in the venous circulation which may not necessarily reflect changes elsewhere in the vascular system. Moreover, changes that occur during a systemic inflammatory response may differ from the isolated and controlled local response seen in these experiments. It would now be important to develop methods to explore the local response to these inflammatory agents in the arterial system. In addition, some of the studies in this thesis have investigated the changes in endothelium-dependent vasodilator tone during acute inflammation. Utilising the same methods, the studies could be extended to examine the change in endothelium-dependent vasoconstrictor factors during an acute inflammatory response.

In summary, using the methods described in this thesis it has been possible to probe some of the mechanisms responsible for eliciting the changes in local vascular reactivity that occur during acute inflammation. The studies have

shown that the vessel wall itself is capable of activating the signal transduction pathways necessary to initiate the vasodilatation seen following exposure to inflammatory stimuli without the need to invoke a systemic response. Moreover, the studies have also shown that although animal models provide insights into pathophysiological mechanisms in acute inflammation, often the results of these studies cannot be extrapolated directly to the human condition. Ultimately, mechanisms need to be explored in human models. The model described here allows this to be done in a controlled fashion and allows coupling of molecular and pharmacological changes.

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Direct measurement of nitric oxide in human beings

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Nitric oxide is a short-lived radical involved in various biological processes. We have used an electrochemical microsensor to detect nitric oxide signals in blood vessels of healthy volunteers. The sensor was inserted into a hand vein, and the vessel was stimulated with acetylcholine or bradykinin. Dose-dependent signals were detected and were attenuated by an inhibitor of nitric oxide synthase. The results provide further evidence that endothelium-derived relaxing factor is nitric oxide and demonstrate a method for monitoring the L-arginine/nitric-oxide pathway in human beings.

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Nitric oxide, synthesised from L-arginine, is involved in cardiovascular control, regulation of platelet function, neurotransmission, and host defence.[1] To explore the role of nitric oxide in man, synthesis blockers, such as N^G monomethyl-L-arginine (L-NMMA) are often used.[2] However, the short half-life of nitric oxide and its instability have precluded direct measurement of its formation in vivo. Electrochemical methods for the detection of nitric oxide have now been described[3,4] and one of these has been developed for use in vivo.[5] We report direct measurement of nitric oxide released in superficial veins in healthy volunteers.

We studied, with the approval of the local ethics committee, 6 subjects (5 male) aged 26–40. Subjects lay supine in a temperature-controlled laboratory (28–29°C). One arm was placed on a support with a cuff around the upper arm inflated to 40 mm Hg to maintain venous distension. Two cannulae were inserted into a hand vein. A 22 G catheter was inserted retrogradely and a 23 G butterfly needle was positioned anterogradely with its tip 10–15 mm from the end of the catheter. The catheter was flushed with 0·5 mL heparin (5000 U/mL) and a nitric oxide sensor mounted on a 22 G needle was placed so its tip protruded 3–5 mm beyond the end of the catheter. A platinum-wire counter-electrode and silver/silver-chloride reference electrode were placed on the skin adjacent to the vein and covered with conductive gel.

Porphyrinic sensors were prepared, and those that showed good catalytic properties, high conductivity of polymeric porphyrin, and integrity of the covering Nafion film were selected.[4,5] Sensors were sterilised with ethylene oxide. Differential pulse amperometry was used to monitor signals. Each sensor's performance was characterised before and after use under static and dynamic conditions (flow of 1·0–1·2 mL/min). Amperometric current was proportional to nitric oxide concentration. Calibration curves were constructed from 2×10^{-8} to 10^{-6} mol/L (detection limit 5×10^{-9} mol/L). The sensor did not generate a signal directly in response to acetylcholine, bradykinin, L-NMMA, L-arginine, heparin, nitrite, or nitrate.

Heparinised (10 000 U/L) physiological saline or drugs (passed through a 0·2 μm bacterial filter) were infused continuously through the butterfly needle (0·5 mL/min). Current was recorded until baseline stabilised. Bradykinin (Sigma, 80 pmol/min for 3 min) was infused (five infusions in 3 subjects) and, in 1 subject, three doses of bradykinin (20, 40, and 80 pmol/min) were infused on two occasions. In 3 further subjects a dose-response curve to acetylcholine (Sigma) was constructed (1·25, 12·5, and 125 nmol/min, each dose for 3 min with 10 min between doses). In 1 subject the highest dose of acetylcholine was infused three times. The deadspace in the infusion system was 40 s. The concentrations of nitric oxide for each experiment were calculated by comparing the current measured from experimental amperogram with the current obtained with nitric oxide solutions.

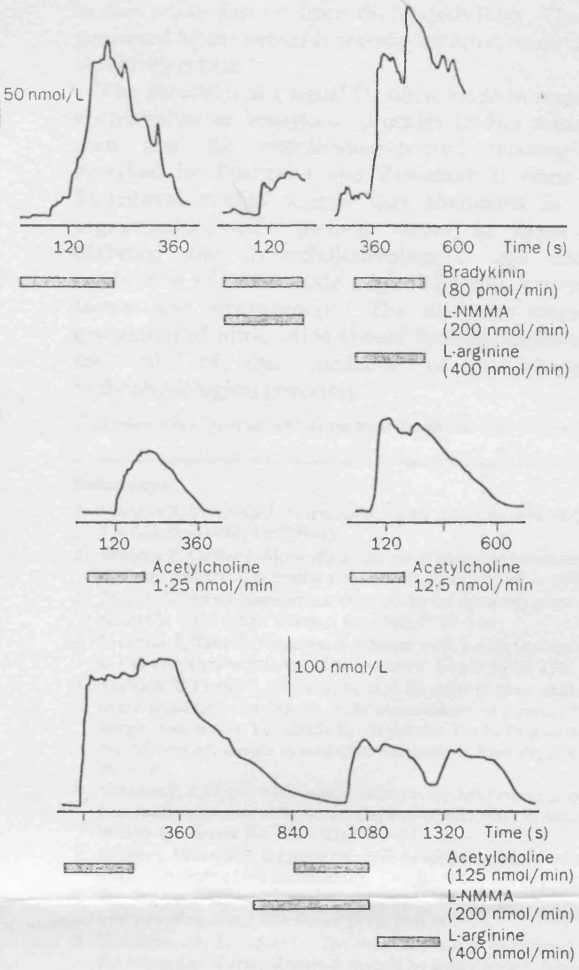

Figure 1: **Amperograms of nitric oxide release during local infusion of bradykinin (upper) and acetylcholine (lower)**

Consistent with previous functional studies in these[6] and other veins, we did not detect basal release of nitric oxide. Bradykinin 80 pmol/min increased the signal from the sensor (mean peak concentration of nitric oxide detected was 124 [SD 19] nmol/L, figure 1). This signal was attenuated by co-infusion of L-NMMA (Wellcome, 200 nmol/min for 5 min), and restored after infusion of L-arginine (Sigma, 400 nmol/min for 6 min). The data for 1 subject in whom we could construct two dose-response curves are shown in figure 2. The rate of increase of nitric oxide concentration was about 0·9 and 1·9 nmol L^{-1} s^{-1} for bradykinin doses of 20 and 80 pmol/min, respectively.

Infusion of acetylcholine caused a dose-dependent signal (figures 1 and 2). The rate of increase of nitric oxide concentration was dose-dependent: acetylcholine 1·25, 12·5, and 125 nmol/min increased nitric oxide concentration by 1·0 (0·2), 3·5 (0·2), and 8·9 (0·3) nmol L^{-1} s^{-1}, respectively. When the highest dose of

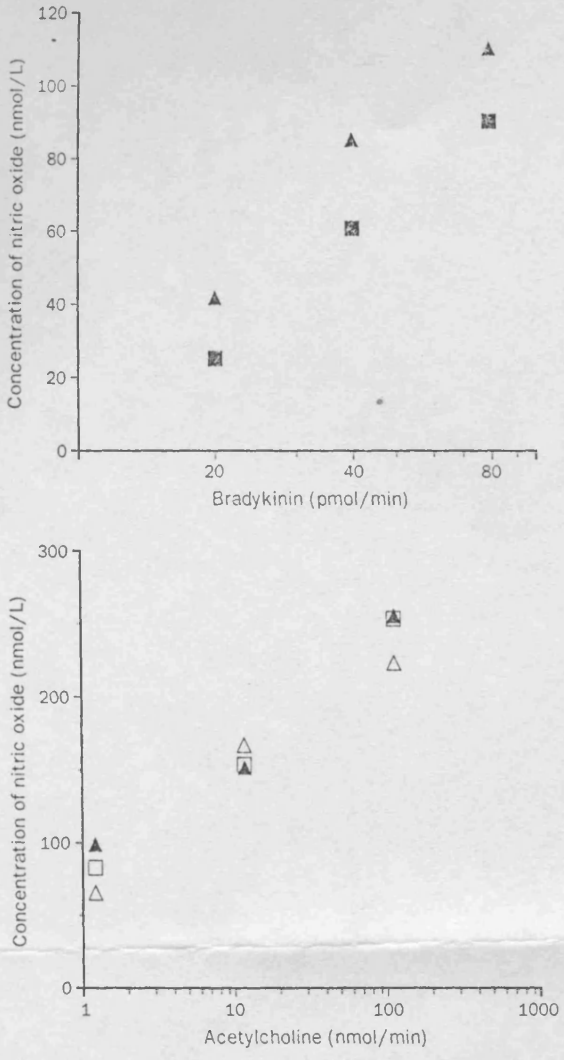

Figure 2: **Dose-response curves of nitric oxide concentration during local infusion of bradykinin (two curves in single subject) and acetylcholine (3 subjects)**

acetylcholine was infused three times in a single experiment in 1 individual, the peak concentrations of nitric oxide detected were 250, 224, and 275 nmol/L. L-NMMA reduced the signal generated in response to a second infusion of acetylcholine. Infusion of L-arginine (400 nmol/min) increased the signal (figure 1).

Insertion of the fine and fragile sensor (diameter about 20 μm) into its catheter resulted in damage to the sensor on three out of thirty occasions. Decrease of current and increase in noise occurred in up to 30% of sensors and was associated with visible clot on the tip of the sensor. None of the sensors that remained active throughout the study was coated with coagulated blood on removal.

Nitric oxide is a short-lived radical and measurement of the kinetics and amount released from tissues presents an analytical problem of direct relevance to clinical investigation. A porphyrinic microsensor was developed to measure nitric oxide release from single cells[4] and we used it to detect nitric oxide in healthy volunteers. A nitric oxide signal was detected in vivo in response to local infusion of acetylcholine or bradykinin, which could be attenuated by L-NMMA. We have previously demonstrated that the dilation produced by these agonists in hand veins is endothelium-dependent[7] and attenuated by L-NMMA,[6] and it is likely that the signal we detected in this study derived from the endothelium. The signal generated by the sensor is specific for nitric oxide[4] and the sensitivity is high.[5]

The detection of a signal for nitric oxide in response to acetylcholine or bradykinin provides further evidence in man that the endothelium-derived relaxing factor described by Furchgott and Zawadzki[9] is nitric oxide. Functional studies suggest that alterations in the L-arginine/nitric-oxide pathway occur in hypertension, diabetes, and hypercholesterolaemia, and decreased production of nitric oxide might be a link between risk factors and atherogenesis.[1] The ability to assess local generation of nitric oxide should facilitate exploration of the role of this mediator in physiological or pathophysiological processes.

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Endothelial "stunning" following a brief exposure to endotoxin: a mechanism to link infection and infarction?

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Abstract

Background: There is an association between infection, inflammation and acute cardiovascular events. In an attempt to explore the mechanism of this association we have developed a model to examine the effects on endothelial function of a brief exposure to endotoxin. **Methods and results:** Endotoxin was instilled into isolated superficial hand veins of healthy volunteers. The vein was isolated by means of two wedges and endotoxin instilled into the isolated segment. After 1 h the contents of the vein were aspirated and the wedges removed. Dose-response curves to bradykinin (a stimulator of nitric oxide synthesis), arachidonic acid (the precursor of prostanoid production) and GTN (a nitric oxide donor) were constructed before and 1 h after endotoxin. Endotoxin caused a glucocorticoid-inhibitable attenuation in the dose-response curves to bradykinin and arachidonic acid ($P < 0.05$). This effect persisted for 48 h and took 7 days to recover. Exposure of saphenous vein to endotoxin in vitro also caused selective impairment of endothelium-dependent relaxation ($P < 0.05$) yet microscopy of the vessels exposed to endotoxin showed no endothelial denudation or structural damage. **Conclusion:** The results demonstrate that a brief local exposure to endotoxin causes endothelial dysfunction that persists for 48 h and takes up to 7 days to recover. The endothelial dysfunction is not due to expression of the inducible isoform of nitric oxide synthase and persists for far longer than the effects of endotoxin on vascular smooth muscle function. We have coined the term endothelial "stunning" to describe the transient endothelial dysfunction and suggest it might provide a mechanism underpinning the association between infection or inflammation and increased cardiovascular risk. Endothelial stunning appears to provide a novel, transient, variable and modifiable potential cardiovascular risk factor.

Keywords: Endothelium; Endotoxins; Myocardial infarction; Inflammation; Nitric oxide; Arachidonic acid

1. Introduction

The vascular endothelium is far more than an inert lining of blood vessels. Endothelial cells sense changes in the local mechanical [1] and chemical environment [2] and transduce these changes into messages understood by the underlying smooth muscle. Numerous vasoactive substances are synthesised by the vascular endothelium and play an important role in cardiovascular homeostasis [3].

Under physiological conditions, the endothelium provides a vasodilator and an antiaggregatory influence on the cardiovascular system and may prevent growth of the underlying smooth muscle cells [4]. Synthesis of nitric oxide and prostacyclin are thought to be important in maintaining this basal vasoprotective state [5,6]. However, in certain diseases endothelial function appears abnormal. An imbalance between endothelium-derived relaxing and contracting factors, between anti- and pro-coagulant mediators or growth-inhibiting and growth-promoting factors could lead to changes in the normal state of the cardiovascular system from one that prevents atheroma, vasospasm and thrombosis to one that supports it. Indeed, endothelial dysfunction is now recognised as a major

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mechanism underlying acute and chronic cardiovascular disease [3].

There is an association between infection or inflammation (for example, a cytokine response) and acute cardiovascular events including myocardial infarction, stroke and the transition from stable to unstable angina [7–10]. However, the mechanisms underlying the association are far from clear. An understanding of the cellular processes linking inflammation and predisposition to acute cardiovascular disease would allow identification of novel targets for treatment. In this study we have explored the possibility that the local inflammatory reaction induced by bacterial endotoxin causes endothelial dysfunction in humans.

2. Methods

Studies were approved by the local ethics committee and performed on male (13) and female (nine) subjects aged 19–38 years. Subjects were included who stated that they were healthy and on no medication, and who gave their informed, written consent. Subjects lay supine in a temperature-controlled laboratory (28–29°C) and the internal diameter of a single vein on the back of the hand was measured at constant distension pressure, as described previously [11–13]. During studies of reactivity drugs or physiological saline were infused continuously into the study vein. In all studies saline was infused for at least 15 min until a stable baseline vein diameter was recorded. Provided subjects are comfortably warm and relaxed, dorsal hand veins have no intrinsic tone [12], and in order to observe dilator responses it is necessary to preconstrict the vessel and we did this with noradrenaline. Dose-response curves to endothelium-dependent and -independent dilators were constructed before and after exposure of the vessel to endotoxin.

2.1. Instillation of endotoxin

To instil endotoxin or control solution (saline), a length of the vein under study was isolated from the circulation by means of two wedges placed 2–3 cm apart on the skin overlying the vessel as described previously [11]. Endotoxin (100 EU in 1 ml saline) or saline (1 ml) was injected into the isolated segment. The calculated local concentration of endotoxin was similar to those that may occur clinically during infection or gut surgery [11]. One hour later the contents of the segment were aspirated and the wedges removed so that the circulation of the blood through the vessel was re-established. All dose-response curves were constructed with the wedges removed and the vessel connected with the circulation and infused continuously with drugs or saline as described above.

2.2. Constrictor dose-response curves

Cumulative dose-response curves to noradrenaline (5–1280 pmol/min, each dose increment a doubling of the previous dose) were constructed before and 1 h after endotoxin as described previously [11].

Table 1
Dose of noradrenaline used and percentage preconstriction of the vein

Study	Time (h) [a]	Dose of noradrenaline (pmol/min)	Percentage preconstriction
Study 1			
ETX/bradykinin	0	84± 22	43±4
	1	296±100 *	44±8
ETX/arachidonic acid	0	84±22	42±6
	1	296±100 *	40±3
Saline/bradykinin	0	160±120	48±3
	1	152±121	44±6
Saline/arachidonic acid	0	160±120	49±4
	1	152±121	47±5
Study 2			
ETX/bradykinin	0	52± 11	52±3
	1	384±227 *	45±6
ETX/GTN	0	52± 11	45±4
	1	384±227 *	47±4
Study 3			
ETX/braydykinin	0	108± 53	48±5
	1	320± 85	52±6
	24	76± 19	51±4
	48	72± 23	54±3
	7 days	68± 24	50±8
ETX/arachidonic acid	0	108± 53	50±5
	1	320± 85	54±3
	24	76± 23	50±3
	48	68± 12	51±8
	7 days	68± 24	47±7
Study 4			
ETX/bradykinin/ steroid	0	40± 10	48±2
	1	56± 28	52±5
ETX/arachidonic acid/steroid	0	40± 10	43±5
	1	56± 28	50±3

[a] Times 0, 1, 6, 24 and 48 h, and 7 days represent the time before (0) and the designated time after instillation of endotoxin or saline.
* $P < 0.05$; P values compared with time 0. ETX = endotoxin.

2.3. Dilator dose-response curves

In all parts of the study we took care to ensure that prior to co-infusion of the vasodilator agent all vessels were preconstricted to approx. 50% of resting diameter by a continuous infusion of noradrenaline (5–1280 pmol/min, doses selected as above; Table 1). Bradykinin (2, 4, 8 pmol/min, each dose for 5 min), arachidonic acid (0.2, 2, and 20 nmol/min, each dose for 5 min) or glyceryltrinitrite (GTN) (1, 2, 4 pmol/min, each dose for 5 min) were co-infused with the noradrenaline and relaxation was recorded. In these vessels bradykinin increases nitric oxide synthesis [13], arachidonic acid stimulates prostanoid production [14] and GTN is a nitric oxide donor that acts directly on smooth muscle.

Study 1: In five subjects a dose-response curve was constructed to bradykinin and arachidonic acid before and 1 h after instillation of endotoxin ($n = 5$) or saline ($n = 5$).

Study 2: In a separate study dose-response curves were constructed to bradykinin and GTN before and 1 h after

instillation of endotoxin ($n = 5$).

Study 3: In a further five subjects dose-response curves to bradykinin and arachidonic acid were constructed before and at 1 h, 24 h, 48 h and 7 days after endotoxin.

In all studies a 10–15 min washout period (infusion of saline and noradrenaline) separated the dose response to different dilator agonists.

Study 4: Five subjects took oral hydrocortisone (100 mg) and endotoxin was instilled 2 h later. Dose-response curves to bradykinin and arachidonic acid were constructed before and 1 h after instillation.

2.4. Measurement of vascular tone in vitro

Specimens of saphenous vein (from patients undergoing coronary artery by-pass surgery) were collected in cold Krebs' solution. Each vessel was freed from connective tissue, cut into rings 3–5 mm long and suspended between two hooks connected to a transducer (Statham UC3, Gould Inc., Cleveland, OH, USA) for the measurement of isometric tension as described previously [15]. Endothelial integrity was determined by assessing the responses to bradykinin. In phenylephrine-preconstricted, endothelium-intact saphenous vein, concentration-response curves were constructed to bradykinin (1 nM–1 mM) and GTN (1 nM–1 mM). After the first concentration-response curve, endotoxin (100 EU/ml, LOT EC-5) or an equal volume of physiological saline was added to the organ bath and allowed to incubate with the saphenous vein rings for 1 h. Concentration-response curves to bradykinin and GTN were then repeated. Studies with arachidonic acid were not undertaken since patients undergoing by-pass surgery would have been taking aspirin, a drug that interferes with the relaxant response to arachidonic acid [14].

2.5. Electron microscopy

Specimens for electron microscopy were assessed by a microscopist blinded to the sample code. Saphenous vein rings were added directly to fixative or incubated for 1 h in either 10 ml of Krebs' solution, or 10 ml of Krebs' with endotoxin (100 EU) prior to fixing. Samples were prepared for scanning for transmission electron microscopy as described previously [16].

2.6. Drugs

Endotoxin (EC-5, 10000 EU/vial) was obtained from USP (Rockville, MD, USA) and stored and prepared as described previously [11] and sodium arachidonate (5 mg/vial) stored under nitrogen was obtained from Sigma (Dorset, UK) and dissolved in 154 ml absolute alcohol and prepared as described previously [14]. Arachidonic acid was always used within 6 h of preparation. Bradykinin was obtained from Clinalpha AG (Laufelfingen, Germany), hydrocortisone (20 mg/tablet) from MSD (Hertfordshire, UK), noradrenaline (2 mg/vial) from Sanofi Winthrop (Guilford, Surrey, UK), ascorbic acid (100 mg/ml) from Evans Medical Ltd. (Horsham, UK), and heparin (100 U/ml) from CP Pharmaceuticals Ltd. (Wrexham, UK). Ascorbic acid was added to noradrenaline stock solutions to prevent auto-oxidation. Heparin (100 U/ml) was added

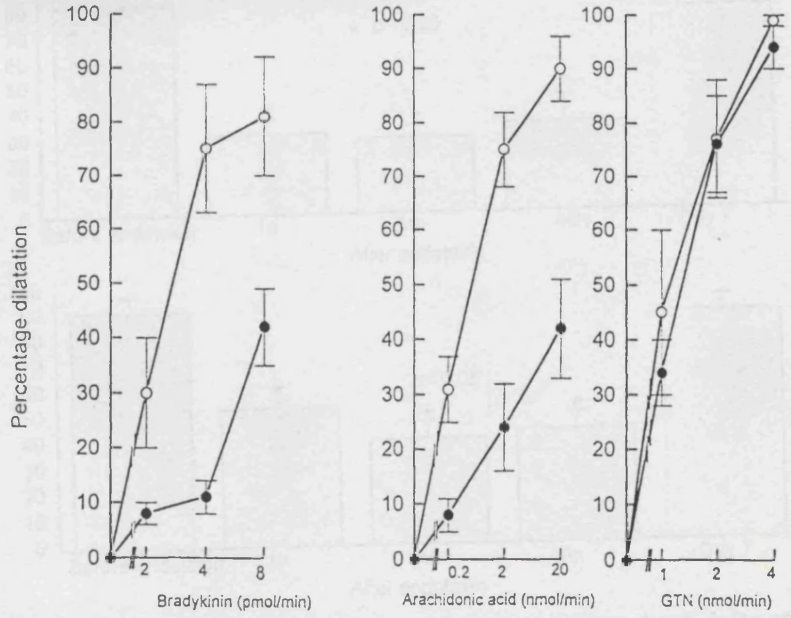


Fig. 1. Dose-response curves to bradykinin, arachidonic acid and GTN before and 1 h after endotoxin. Vessels were preconstricted to approx. 50% of resting diameter by a continuous infusion of noradrenaline (5–1280 pmol/min). Then bradykinin (2, 4, 8 pmol/min, each dose for 5 min), arachidonic acid (0.2, 2, and 20 nmol/min, each dose for 5 min) or GTN (1, 2, 4 pmol/min, each dose for 5 min) was co-infused with the noradrenaline and relaxation was recorded. Studies were undertaken before and 1 h after endotoxin was instilled. ○, before endotoxin; ●, 1 h after endotoxin.

to the endotoxin solution before administration to prevent thrombus formation.

2.7. Calculations and statistics

Changes in vein size were measured in arbitrary units and converted to millimetres following calibration of the transducer at the end of each experiment. The response of the resting vein to drugs is expressed as a reduction in diameter from that measured during infusion of saline alone. The response of the noradrenaline-preconstricted vein to drugs is expressed as percentage reversal of the induced constriction. Results are compared using Student's *t*-test for paired data or analysis of variance of the means as appropriate; $P < 0.05$ is considered statistically significant.

3. Results

3.1. Effects of endotoxin on noradrenaline responses

As we have reported previously [11], instillation of endotoxin decreased the contractile response to noradrenaline such that the dose of noradrenaline producing 48 ± 7% ($n = 15$) constriction before endotoxin produced only a 12 ± 3% constriction 1 h after endotoxin ($P < 0.05$), and the maximal constriction achieved was reduced from 87 ± 6% to 53 ± 3%. In our previous study [11], we showed that the hyporesponsiveness to noradrenaline disappeared by 4 h, and in this study the response to noradrenaline was normal at 24 h, 48 h and 7 days after endotoxin.

3.2. Effects of endotoxin on endothelium-dependent relaxation

Exposure to endotoxin attenuated the dilator response to bradykinin ($p = < 0.05$) and arachidonic acid ($p = < 0.05$) whereas the dilator response to GTN was unaffected (Fig. 1). In contrast, after instillation of saline there was no significant change in the dose-response curves to any dilator. In subjects receiving endotoxin, dilatation to the highest dose of each drug before endotoxin (time 0) was: bradykinin: 81 ± 6% ($n = 15$); arachidonic acid: 90 ± 6% ($n = 10$); and GTN: 99 ± 2% ($n = 5$); 1 h after endotoxin, dilatation to the highest dose of each drug was: bradykinin: 40 ± 4% ($n = 10$; $P < 0.05$), arachidonic acid: 42 ± 9% ($n = 10$; $P < 0.05$); and GTN: 94 ± 4% ($n = 5$). In subjects receiving saline dilatation to the highest dose of each drug before saline (time 0) was: bradykinin: 79 ± 8% ($n = 5$); arachidonic acid: 83 ± 3% ($n = 5$); and GTN: 89 ± 6% ($n = 5$); 1 h after saline, dilatation to the highest dose of each drug was: bradykinin: 68 ± 5% ($n = 5$), arachidonic acid: 79 ± 9% ($n = 5$); and GTN: 88 ± 6% ($n = 5$).

The endotoxin-induced attenuation in the response to bradykinin and arachidonic acid (Fig. 2) persisted for at least 48 h and returned to pre-endotoxin values by 7 days. Before endotoxin, dilatation to the highest dose of drug was: bradykinin 82 ± 7% ($n = 5$), arachidonic acid 92 ± 6% ($n = 5$). One hour after endotoxin dilatation to the

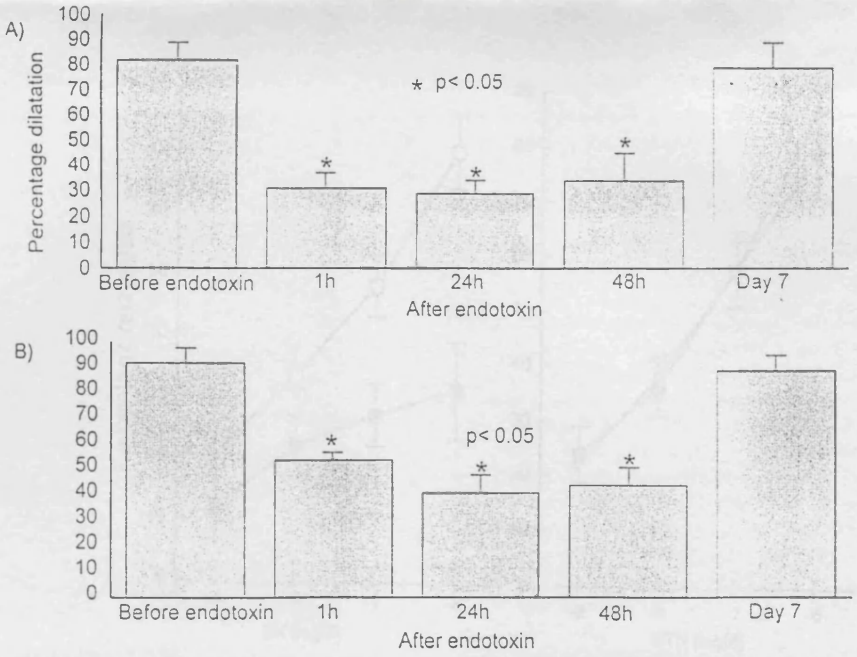


Fig. 2. Maximum dilatation to bradykinin (A) and arachidonic acid (B) at 1 h, 24 h, 48 h and 7 days after endotoxin. In five subjects dose-response curves to bradykinin and arachidonic acid were constructed before and at 1 h, 24 h, 48 h and 7 days after endotoxin. At each time point the maximal dilatation achieved to bradykinin and arachidonic acid is shown.

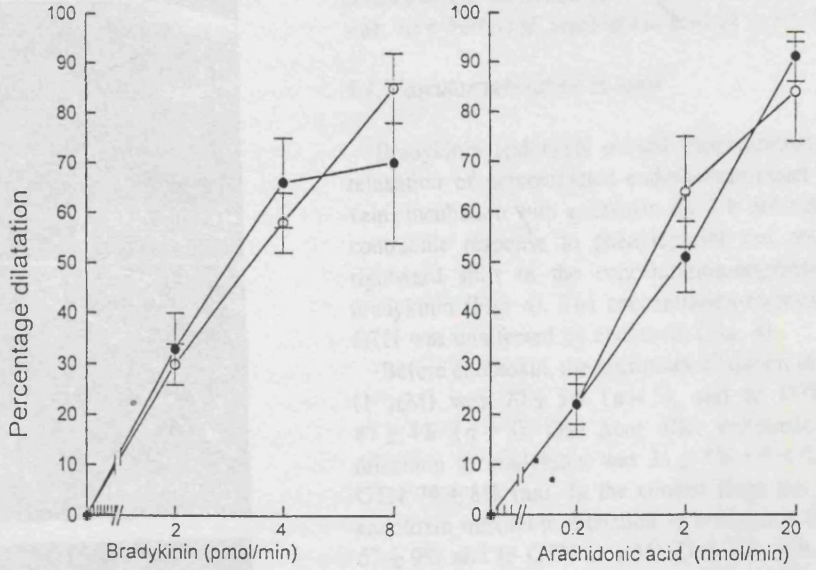


Fig. 3. Effects of hydrocortisone on the response to endotoxin. In five subjects oral hydrocortisone (100 mg) was given. Endotoxin was instilled into vein 2 h later. Dose-response curves to bradykinin and arachidonic acid were constructed before and 1 h after instillation. ○, before endotoxin; ●, after endotoxin.

same dose of drug was: bradykinin 31 ± 6% ($n = 5$; $P < 0.05$), arachidonic acid 54 ± 3% ($n = 5$; $P < 0.05$); 24 h later: bradykinin 29 ± 5% ($n = 5$; $P < 0.05$), arachidonic acid 41 ± 7% ($n = 5$; $P < 0.05$); 48 h later: bradykinin 34 ± 11% ($n = 5$; $P < 0.05$), arachidonic acid 44 ± 7% ($n = 5$; $P < 0.05$); and 7 days later: bradykinin 79 ± 10% ($n = 5$; ns), arachidonic acid 89 ± 6% ($n = 5$; ns).

3.3. Effects of hydrocortisone

Hydrocortisone (100 mg) given 2 h before the stu inhibited the effects of endotoxin (Fig. 3). Before end toxin, dilatation to the highest dose of each drug used wa bradykinin 85 ± 6% ($n = 5$), arachidonic acid 84 ± 10 ($n = 5$). Following hydrocortisone treatment, 1 h aft

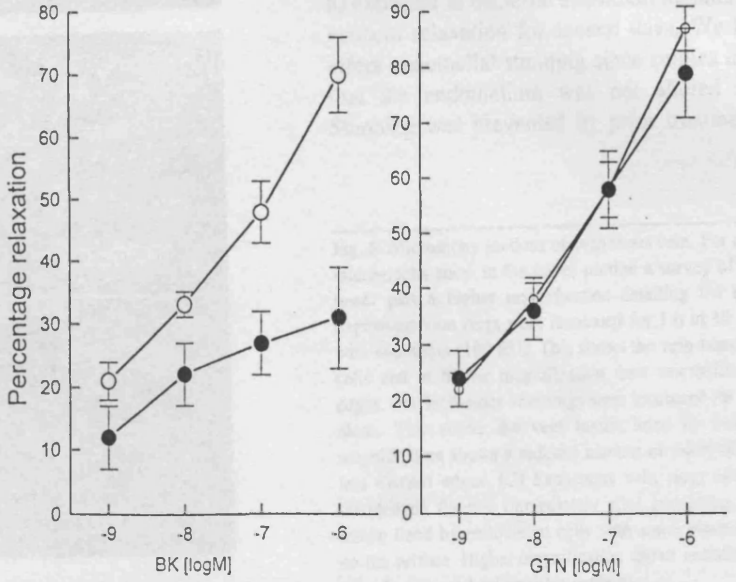
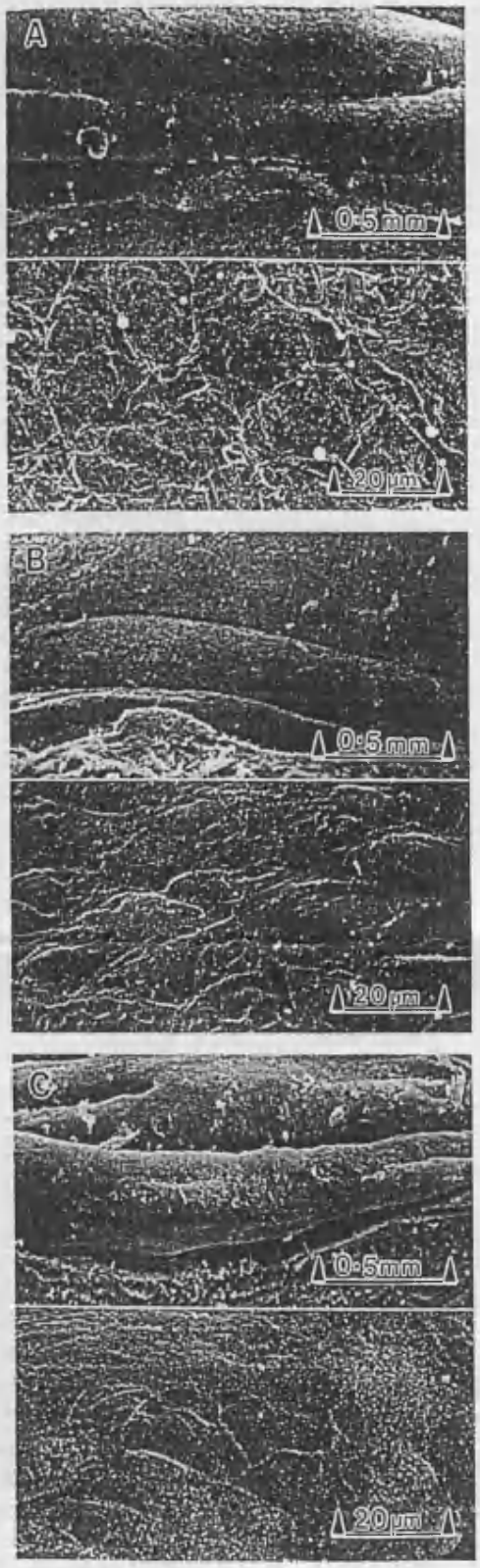


Fig. 4. Effects of endotoxin on responses of saphenous vein in vitro. In preconstricted, endothelium-intact saphenous vein, concentration-response curve were constructed to bradykinin (1 nM – 1 μM) and GTN (1 nM – 1 μM). After the first concentration-response curve endotoxin (100 EU, LOT EC-5) wa added to the organ bath and allowed to incubate with the saphenous vein rings for 1 h. Concentration-response curves to bradykinin and GTN were ther repeated. ○, before endotoxin; ●, 1 h after endotoxin.



endotoxin the dilatation to the same dose of bradykinin was: 70 ± 16% (ns), arachidonic acid 91 ± 5% (ns).

3.4. Vascular relaxation in vitro

Bradykinin and GTN caused concentration-dependent relaxation of precontracted endothelium-intact saphenous vein. Incubation with endotoxin for 1 h did not affect the contractile response to phenylephrine but resulted in a rightward shift in the concentration-response curve to bradykinin (Fig. 4). The concentration-response curve to GTN was unaffected by endotoxin (Fig. 4).

Before endotoxin, the maximum dilatation to bradykinin (1 μM) was 70 ± 5% ($n = 5$), and to GTN (1 μM) 87 ± 4% ($n = 5$). One hour after endotoxin maximum dilatation to bradykinin was 31 ± 4% ($P < 0.05$) and to GTN 79 ± 8% (ns). In the control rings not exposed to endotoxin maximum dilatation to bradykinin (1 μM) was 67 ± 9% and to GTN (1 μM) 77 ± 7%; 1 h later maximum dilatation to bradykinin was 72 ± 11% (ns) and to GTN 67 ± 4% (ns).

3.5. Electron microscopy

Electron microscopy (Fig. 5) detected no clear differences between the endotoxin-treated vein ($n = 5$), the vein that was allowed to remain in Krebs' for 1 h before adding fixative ($n = 5$) and the vein that had been added immediately to fixative ($n = 5$).

4. Discussion

The results of the present study suggest that a brief (1 h) exposure to bacterial endotoxin impairs endothelium-dependent relaxation for several days. We have termed this effect endothelial stunning since studies in vitro suggested that the endothelium was not altered morphologically. Stunning was prevented by prior treatment with a gluco-

Fig. 5. Microscopy sections of saphenous vein. For each of the groups the micrographs show in the upper portion a survey of the lumen and in the lower part a higher magnification detailing the endothelial cells. (A) Saphenous vein rings were incubated for 1 h in 10 ml of Krebs' solution with endotoxin (100 EU). This shows the vein lumen lined by endothelial cells and at higher magnification their microvillus surface and ridged edges. (B) Saphenous vein rings were incubated for 1 h in Krebs' solution alone. This shows the vein lumen lined by endothelial cells; higher magnification shows a reduced amount of microvilli on their surface and less distinct edges. (C) Saphenous vein rings added directly to a glutaraldehyde fixative immediately after harvesting. This shows the vein lumen lined by endothelial cells with some plasma protein and red cells on the surface. Higher magnification shows endothelial cells with distinct ridged edges and microvillus surface.

corticoid, indicating that locally generated inflammatory mediators contribute to the effect. Endothelial dysfunction promotes thrombosis and vasospasm [3,6], and if this effect also occurs in arteries, transient stunning of the endothelium would provide a possible mechanism underlying the epidemiological association between infection or inflammation and acute cardiovascular events [7–10].

Using a system in which drugs are given into a single superficial vein in very low doses sufficient only to produce changes in the study vessel, we found that endotoxin (in concentrations similar to those that may occur clinically [17,18]) attenuated the dilator response to bradykinin (an endothelium-dependent dilator that works by activating the L-arginine:NO pathway [13]) and arachidonic acid (the precursor of vasoactive prostanoids [14,19]). The response to GTN, an endothelium-independent nitric oxide donor, was unaffected. The attenuation of endothelium-dependent dilatation was greatest 24 h after exposure to endotoxin but was still significant 48 h later. By 7 days the response had returned to normal. Studies in vitro using saphenous vein demonstrated a similar shift in the concentration-response curves to bradykinin after endotoxin, whilst the concentration-response curves to GTN again were unaltered. This functional change in endothelium-dependent relaxation was not associated with endothelial abnormalities on electron microscopy. We have previously shown that endotoxin attenuates the constrictor response to noradrenaline and that this effect lasts only for 3 h [11]. This short-lived response is likely to be relevant to certain aspects of the hypotension and vasodilatation that occurs in septic shock [20,21] and in these vessels is not due to expression of the inducible isoform of nitric oxide synthase [11]. However, the present study gives insight into the cardiovascular changes that might occur in a wide variety of patients who have endotoxaemia [22–24] without developing any clinically obvious sepsis. Our findings suggest that the vascular smooth muscle recovers rapidly from the direct effects of endotoxin [11] whereas the endothelium does not.

Possible explanations for the reduced relaxant response observed after endotoxin include structural damage to endothelium or smooth muscle, functional antagonism due to differing degrees of preconstriction in different parts of the study, or induction of biochemical and pharmacological changes in the vessel wall. The studies with saphenous vein in vitro suggest that endothelial denudation is unlikely to account for the changes we have seen. Damage to the smooth muscle is also unlikely since the relaxant response to the endothelium-independent dilator GTN was unaltered by endotoxin. Similarly functional antagonism would not explain our findings since the effect was specific for bradykinin and arachidonic acid and the degree of preconstriction was the same for all parts of the study. Thus the most likely explanation of our findings is that a brief exposure to endotoxin induces endothelial dysfunction by stimulating biochemical and pharmacological changes within the endothelium. This interpretation is strengthened by the finding that hydrocortisone, a drug that inhibits the production of a wide variety of inflammatory mediators and cytokines [25,26], prevented the endotoxin-induced abnormality of endothelium-dependent relaxation. Mechanisms underlying the pharmacological changes induced by endotoxin or cytokines might include an effect on the stability of mRNA for the enzymes nitric oxide synthase and cyclooxygenase [27,28], changes in the coupling of receptor stimulation to mediator production (for example G protein related pathways [29]) or alterations in the stability or function of the enzymes themselves. Decreased endothelium-dependent relaxation after exposure to endotoxin has also been observed in some studies [30,31] in vessels in vitro or in animals. Indeed, even in conditions of gross experimental endotoxic shock, when the inducible isoform of nitric oxide synthase (iNOS) is expressed [32,33] and generates nitric oxide in amounts sufficient to produce profound vasodilatation and hypotension, decreased "physiological" endothelium-dependent dilatation has been reported [34]. These changes observed in animals might be due to an effect of nitric oxide generated from iNOS on the expression [34] or function of the normal constitutive, endothelial nitric oxide synthase. However, functionally active iNOS is not expressed in response to local administration of endotoxin in the veins we have studied [11], yet endothelium-dependent dilatation is markedly diminished. Furthermore, our results in humans clearly indicate that the endotoxin-induced abnormality is not confined to the endothelial L-arginine:nitric oxide pathway but extends to production of dilator prostanoid(s) and persists long after the smooth muscle changes have returned to normal. The effect appears to be generated locally within the vessel wall and the finding that it was reproducible in vitro indicates that it is not dependent upon the presence of circulating inflammatory cells. It remains to be determined whether other bacterial toxins (for example, staphylococcal toxin) or inflammatory cytokines that are elevated postoperatively [9,35] or during the process of unstable angina and myocardial infarction [23] also stun the endothelium. Glucocorticoids (or possibly anti-endotoxin or anti-cytokine antibodies) should prevent the development of endothelial stunning if administered before the inflammatory process has started and it would now be important to identify agents that might reverse established stunning. Arterial endothelium differs from venous endothelium and it would be important to extend these studies to determine whether arterial endothelium is also affected by exposure to endotoxin.

There is a largely unexplained association between infection or inflammation and the subsequent development of vascular pathology including myocardial infarction and stroke [8,10]. An immediate preceding febrile respiratory infection is a major risk factor for stroke in young and middle-aged adults [9] and transient endotoxaemia often occurs postoperatively [24,35,36], a time when the incidence of myocardial infarction and stroke rises. If other

vessels (in particular, clinically relevant arteries) behave similarly to the veins we have studied, it is possible that sufficient bacterial toxins or inflammatory cytokines may be present in a variety of conditions to stun the endothelium for several days or longer, and the loss of physiological nitric oxide and prostanoid-mediated effects would predispose to vasospasm, thrombosis and vessel occlusion [37].

Many studies exploring predisposition to cardiovascular disease have investigated chronic "stable" factors and their effects on endothelial function. Our studies in healthy volunteers indicate that endothelial function may alter on a day-to-day basis and provide a mechanism to link infection or inflammation to increased risk of an acute cardiovascular event. We suggest the existence of a novel variable and potentially modifiable cardiovascular risk factor, that of transient "endothelial stunning".

Acknowledgements

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Local Venous Responses to Endotoxin in Humans

Kiran Bhagat, BSc, MRCP; Joe Collier, MD, FRCP; Patrick Vallance, MD, FRCP

Background Septic shock is characterized by arterial and venous dilatation and decreased responsiveness to vasoconstrictors. We have developed a method to explore the effects and mechanisms of action of administration of endotoxin into a blood vessel in vivo.

Methods and Results Endotoxin was instilled into a dorsal hand vein for 1 hour and then removed. A dose-response curve to norepinephrine was constructed before and 1, 2, 3, and 4 hours after endotoxin. In a separate study, dose-response curves to norepinephrine were constructed in two separate veins on the same hand, only one of which received endotoxin. Sympathetic-mediated venoconstrictor responses were also studied. Cyclooxygenase inhibitors, nitric oxide synthase inhibitors, and hydrocortisone were used to explore the mechanisms of the effects seen. Endotoxin caused a rightward shift in the dose-response curve to norepinephrine. The effect was greatest at 1 hour (maximal constriction: before endotoxin, 87±4%; after endotoxin, 52±8%; occlusion n=4; $P<.05$) and returned to normal by 4 hours. In addition, deep-breath venoconstrictor responses were abolished in the endotoxin-treated vein. Instillation of endotoxin daily for 3 days resulted in the development of tolerance (maximal constriction to norepinephrine after endotoxin: day 1, 39±6%; day 2, 67±7%; day 3, 85±7%). Cyclooxygenase and/or nitric oxide synthase inhibitors did not alter the response to endotoxin, whereas prior administration of hydrocortisone abolished the effects.

Conclusions Instillation of endotoxin caused a glucocorticoid-inhibitable hyporesponsiveness to the constrictor effects of norepinephrine and abolished sympathetically induced and drug-induced venoconstriction. This acute response does not appear to be mediated by nitric oxide or prostanoids. Direct vascular tolerance to endotoxin occurs on repeated administration. (***Circulation.* 1996;94:490-497.**)

Key Words • norepinephrine • shock • veins •

Septic shock carries a high mortality. Characteristic hemodynamic changes include hypotension due to arterial and venous dilatation and impaired cardiac contractility, but there are also profound changes in metabolic, respiratory, hematologic, and host-defense functions.[1-3] Decreased peripheral responsiveness to vasoactive agents contributes to the progressive decline of the systemic blood pressure, which ultimately leads to tissue hypoperfusion and circulatory failure.[1-4]

Bacterial wall lipopolysaccharide (endotoxin) is considered an important etiologic agent in the pathophysiology of septic shock[5] and has been administered to animals to produce a model of the human condition.[6-8] An understanding of the mechanisms by which endotoxin produces changes in vascular behavior is likely to lead to novel therapies. Two major approaches to the exploration of the vascular effects of endotoxin have been adopted: studies in vitro have focused on functional, biochemical, and molecular changes induced by direct exposure of cells or blood vessels to endotoxin,[9-12] whereas studies in vivo have assessed vascular changes after initiation of a generalized inflammatory response induced by systemic administration of endotoxin.[13,14] Two phases in the response to endotoxin have been revealed: an acute phase that occurs over 5 to 90 minutes followed by a delayed phase that begins 3 to 4 hours after administration of endotoxin and lasts for up to 24 hours.[14,15] With repeated administration, tolerance of the effects of endotoxin develops.[10,16,17]

The proposed mechanisms that underlie the acute vascular effects of endotoxin include enhanced synthesis of bradykinin,[11,13] NO,[4,8] prostanoids,[12] platelet-aggregating factor,[18] cytokines[19,20] or leukotrienes,[21] and direct effects of endotoxin on the vascular endothelium.[22] Some studies have shown increased production of the vasodilator mediator NO in the vessel wall due to expression of an inducible isoform of NO synthase,[23] and enhanced production of vasodilator prostanoid may occur after induction of COX-II.[24] Most studies have used arterial vessels[25]; however, venodilatation is an important component of the pathophysiology of septic shock and could contribute to the changes in cardiac filling pressure and cardiac output. It is unclear whether the mechanisms and action of endotoxin are similar in veins, resistance vessels, and conduit arteries.

Systemic administration of endotoxin to healthy human volunteers produces cardiovascular changes similar to those seen in animal models; arterial[15,25] and venous[26,27] dilatation and hypotension are seen within 60 minutes and persist for up to 8 hours,[15,27] but it is difficult to dissect the mechanisms in whole-body studies. Human vessels in vitro show inconsistent responses to endotoxin,[28] and it is possible that the vascular responses seen in vivo are dependent on involvement of other cell types or tissues, increased concentrations of cytokines circulating in blood,[20] infiltration of inflammatory cells into the vessel wall,[23] or neurohumoral effects of systemically administered endotoxin.[29] One approach to gaining insight is to investigate the local vascular effect of endotoxin in vivo. This is what we have attempted in the experiments reported here. We used superficial hand veins to explore the mechanisms of the changes seen and examined the phenomenon of tolerance to endotoxin.

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Selected Abbreviations and Acronyms

COX-II = cyclooxygenase II
EU = endotoxin units
iNOS = inducible nitric oxide synthase
L-NMMA = N^G-monomethyl-L-arginine
NO = nitric oxide

Methods

Studies were approved by the local research ethics committee and were performed on male (14) and female (18) subjects aged 19 to 38 years. Subjects who were included gave their informed, written consent and stated that they were healthy and were not taking any medication. Throughout the study, subjects lay supine in a temperature-controlled laboratory (28°C to 30°C) with one hand placed on an angled support above the level of the heart.

Assessment of Venous Response to Norepinephrine

To assess the responses to norepinephrine in a single vein on the back of the hand, a congesting cuff was placed around the upper arm and inflated to 40 mm Hg. Drugs or physiological saline (0.9%) were infused continuously (0.25 mL/min) through a 23-gauge needle inserted into the study vein. A lightweight probe was placed 5 to 10 mm downstream from the tip of the needle on the skin overlying the summit of the vessel. We assessed internal venous diameter every 5 minutes by recording the linear displacement of the probe when the pressure in the congesting cuff was lowered from 40 to 0 mm Hg and then inflated to 40 mm Hg again.[30] The diameter of a vessel under constant distension pressure is determined by the state of contraction of the smooth muscle (Laplace's relationship), and contraction leads to a reduction in diameter.

Baseline recordings of vein size were made for 10 minutes during infusion of saline. The continuous infusion of saline (0.25 mL/min) during periods of cuff inflation does not by itself alter venous diameter,[31,32] because the venous outflow to the limb is not occluded but simply maintained at constant pressure. Cumulative dose-response curves to norepinephrine (5 to 1280 pmol/min, with each dose increment representing a doubling of the previous dose) were constructed. Each dose was infused for 5 minutes, and doses were increased until a maximal response was achieved (no further constriction despite a doubling in dose). The response to norepinephrine varies between subjects but is consistent and reproducible within a single study in an individual.[33] To limit the duration of the study, four doses of norepinephrine were selected for repeat dose-response curves in a single study; these doses produced 0%, 20% to 40%, 40% to 70%, and 70% to 100% constriction on the first occasion (the doses varied between individuals and for clarity were designated doses A, B, C, and D). Maximal constriction refers to the response to dose D unless otherwise stated. Vein size was recorded in the same place as on the first occasion. Dose-response curves to norepinephrine were constructed before and after local administration of endotoxin.

Single Deep Breath as a Venoconstrictor Stimulus

We induced sympathetic venoconstriction by asking subjects to take a single deep inspiration over a period of 5 seconds, to hold this for a comfortable period (approximately 10 seconds), and then to breathe out slowly before they resumed normal breathing. The transient constriction evoked by this maneuver is blocked by drugs that inhibit the sympathetic nervous system.[34] Two adjacent dorsal hand veins were studied simultaneously. One vein was isolated and received endotoxin while the other was left unoccluded. Deep-breath maneuvers were performed before and 1 hour after endotoxin was instilled.

Instillation of Endotoxin

To instill endotoxin or control solution (saline), a length of the vein under study was isolated from the circulation by means of two wedges placed 2 to 3 cm apart on the skin overlying the vessel.[35] The wedges were weighted to occlude the inflow and outflow to the isolated segment. Endotoxin (100 EU in 1 mL saline) or saline (1 mL) was injected into the isolated segment, and although we did not assess it formally, there appeared to be no significant leakage (the vessel stayed distended despite deflation of the upper arm cuff). This dose of endotoxin gives a calculated local concentration of about 20 ng/mL, similar to that reported in the blood of patients with severe sepsis.[36] One hour later, the contents of the segment were aspirated and the wedges removed so that the circulation of the blood through the vessel was reestablished. At this stage, dose-response curves to norepinephrine were repeated.

Effects of Endotoxin: Time Course (Study 1)

In 12 subjects, the time course of the response to endotoxin was explored. Dose-response curves were constructed to norepinephrine before and at 1 and 2 hours after exposure to endotoxin or saline in 4 subjects, at 3 hours (after endotoxin only) in 4 subjects, and at 4 hours (after endotoxin only) in 4 subjects. In 3 additional subjects, deep breath–induced venoconstriction was studied before and 1 hour after endotoxin. For deep-breath studies, two adjacent veins were compared. One was occluded and received endotoxin while the other was left unoccluded. Deep-breath studies were performed in both veins simultaneously, before and 1 hour after endotoxin. At the end of the study, phentolamine hydrochloride (25 nmol/min for 20 minutes) was infused into the control vein and the deep-breath response repeated.

In all subsequent studies, the response to endotoxin was assessed at 1 hour after endotoxin, and to study the response to drugs that might inhibit or reverse the effects of endotoxin, we selected subjects who in preliminary studies demonstrated a large response to endotoxin (arbitrarily defined as >40% suppression of contraction to norepinephrine).

Effects of Endotoxin: Local or Systemic Effect? (Study 2)

To determine whether endotoxin was producing a local rather than a systemic effect, dose-response curves to norepinephrine were constructed simultaneously in two adjacent veins on the same hand (n=3). One vein was isolated and received endotoxin as before, while the other was left unoccluded. Dose-response curves to norepinephrine were constructed in both veins simultaneously, before and 1 hour after one vein was exposed to endotoxin.

Effects of Repeated Administration of Endotoxin (Study 3)

In five subjects, the effects of daily instillation of endotoxin into a single vein for 3 days were explored. On each day, dose-response curves to norepinephrine were constructed before and 1 hour after endotoxin. To determine whether the effects of repeated exposure to endotoxin initiated a local or systemic response, an adjacent vein on the same hand (ie, a vein that had not been exposed to endotoxin previously) was exposed to endotoxin on day 3, and a dose-response curve to norepinephrine was constructed before and 1 hour after endotoxin.

Effects of Inhibition of Cyclooxygenase and/or NO Synthase on the Response to Endotoxin (Study 4)

Five subjects were given soluble aspirin (1 g, a dose that inhibits the dilation to arachidonic acid[37]), and the response to endotoxin was determined 2 hours later. Dose-response curves were established to norepinephrine before and 1 hour after exposure to endotoxin. In a separate set of studies in five subjects, a dose-response curve to norepinephrine was constructed before and 1 hour after exposure to endotoxin, and at the end of the second dose-response curve, L-NMMA (100 nmol/min for 10 minutes) was coinfused with the same dose of norepinephrine that pro-

Basal Vein Size (ID at Constant Distension Pressure) for Each Study

Study	Vein Size, mm
1	1.8±0.5
2	1.8±0.4
3	1.6±0.3
4	1.8±0.3
5	1.4±0.3
6	1.7±0.2

duced the maximal constriction before endotoxin. The combined effects of aspirin and L-NMMA were also studied. Five subjects were given oral aspirin (1 g) 2 hours before the study, and the response to norepinephrine was determined before and at 1 hour after exposure to endotoxin. In addition, immediately after the second dose-response curve to norepinephrine had been constructed, L-NMMA (100 nmol/min) was coinfused with norepinephrine and the dose-response curve repeated for a third time.

Effects of Hydrocortisone on Response to Endotoxin and to Development of Endotoxin Tolerance (Study 5 and Study 6)

Five subjects received hydrocortisone (100 mg) 2 hours before the study. Dose-response curves to norepinephrine were constructed before and 1 hour after exposure to endotoxin (study 5).

To determine the effects of the glucocorticoid (study 6) on repeated daily dosing of endotoxin (for 3 days, as above), five subjects were given hydrocortisone (100 mg) 2 hours before the study on day 1 and day 2. On day 3, no steroid was given. Dose-response curves to norepinephrine were constructed before and 1 hour after endotoxin.

Drugs

Endotoxin (EC-5, 10 000 EU/vial) was obtained from USP. Vials were stored at −4°C. The endotoxin was rehydrated with 20 mL sterile saline (0.9%) to give a solution of 1000 EU/mL. The vial was then shaken for a minimum of 20 minutes. The solution was divided into aliquots and stored at −20°C for up to 10 weeks. To determine activity of the endotoxin, dose-response curves to endotoxin were constructed at four weekly intervals in J774 murine macrophage cell lines, and nitrite production was determined as described previously.[32] All endotoxin was filtered through a 0.8-μm bacterial filter (Acrodisc PF, Gelman Sciences). Dispersible aspirin was obtained from Aspar Pharmaceuticals Ltd, L-NMMA from Welcome Foundation Limited, hydrocortisone (20 mg) from MSD, norepinephrine (2 mg/vial) from Sanofi Winthrop, ascorbic acid (100 mg/mL) from Evans Medical Ltd, heparin (100 U/mL) from CP Pharmaceuticals Ltd, and phentolamine (10 mg/mL) from Ciba. Ascorbic acid was added to norepinephrine stock solutions to prevent auto-oxidation. Heparin (100 U) was added to the endotoxin solution before administration to prevent thrombus formation.

Calculations and Statistics

Vein size was measured in arbitrary units and converted to millimeters after calibration of the transducer at the end of the experiment. The response of the resting vein to drugs is expressed as a percentage reduction in diameter from that measured during infusion of saline alone. Results were compared by use of Student's t test for paired data or ANOVA of the means as appropriate; a value of $P<.05$ was considered statistically significant.

Results

Local instillation of endotoxin into the isolated vein had no effect on resting vein size and caused no local adverse or systemic effects. The average basal vein size (ID at constant pressure) for each study is shown in the Table. In each subject, norepinephrine produced a dose-dependent

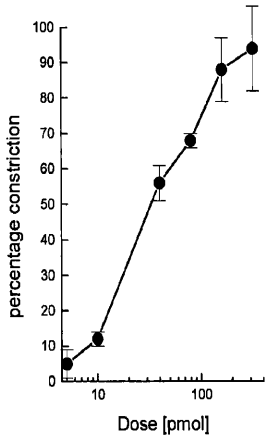

FIG 1. Dose-response curve to norepinephrine; n=7.

venoconstriction (Fig 1) but as is recognized,[33] there was variability in the response between individuals, with the dose that produced maximal constriction ranging from 40 to 1280 pmol/min.

Effects of Endotoxin: Response and Time Course (Study 1)

Local instillation of endotoxin caused a rightward shift in the dose-response curve to norepinephrine (for example, the dose of norepinephrine that produced a 40±3% constriction before endotoxin produced a 6±3% constriction 1 hour after endotoxin) and suppressed the maximal constriction achieved (Fig 2). In seven subjects, the dose of norepinephrine given after endotoxin was increased in an attempt to produce full constriction. In these subjects, maximal constriction (89±11%) was produced in response to 623±64 pmol/min before endotoxin, but 1 hour after endotoxin, the constriction reached a plateau at 52±14%, and even doses up to 2560 pmol/min had no additional effect. Attenuation of the norepinephrine response was seen at 1, 2, and 3 hours after endotoxin, but by 4 hours,

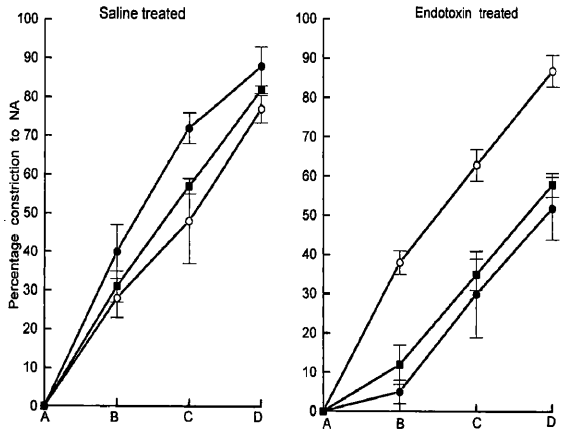

FIG 2. In four subjects, dose-response curves to norepinephrine (NA in figure) were constructed before and at 1 and 2 hours after the vein was exposed to endotoxin (n=4) or saline (n=4). For the repeat dose-response curves to norepinephrine at 1 and 2 hours after saline or endotoxin, 4 doses of norepinephrine were selected that produced 0%, 20% to 40%, 40% to 70%, and 70% to 100% constriction on the first occasion (designated A, B, C, and D). Left: A, 23±19 pmol/min; B, 55±40 pmol/min; C, 208±148 pmol/min; and D, 425±292 pmol/min. Right: A, 6±2 pmol/min; B, 41±22 pmol/min; C, 93±41 pmol/min; and D, 220±60 pmol/min. ○, before saline or endotoxin; ●, 1 hour after saline or endotoxin; ■, 2 hours after saline or endotoxin.

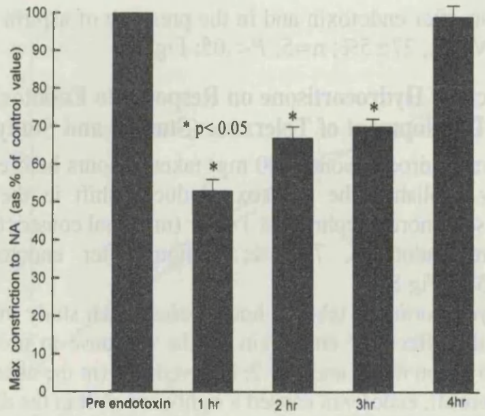

FIG 3. In 12 subjects, the time course of the response to endotoxin was explored. Dose-response curves were constructed to norepinephrine (NA in figure) before and at 1, 2, 3, and 4 hours after exposure to endotoxin (n=4 at each time point). Results are expressed as maximal percentage constriction to norepinephrine relative to the initial control value for that study.

the venoconstrictor potency of norepinephrine was fully restored (Fig 3). The maximal constriction to norepinephrine (dose D) before endotoxin was 87±4% and 1 hour later was 52±8% ($P<.05$). In contrast, the maximal constriction to norepinephrine 4 hours after endotoxin was back to 99±0.2% of the control value (P=NS; Fig 3). The effect was specific for endotoxin, because there was no change in the dose-response curve to norepinephrine at 1 or 2 hours after instillation of saline (maximal constriction: before saline, 77±4%; 1 hour later, 88±5%; 2 hours later, 82±2%; Fig 2).

The potential physiological significance of attenuation of the constrictor response to norepinephrine is illustrated in the three subjects who took part in the study of deep-breath responses. A deep breath produced simultaneous transient venoconstriction in both veins (13±4% constriction; Fig 4). After endotoxin, the constrictor deep-breath response was abolished in the treated (0% constriction) but

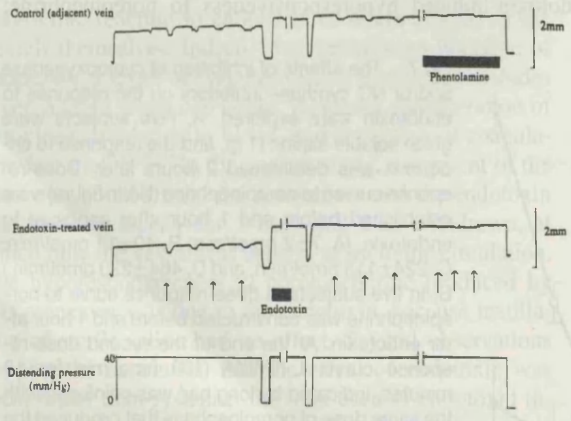

FIG 4. In three subjects, sympathetically mediated venoconstriction responses were assessed simultaneously in two adjacent veins. The control vein (top) was left unoccluded while the other vein was isolated and received endotoxin (bottom). Deep-breath (↑) venoconstrictor responses were performed simultaneously in both veins before and 1 hour after instillation of endotoxin. A deep breath produced simultaneous transient venoconstriction in both veins. After endotoxin, the constrictor deep-breath response was abolished (bottom). Finally, in the control (untreated) vein, phentolamine (25 nmol/min for 20 minutes) was infused, and the deep-breath response was assessed again (top).

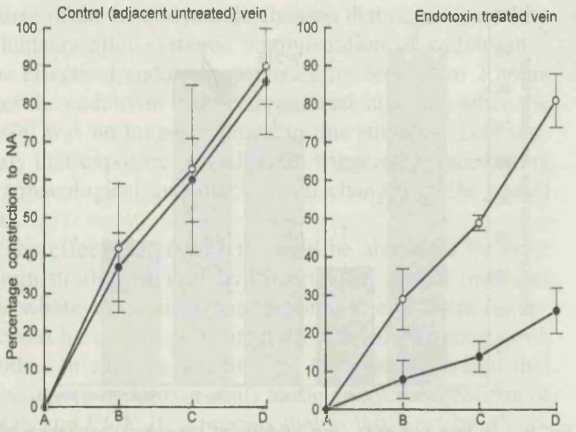

FIG 5. In three subjects, dose-response curves were constructed simultaneously in two adjacent veins. The control vein was left unoccluded while the other vein was isolated and received endotoxin. Dose-response curves to norepinephrine (NA in figure) were constructed in both veins before and 1 hour after one vein was exposed to endotoxin. For the repeat dose-response curves to norepinephrine at 1 and 2 hours after saline or endotoxin, four doses of norepinephrine were selected that produced 0%, 20% to 40%, 40% to 70%, and 70% to 100% constriction on the first occasion (designated A, B, C, and D). Left: A, 4±1 pmol/min; B, 10±0 pmol/min; C, 40±0 pmol/min; and D, 107±27 pmol/min. Right: A, 7±2 pmol/min; B, 30±10 pmol/min; C, 67±14 pmol/min; and D, 160±0 pmol/min. ○, before endotoxin; ●, 1 hour after endotoxin.

not in the control vein (11±3% constriction). Infusion of phentolamine (25 nmol/min for 20 minutes) into the control vein abolished the deep-breath response in this vessel (Fig 4), which confirmed that the constriction produced by this maneuver was due to sympathetic nervous system activation and norepinephrine release.

Effects of Endotoxin: Local or Systemic Effect? (Study 2)

In experiments that compared two adjacent veins on the same hand, there was a rightward shift of the norepinephrine dose-response curve in the vein that received endotoxin, and there was suppression of maximal constriction at 1 hour (maximal constriction: before endotoxin, 81±7%; after endotoxin, 26±6%; $P<.05$). In contrast, in the control vein, there was no change in the response to norepinephrine at the same time points (maximal constriction: time 0, 86±13%; 1 hour after instillation of endotoxin into the adjacent vein, 90±10%; Fig 5).

Effects of Repeated Administration of Endotoxin (Study 3)

Instillation of endotoxin into the same vein on 3 consecutive days resulted in the development of tolerance to the effects of endotoxin, so that by day 3, there was no shift in the dose-response curve to norepinephrine after endotoxin (maximal constriction before endotoxin was 91±6%, 98±2%, and 94±4% on days 1, 2, and 3, respectively; 1 hour after instillation of endotoxin, maximal constriction was 32±11% [$P<.001$], 67±6% [$P<.05$], and 85±7% [P=NS], respectively; Fig 6). In contrast, on day 3, the adjacent vein that had not been exposed to norepinephrine previously still demonstrated sensitivity to the effects of endotoxin (maximal constriction: before endotoxin, 94±4%; 1 hour after endotoxin, 39±6%; Fig 6).

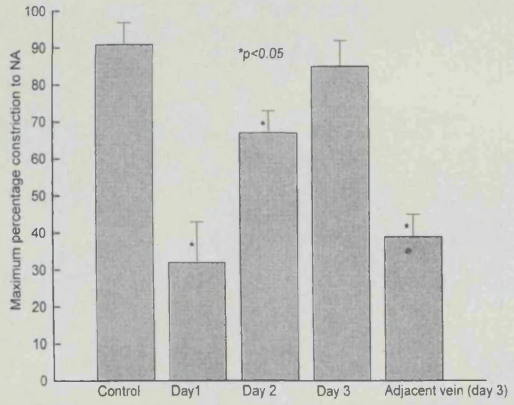

FIG 6. In five subjects, the effects of repeated instillation of endotoxin for 3 days into a single vein were explored. On each day, dose-response curves to norepinephrine (NA in figure) were constructed before and 1 hour after endotoxin. To determine whether the effects of repeated exposure to endotoxin initiated a local or systemic response, on the third day, an adjacent vein on the same hand was exposed to endotoxin and a dose response to norepinephrine was constructed before and 1 hour after endotoxin.

Effects of Cyclooxygenase Inhibitors and/or NO Synthase Inhibitors on the Response to Endotoxin (Study 4)

Neither the cyclooxygenase inhibitor aspirin nor the NO synthase inhibitor L-NMMA modified the shift in the norepinephrine response induced by endotoxin (Fig 7).

Coinfusion of L-NMMA for 10 minutes with a dose of norepinephrine (124±66 pmol/min) that produced maximal constriction before endotoxin did not alter vein size (maximal constriction: before endotoxin, 88±11%; after endotoxin, 30±10%; after endotoxin and in the presence of L-NMMA for 10 minutes, 31±10%; $P<.05$; Fig 7).

Similarly, the combination of aspirin (1 g) taken 2 hours before the study plus coinfusion of L-NMMA with norepinephrine did not affect the endotoxin-induced dose-response shift to norepinephrine (maximal constriction: before endotoxin, 90±5%; 1 hour after endotoxin, 33±5%; 1 hour after endotoxin and in the presence of aspirin and L-NMMA, 27±5%; n=5; $P<.05$; Fig 7).

Effects of Hydrocortisone on Response to Endotoxin and Development of Tolerance (Study 5 and Study 6)

Oral hydrocortisone (100 mg) taken 2 hours before the study abolished the endotoxin-induced shift in the response to norepinephrine at 1 hour (maximal constriction: before endotoxin, 77±4%; 1 hour after endotoxin, 70±5%; Fig 8).

Hydrocortisone taken 2 hours before each study inhibited the effects of endotoxin on the response to norepinephrine on day 1 and day 2, but on day 3 (in the absence of steroid), endotoxin caused a rightward shift in the dose-response curve to norepinephrine (maximal constriction before endotoxin was 89±5%, 99±1%, and 100±0% on days 1, 2, and 3, respectively; 1 hour after endotoxin, maximal constriction was 99±1%, 97±3%, and 40±6%, respectively; $P<.05$; Fig 9).

The in vitro endotoxin assay (nitrite production by J774 cells[38]) demonstrated that the endotoxin solution retained full activity throughout the period of the study.

Discussion

Injection of endotoxin into animals or humans causes systemic arterial[15] and venous[27] dilation and a fall in blood pressure. These effects are similar to the changes seen in patients with septic shock. The results of the present study provide direct evidence for a local vascular action of endotoxin in human veins in vivo and demonstrate that this effect is suppressed by a glucocorticoid. Using a system in which drugs are given into a single superficial vein in very low doses sufficient only to produce changes in the study vessel, we found that endotoxin attenuates the constrictor response to norepinephrine, causing a shift in the dose-response curve and suppressing the maximal constriction achieved. This effect was greatest 1 hour after exposure to the endotoxin and had waned by 4 hours, by which time the norepinephrine response had fully returned. Pretreatment with oral hydrocortisone abolished the endotoxin-induced hyporesponsiveness to norepinephrine;

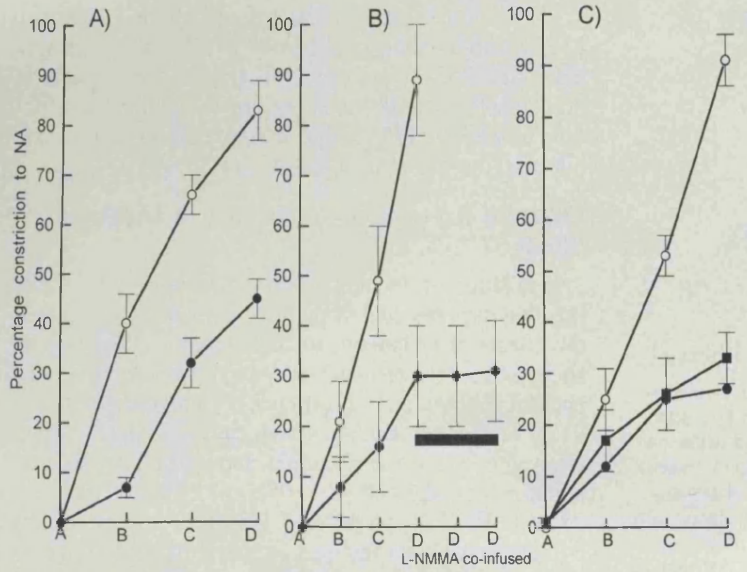

FIG 7. The effects of inhibition of cyclooxygenase and/or NO synthase inhibitors on the response to endotoxin were explored. A, Five subjects were given soluble aspirin (1 g), and the response to endotoxin was determined 2 hours later. Dose-response curves to norepinephrine (NA in figure) were established before and 1 hour after exposure to endotoxin. (A, 7±2 pmol/min; B, 40±11 pmol/min; C, 224±113 pmol/min; and D, 464±220 pmol/min.) B, In five subjects, a dose-response curve to norepinephrine was constructed before and 1 hour after endotoxin. At the end of the second dose-response curve, L-NMMA (100 nmol/min for 10 minutes, indicated by long bar) was coinfused with the same dose of norepinephrine that produced the maximal constriction before endotoxin. (A, 7±2 pmol/min; B, 23±7 pmol/min; C, 41±9 pmol/min; and D, 124±66 pmol/min.) C, The combined effects of L-NMMA and aspirin were studied in five subjects. All subjects received oral aspirin (1 g) 2 hours before the study, and the response to norepinephrine was determined before and 1 hour after exposure to endotoxin. In addition, immediately after the second dose-response curve to norepinephrine had been constructed, L-NMMA (100 nmol/min) was coinfused with norepinephrine and the dose-response curve repeated for a third time. (A, 8±1 pmol/min; B, 32±5 pmol/min; C, 80±18 pmol/min; and D, 173±32 pmol/min.) ○, before endotoxin; ●, 1 hour after endotoxin; ■, 1 hour after endotoxin with L-NMMA coinfused.

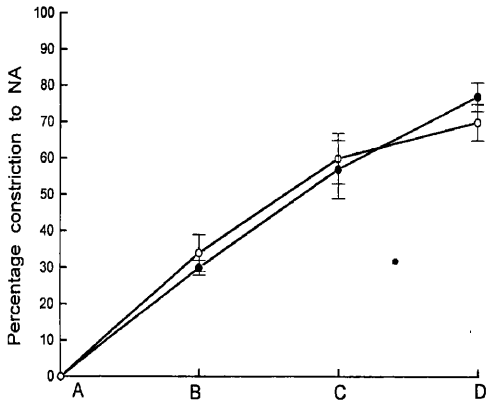

FIG 8. In five subjects, oral hydrocortisone (100 mg) was taken 2 hours before the study and the response to endotoxin determined 2 hours later. Dose-response curves to norepinephrine (NA in figure) were established before and 1 hour after exposure to endotoxin. A, 6±1 pmol/min; B, 28±11 pmol/min; C, 57±22 pmol/min; and D, 133±39 pmol/min. ○, before endotoxin; ●, 1 hour after endotoxin.

however, the local vasoactive mediators NO and prostaglandins appeared not to contribute to the changes seen. Repeated exposure to endotoxin induced tolerance.

Under the experimental conditions used, provided subjects are comfortably warm and relaxed, superficial hand veins have no intrinsic tone and cannot be dilated further.[32] To see a dilatation, it is necessary to preconstrict the vein, and we did this with norepinephrine (a situation similar to a vessel in an organ bath in vitro). Norepinephrine released from sympathetic nerves is the major determinant of venous tone,[39] and our study suggests that systemic administration of endotoxin inhibits contractions induced by neuronally derived norepinephrine and thereby causes venodilation. This suggestion is directly supported by the observation that the venoconstriction produced by activation of the nervous system after a deep breath was taken was abolished by treatment with endotoxin.

The hypotensive and vasodilator effects of endotoxin in healthy volunteers have been reported previously,[15] but it has not been determined whether these effects are due to a systemic reaction to endotoxin or direct effects on the vessels themselves. Indeed, after intravenous injection of endotoxin, a series of systemic effects occurs that includes fever, altered white cell count, increased concentration of cytokines circulating in blood, and activation of coagulation factors.[10,15,19,20] In the present study, a segment of the vein was isolated from the circulation, and the endotoxin was instilled locally for 1 hour and then withdrawn, at which time the vessel was opened again to the circulation. The hyporesponsiveness to norepinephrine produced by this maneuver was due to the endotoxin, because instillation of sterile saline had no effect. Several observations support the conclusion that the effect of endotoxin was local rather than systemic: the total dose of endotoxin instilled and then removed was less than the minimum pyrogenic dose[40]; subjects experienced no systemic symptoms; and, most conclusively, reactivity of an adjacent, untreated vein on the same hand was unaltered. However, it remains possible that the blood cells trapped within the isolated segment contributed to the response to endotoxin.

Hyporesponsiveness to norepinephrine was evident 1 hour after endotoxin and persisted for at least 3 hours. This rapid effect is consistent with the acute effects of endotoxin seen in certain animal models[25] and with the time course of the hemodynamic changes that occur in healthy volunteers after systemic administration of endotoxin.[15] The effects of endotoxin persisted for more than 2 hours after the endotoxin had been removed, at a time when the vessel was no longer exposed to this stimulus. This suggests that exposure to endotoxin triggered longer-lasting pharmacological and biochemical changes in the vessel wall.

The effects of endotoxin could be abolished by prior administration of oral hydrocortisone, which indicates that whatever mediates the response to endotoxin is suppressed by an anti-inflammatory dose of glucocorticoid. Studies in animals and in vitro have demonstrated that exposure to endotoxin leads to the delayed expression of iNOS and COX-II, a process that is inhibited by glucocorticoids.[41,42] Furthermore, the blocking of the activity of iNOS or COX[43] reverses many of the vascular effects of endotoxin, which indicates that the products of these enzymes, NO and prostanoids, contribute to the changes seen. However, in the hand veins, although glucocorticoids were effective, the NO synthase inhibitor L-NMMA (which should inhibit NO synthesis within minutes) and the cyclooxygenase inhibitor aspirin had no effect on the hyporesponsiveness to norepinephrine. Thus, NO or prostanoids are unlikely to contribute to the response to endotoxin that we detected in these veins. This finding is consistent with certain previous studies of the acute response to endotoxin in animals[42] and a study of the delayed effects of endotoxin on human saphenous veins in vitro.[28] In addition, a biochemical study[44] on human saphenous vein vascular smooth muscle cells suggested that interleukin-1 might elevate the concentration of cGMP independent of NO generation. It is unlikely in the present study that insufficient doses of the blocking agents were used, because we have demonstrated previously that the dose of L-NMMA used in the present study attenuates the response to endothelium-dependent dilators[45] and that the dose of aspirin used inhibits dilation to arachidonic acid.[37] Thus, the mechanism of the change

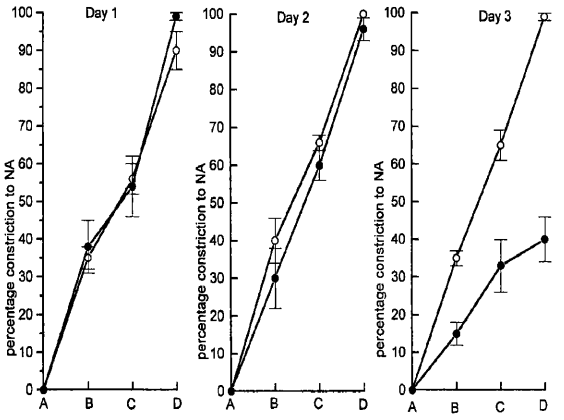

FIG 9. On 2 consecutive days, five subjects were given oral hydrocortisone (100 mg) 2 hours before the study. Dose-response curves to norepinephrine (NA in figure) were constructed before and 1 hour after endotoxin. On day 3, no steroid was given, and the dose-response curve to norepinephrine was established before and 1 hour after endotoxin. Day 1: A=7±1 pmol/min; B=18±2 pmol/min; C=36±4 pmol/min; and D=88±20 pmol/min. Day 2: A=9±1 pmol/min; B=36±11 pmol/min; C=72±23 pmol/min; and D=144±46 pmol/min. Day 3: A=8±1 pmol/min; B=28±5 pmol/min; C=56±10 pmol/min; and D=112±20 pmol/min. ○, before endotoxin; ●, 1 hour after endotoxin.

we have observed in the veins remains uncertain, but the possibilities include direct stimulation of guanylate cyclase,[44] local generation of platelet-aggregating factor, leukotrienes, cytokines, isoprostanes, or other glucocorticoid-suppressible inflammatory mediators.[18-20,46] Further studies with specific antagonists of these mediators are now needed. It is also important to develop methods to study the direct local actions of endotoxin on the arterial circulation in humans (resistance vessels and conduit vessels).

By 4 hours, the effects of endotoxin had disappeared. This contrasts with the effects seen when endotoxin is administered systemically, in which case the hypotension persists for 8 hours or longer.[15] The reasons for this are not known but might include persistence of endotoxemia after systemic administration, differences between arteries and veins or between vascular beds, or the contribution of systemic rather than direct local effects of endotoxin to the delayed and more long-lasting response to endotoxin. The chronic vascular response to endotoxin seems to be due in large part to expression of iNOS[4,23,43] in the vessel wall, a process that takes longer than 2 to 3 hours and might be due to a systemic response, with infiltration into the vessel wall by activated circulating macrophages.[23] Clearly, such a systemic response would have been absent in the present study, and it now should be possible to dissect which mechanisms are generated locally and which are systemic in origin.

Repeated daily exposure to endotoxin for 1 hour for 3 consecutive days induced tolerance to the effects of endotoxin by the third day. Again, this effect appeared to be a local phenomenon, because at a time when the treated vein was tolerant to endotoxin, an adjacent vein on the same hand was not. Interestingly, tolerance to endotoxin on day 3 did not occur when the biological response (hyporesponsiveness to norepinephrine) on days 1 and 2 had been blocked by prior administration of hydrocortisone. These results suggest that tolerance occurs as a result of a downregulation or depletion of pathways that mediate the response to endotoxin rather than downregulation of the endotoxin receptors. This is consistent with studies performed in vitro on macrophage and monocyte cell lines that demonstrated that tolerance occurred as a result of decreased, endotoxin-induced expression of mRNA for tumor necrosis factor and decreased G protein function,[47] with no alteration of endotoxin receptors.[48] Our results do not support the suggestion that systemic elevation of endogenous glucocorticoids is the mechanism of vascular tolerance to endotoxin.[49] There is interest in the possibility that inducing endotoxin tolerance could be used therapeutically in high-risk patients.[50] Our results suggest that tolerance to the vascular effects of endotoxin is achievable but indicate that it may not be possible to induce tolerance without first eliciting the biological response.

We have developed a safe and reproducible model that allows the study of the acute local effects of endotoxin on the venous wall in situ. To the best of our knowledge, similar experiments have not been undertaken previously in animals or humans. The model permits exploration of the role of inflammatory mediators and their effects on constrictor or dilator responses and might also be extended to explore the effects of endotoxin on endothelial function, blood cell/vessel wall interactions, or changes in permeability. The finding that endotoxin produces an acute venous hyporesponsiveness to the constrictor effects of norepinephrine or activation of the sympathetic nervous system might account for the changes in cardiac filling pressure that occur in septic shock.[1,2] This condition is associated with profound venodilatation despite high circulating levels of catecholamines and activation of the sympathetic nervous system (the major determinant of venous tone).[51] The observation that the hyporesponsiveness to norepinephrine is inhibited acutely by glucocorticoids but appears not to involve prostanoids or NO suggests that other unidentified mediators contribute to the overall vascular response to endotoxin. This suggestion is supported by the finding that animals that lack the gene that encodes for iNOS still show a degree of hypotension in response to endotoxin.[52] Considerable species variability in the response to and effects of endotoxin has been reported,[25] and it should now be possible to explore the local mechanisms of action of endotoxin in detail in humans.

Acknowledgment

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Vasodilatation to Arachidonic Acid in Humans

An Insight Into Endogenous Prostanoids and Effects of Aspirin

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Background Human endothelial and vascular smooth muscle cells synthesize prostanoids. Several of these have been implicated in the physiological and pathophysiological regulation of vascular tone; however, there is no direct evidence that human blood vessels synthesize sufficient prostanoid to alter vessel tone.

Methods and Results We explored the effects of local infusions of arachidonic acid on the tone of preconstricted superficial hand veins in healthy volunteers. Aspirin was used to assess the contribution of prostanoids to the responses seen. Local infusion of arachidonic acid produced a dose-dependent dilatation of preconstricted veins. This was abolished by local infusion of aspirin. Oral aspirin was also effective: a high (anti-inflammatory) dose of aspirin (1 g) taken 2 hours before the experiment blocked the arachidonic acid–induced venodilatation; however, a low (cardioprotective) dose of aspirin (75 mg) did not. Unlike the responses to arachidonic acid, responses to glyceryltrinitrate and bradykinin were unaltered by aspirin (1 g). Ex vivo platelet aggregation was inhibited by aspirin in both high and low doses. Aspirin (1 g) inhibited arachidonic acid–induced venodilatation for up to 5 days. The time course was similar for vascular and platelet effects.

Conclusions The present findings demonstrate that local generation of prostanoids in a human vessel in vivo alters vascular tone. The predominant prostanoid synthesized is a dilator and its synthesis can be blocked by an anti-inflammatory but not a cardioprotective dose of aspirin. The results suggest that selective inhibition of platelet aggregation by oral aspirin might be a function of dose rather than the interval between doses. (*Circulation.* 1995;92:2113-2118.)

Key Words • vasodilation • aspirin

Production of prostaglandins by vascular cells was first demonstrated over 20 years ago.[1] It is now recognized that endothelial cells and smooth muscle cells have the capacity to synthesize constrictor and dilator prostanoids from the common precursor arachidonic acid. Indeed, continuous synthesis and release of prostacyclin is thought to contribute to the thromboresistant and vasodilator properties of the blood vessel wall and oppose the vasoconstrictor and prothrombotic effects of platelet-derived thromboxane.[1,2] Studies in vitro and in vivo have demonstrated the capacity of human blood vessels to produce prostanoids; prostacyclin and thromboxane are synthesized by human endothelial cells in culture, and circulating metabolites of these prostanoids are found in plasma and urine.[3] In healthy volunteers, prostacyclin synthesis can be stimulated further by the vasodilator bradykinin.[4]

Abnormalities of vascular prostanoid synthesis have been implicated in a number of pathophysiological states ranging from septic shock[5] to hypertension[6] and unstable angina.[7] In experimental models of hypertension, a prostanoid endothelium–derived constrictor factor has been described,[8] and in unstable angina and myocardial infarction, a shift in the balance of locally generated prostacyclin and thromboxane is thought to contribute to vasospasm and vessel occlusion. The potential importance of prostanoids in vascular physiology and pathophysiology is highlighted by the efficacy of aspirin; this drug irreversibly inhibits cyclooxygenase, the enzyme that converts arachidonic acid to endoperoxide[9] and reduces mortality and morbidity in cardiovascular disease. When used as a prophylactic agent in patients with cardiovascular disease, aspirin is given in low doses[10] and/or at long dose intervals in an attempt to inhibit thromboxane production in platelets while sparing the synthesis of prostacyclin in endothelial cells.[3]

Despite these observations, there is no direct evidence in vivo for the production from arachidonic acid of vasoactive amounts of prostanoids in human blood vessel under resting conditions. Indeed, aspirin or other inhibitors of cyclooxygenase appear to produce constriction in the renal vascular bed but not elsewhere, suggesting that basal release of prostanoids does not contribute significantly to resting vascular tone. However, the supply of endogenous arachidonic acid is rate limiting for prostanoid synthesis in many cell types,[1] and addition of exogenous arachidonic acid has been shown to produce constriction or dilatation of isolated blood vessels from animals.[8,11-14] In the present study we have explored the effects of local infusions of arachidonic acid on the tone of single superficial dorsal hand veins in vivo in an attempt to determine whether there is a prostanoid pathway in human vessels in vivo that is capable of altering vascular tone and that is rate limited by availability of substrate. We used cyclooxygenase inhibitors to determine the contribution of prostanoids to the responses seen and developed the experimental system to study directly the dose response and time course of the vascular effects of aspirin.

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Methods

Studies were approved by the local ethics committee and were performed on male (n=15) and female (n=6) subjects aged 19 to 38 years. Subjects were included who stated that they were healthy and on no medication and who gave their written informed consent. Subjects lay supine in a temperature-controlled laboratory (28°C to 29°C) with one hand placed on an angled support above the level of the heart. A congesting cuff was placed around the upper arm and inflated to 40 mm Hg. Drugs or physiological saline (0.9%) were infused continuously (0.25 mL/min) through a 23-gauge "butterfly" needle placed in a dorsal hand vein. The diameter of the infused vein was measured 5 to 10 mm downstream from the tip of the infusion needle by recording the linear diplacement of a lightweight probe placed on the skin overlying the summit of the vessel when the pressure in the congesting cuff was lowered from 40 to 0 mm Hg.[15,16] In all studies, saline was infused for at least 15 minutes until a stable baseline vein diameter was recorded. Provided subjects are comfortably warm and relaxed, dorsal hand veins have no intrinsic tone,[16] and in order to observe dilator responses, it is necessary to preconstrict the vessel. In this study (unless stated othewise), after baseline recordings of stable vein size had been made, veins were preconstricted to between 30% and 50% of resting diameter by a continuous infusion of norepinephrine (20 to 160 pmol/min) for the remainder of the study. Study drugs were coinfused with norepinephrine. Measurements of vein size were made every 5 minutes.

Effects of Arachidonic Acid: Dose Response, Reproducibility, and Effects of Local Aspirin

In 3 subjects, three doses of arachidonic acid (200 pmol/min, 2 nmol/min, and 20 nmol/min, each dose for 5 minutes) were infused into a preconstricted vein. Dose increments were made at 10-minute intervals. To determine the reproducibility of the response within a single experiment, 6 subjects were given two infusions of arachidonic acid (20 nmol/min for 5 minutes) 30 to 45 minutes apart and when a stable norepinephrine constriction was reestablished. In a separate study in the same 6 volunteers, arachidonic acid (20 nmol/min for 5 minutes) was infused into a preconstricted vein before and after local infusion of aspirin (18 μmol/min for 30 minutes). The effects of arachidonic acid (20 nmol/min for 5 minutes) on unconstricted veins (n=3) and arachidonic acid vehicle on preconstricted vessels (n=3) also were determined.

Effects, Dose Response, and Time Course of Oral Aspirin and Paracetamol

Subjects were given soluble aspirin (75 mg, n=6, or 1 g, n=3) or paracetamol (1 g, n=5), and the response to arachidonic acid (20 nmol/min) was determined 2 hours later. In the subjects taking 1 g aspirin, glyceryltrinitrate (GTN; 20 pmol/min) was infused for 5 minutes at the end of the study to determine whether the preconstricted vein would relax to an agent that does not utilize the prostanoid pathway. In a further study, 10 subjects were given high-dose aspirin (1 g). In 5, the response to arachidonic acid was determined at 0 (predose), 2, 6, and 24 hours and in 5 at 0 (predose), 2, and 120 hours after aspirin. Response was determined as the maximal change in vein diameter within 15 minutes of starting the infusion of arachidonic acid. The effects of oral aspirin (1 g) on the venodilator drugs bradykinin and GTN also were studied. In 5 subjects, dose-response curves were constructed to bradykinin (2, 4, and 8 pmol/min; each dose for 5 minutes) and GTN (1, 2, and 4 pmol/min; each dose for 5 minutes) before and 2 hours after oral aspirin (1 g).

Platelet Studies

Blood was drawn for platelet aggregometry from subjects taking part in the studies of oral aspirin (75 mg, n=5; 1 g, n=13) or paracetamol therapy (1 g, n=5). Immediately before taking the drug and 2.5 hours later, 15 mL of venous blood was drawn into a syringe containing 2.5 mL of 3.15% (wt/vol) trisodium citrate. Blood was centrifuged at 200g for 20 minutes to obtain platelet-rich plasma. One-milliliter aliquots of platelet-rich plasma were placed in cuvettes, and the responses to 10 μL of collagen and 300 μmol/L arachidonic acid were determined using a dual-channel optical aggregometer (Chronolog Corp). One milliliter of platelet-rich plasma was placed in an Eppendorf microtube and centrifuged at 1300g for 5 minutes to produce a supernatant of platelet-poor plasma, which was used as a control. The maximal extent of aggregation was used for data analysis, and results after drug administration were compared with control (predrug) values. The predrug aggregatory response was taken as 100%.

Drugs

Sodium arachidonate (5 mg per vial) stored under nitrogen was obtained from Sigma. Vials were stored at −20°C, and a single vial was used for each study. Sodium arachidonate (5 mg) was dissolved in 154 μL absolute alcohol to produce a stock solution of 1 mmol/L. Subsequent dilution was in saline, and the final concentration of alcohol in the infusate was 0.0001%. Arachidonic acid was always used within 6 hours of preparation. Lysine acetylsalicylate (1 g per vial) was obtained from Synthe Labo; paracetamol (500 mg) and norepinephrine (2 mg per vial) from Sanofi Winthrop; dispersible aspirin from Aspar Pharmaceuticals Ltd; GTN (5 mg per vial) from Dupont Pharma; bradykinin (50 μg per vial) from Clinalfa AG; ascorbic acid (100 mg/mL) from Evans Medical Ltd; and collagen reagent from Chrono-Log. Ascorbic acid 0.5 mL was added to norepinephrine stock solutions to prevent auto-oxidation. All drugs were prepared fresh on the day of the experiment and dissolved in sterile physiological saline solution.

Calculations and Statistics

Changes in vein size were measured in arbitrary units and converted to millimeters after calibration of the transducer at the end of each experiment. The response of the resting vein to drugs is expressed as a reduction in diameter from that measured during infusion of saline alone. The response of the norepinephrine-preconstricted vein to drugs is expressed as percentage reversal of the induced constriction. Results are compared using the Student's t test for paired data or ANOVA of the means as appropriate; $P<.05$ is considered statistically significant.

Results

Local infusion of arachidonic acid caused no adverse or systemic effects, and subjects did not report any discomfort. In 7 subjects, after the infusion of arachidonic acid, a flare appeared on the skin overlying the vein, and this persisted for up to 20 minutes. The internal diameters of veins and the degree of preconstriction for each part of the study are shown in the Table.

Effects of Arachidonic Acid: Dose Response and Reproducibility

In the initial studies in 3 subjects, local infusion of arachidonic acid produced a dose-dependent venodilatation such that 200 pmol/min, 2 nmol/min, and 20 nmol/min (each dose for 5 minutes) resulted in dilatation of 27±3%, 42±3%, and 87±10%, respectively. The lower two doses of arachidonic acid produced transient dilatations (lasting less than 10 minutes), whereas the duration of dilatation in response to the highest dose was in the order of 15 to 25 minutes (Figs 1 and 2). For all subsequent studies, the highest dose of arachidonic acid was used. Arachidonic acid (20 nmol/min for 5 minutes) produced identical responses when infused

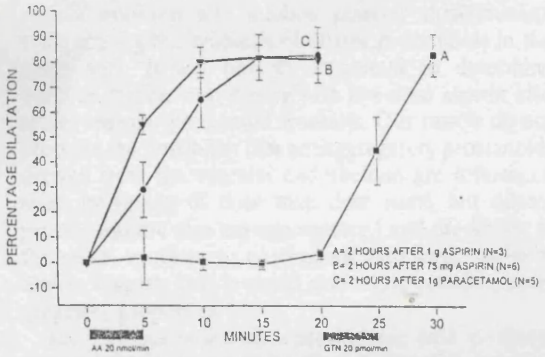

FIG 3. Plot shows effects of oral cyclooxygenase inhibitors on the response to arachidonic acid (AA). Three subjects received 1 g of oral aspirin (A), 6 received 75 mg (B), and 5 received 1 g of paracetamol (C). The response to arachidonic acid was determined 2 hours later. In subjects receiving high-dose aspirin, a 5-minute infusion of glyceryltrinitrate (GTN) was given at the end of the experiment. Low-dose aspirin and paracetamol did not affect the dilatation to arachidonic acid, whereas the response was abolished with 1 g of oral aspirin.

inhibitory effects of oral aspirin on arachidonic acid–induced venodilatation is shown in Fig 4. Oral aspirin (1 g) inhibited arachidonic acid–induced venodilatation at 2, 6, and 24 hours (14±4%, 15±3%, and 30±7% dilatation, respectively). However, 5 days after aspirin, the dilator response to arachidonic acid had returned to 74±11% of the control dilatation (NS).

Effects of Oral Aspirin on the Response to GTN and Bradykinin

Oral aspirin (1 g) did not alter the constrictor response to norepinephrine (Table). The response to bradykinin and GTN was unaltered by oral aspirin (Fig 5). The dilatation to bradykinin 2, 4, and 8 pmol/min was 34±5%, 45±6%, and 65±13% before aspirin and 38±2%, 60±7%, and 73±5% 2 hours after oral aspirin. The dilatation to GTN 1, 2, and 4 pmol/min was 44±13%, 80±6%, and 92±6% before aspirin and 34±65%, 65±8%, and 88±6% 2 hours after oral aspirin.

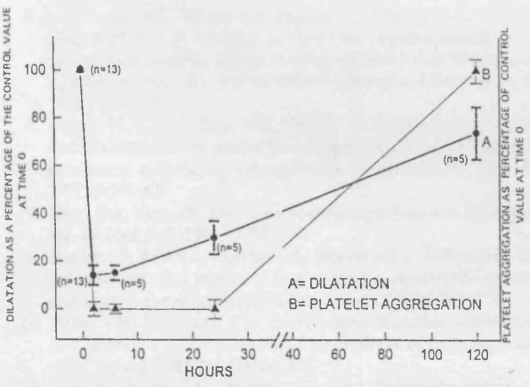

FIG 4. Plot shows time course of the effects of 1 g aspirin on the response to arachidonic acid. All subjects (n=13) received 1 g of oral aspirin. For studies of dilatation (●), all 13 subjects were studied at 2 hours. Five were also studied at 6, 24, and 120 hours. At 6 and 24 hours, there was still a significant ($P<.05$) degree of inhibition to arachidonic acid–induced venodilatation, but by 120 hours the response had returned to 74±11% of the control value (NS). Platelet aggregation studies (▲) showed a similar time course of recovery, reaching the control value at 120 hours.

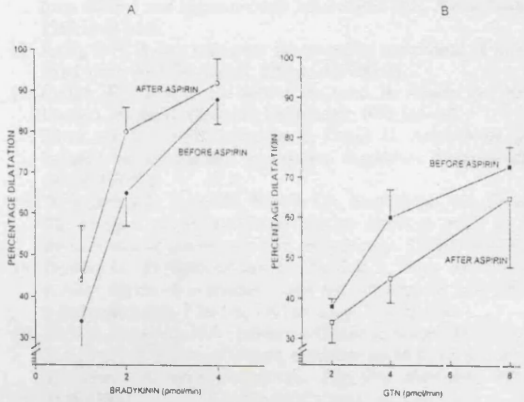

FIG 5. In 5 subjects dose-response curves constructed to bradykinin (A) (2, 4, and 8 pmol/min, each dose for 5 minutes) and glyceryltrinitrate (GTN; B) (1, 2, and 4 pmol/min, each dose for 5 minutes) before (filled symbols) and 2 hours after (open symbols) oral aspirin (1 g). Oral aspirin did not affect the response to bradykinin or glyceryltrinitrate.

Platelet Studies

Arachidonic acid–induced platelet aggregation was inhibited by low- (n=6) and high-dose aspirin (n=13) but not by paracetamol (n=5) (Fig 6). The inhibitory effect of aspirin (1 g) was still evident at 24 hours but not at 120 hours (Fig 6). The time course of the effect of aspirin on arachidonic acid–induced platelet aggregation was similar to the time course of the effect of aspirin on arachidonic acid–induced venodilatation (Fig 6). Collagen-induced platelet aggregation was not significantly affected by aspirin or paracetamol.

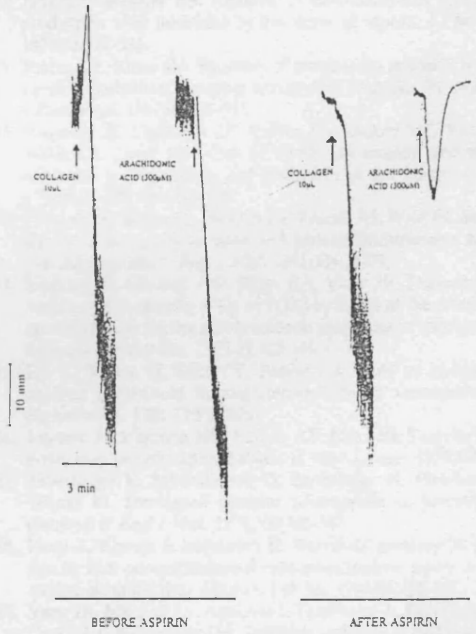

FIG 6. Tracings of platelet aggregation before and after 75 mg aspirin: platelet response to 300 µmol/L arachidonic acid or 10 µL collagen before and 2 hours after 75 g oral aspirin. Low-dose aspirin completely abolished aggregation to arachidonic acid. Collagen-induced aggregation was not significantly affected.

the dose given rather than interval of dosing; low-dose aspirin preferentially inhibits platelet thromboxane while sparing the synthesis of dilator prostanoids in the vessel wall. It will now be important to determine whether chronic oral dosing with low-dose aspirin also spares vascular prostanoid synthesis. Our results do not preclude the possibility that antiaggregatory prostanoids derived from the vascular endothelium are influenced more by timing of dose than dose itself, but dilator prostanoids are also antiaggregatory,[1] and the ability of the vessel to dilate to arachidonic acid after low-dose aspirin suggests that it would also retain certain antiaggregatory properties.

The demonstration that arachidonic acid produces aspirin-inhibitable vasodilatation in humans provides direct evidence for functional effects of locally generated prostanoids in the vessel wall. The relative simplicity and safety of the experimental system and the reproducibility of the response to arachidonic acid suggest that it may be possible to use this system to explore the role of locally generated prostanoids in disease states such as hypertension,[6,35] diabetes,[36,37] and liver disease,[38] where endothelium-derived prostanoids have been implicated, or in conditions of chronic inflammation when the inducible isoform of cyclooxygenase is expressed.[39]

Acknowledgment

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Nitric oxide 9 years on

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J R Soc Med 1995;**88**:667-673 NITRIC OXIDE AND ITS CLINICAL IMPLICATIONS, RSM CONFERENCE, 11 JUNE 1996

Until the 1970s, the study of amine mediators such as noradrenaline, histamine and acetylcholine dominated pharmacological research. The discovery of the prostaglandins heralded a decade of research in which lipid mediators took pride of place. But since 1987 the research community has had to contend with yet another class of biological mediators—gases.

Nitric oxide is the first endogenously synthesized gas shown to have a physiological mediator function and in the past 8 years over 15 000 papers have been published on the molecular biology, biochemistry, pharmacology and clinical significance of this most unexpected of mediators. This review is based on a meeting at which some of the major contributors to this area of research distilled the welter of data to present an update and overview of the biology of NO in the cardiovascular, immune and nervous systems. The main speakers are listed at the end.

DISCOVERY OF NO

The discovery of NO is a story of serendipity, a sharp eye and imaginative, adventurous research. Amines and gases came together in the New York laboratory of Robert Furchgott, SUNY Health Center, Brooklyn[1]. He was investigating mechanisms of contraction and relaxation of isolated blood vessels when he noticed that one of his technicians always found that acetylcholine relaxed the vessels whereas the other found that it caused contraction. The answer to this paradox lay in the fragile monolayer of endothelial cells that lines the luminal surface of blood vessels. One technician (like countless investigators before) handled the vessel roughly and inadvertently rubbed off the endothelium. The other was careful and (equally inadvertently) kept the endothelium intact. Furchgott understood what he saw and went on to demonstrate that the presence of an intact endothelium is a prerequisite for the relaxant effects of acetylcholine, bradykinin, substance P and various other mediators. By separating the endothelium from the smooth muscle he showed that a labile factor is released from the endothelium and it is this factor that relaxes the smooth muscle.

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6 years after his discovery of endothelium-derived relaxing factor (EDRF), Furchgott surprised vascular pharmacologists again. He suggested that EDRF was an inorganic gas, nitric oxide—a substance hitherto thought of as a pollutant, a product of electrochemical storms, a toxic contaminant of cylinders of nitrous oxide. But within a year, Moncada and co-workers[2] demonstrated that vascular endothelial cells generate NO, showed that the amounts generated account for the biological activity of EDRF, identified the substrate for NO synthesis (L-arginine), and synthesized an inhibitor of NO formation (L-NMMA) (Figure 1). Drawing on the technology developed by scientists studying pollution, they adapted a chemiluminescence machine built for detecting NO released in car exhaust, increased the sensitivity, and attached it to columns of endothelial cells grown onto miniaturized glass beads. Upon stimulation of the endothelial cells NO was detected and the amount was sufficient to relax vascular smooth muscle.

HOW DOES NO ACT?

Before the chemical identity of EDRF was known, it was recognized that endothelium-dependent relaxation of blood vessels was associated with an increase in the levels of cyclic GMP in the vascular smooth muscle[3]. cGMP is produced upon activation of guanylate cyclase, a magnesium-sensitive

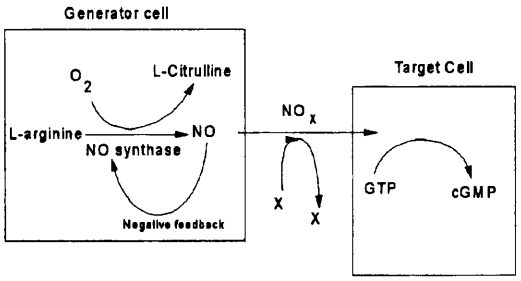

Figure 1 **Synthesis of nitric oxide and production of physiological effects.** Nitric oxide synthase catalyses synthesis of nitric oxide from L-arginine and molecular oxygen. L-citrulline is the byproduct. Nitric oxide itself might inhibit the activity of nitric oxide synthase by interacting with the haem moiety of this enzyme. Physiological effects are produced after nitric oxide binds to the haem moiety of guanylate cyclase and activates this enzyme to produce cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP) in target and generator cells. Carrier molecules (X) that stabilize nitric oxide have been proposed.

protein that exists as a heterodimer containing two haem molecules. Under basal conditions, guanylate cyclase has no significant activity, but NO binds to haem with a high affinity and this binding initiates a conformational change in the ferrous haem complex to cause a greater than 400-fold activation of the enzyme. Interestingly, carbon monoxide, another endogenously synthesized gas proposed as a biological mediator working through cGMP, causes only a 4.4-fold activation of the enzyme. However, the high affinity binding of NO to the haem moiety of guanylate cyclase presents a theoretical problem: how is the enzyme deactivated? It appears that the configuration of the haem molecules within the heterodimer means that the dissociation rate of NO is far faster than initially predicted, allowing for rapid deactivation of the enzyme.

SYNTHESIS OF NO

It is now clear that the endothelium-dependent relaxation described by Furchgott is just one of the myriad mediator functions of NO (see below). Three NO synthases have been described—an endothelial isoform (eNOS), a neuronal isoform (nNOS) in 'nitrergic' nerves and a macrophage or inducible isoform (iNOS). NOSs cleave a nitrogen from one of the guanidino nitrogens of the semi-essential aminoacid L-arginine, and combine it with oxygen from molecular oxygen to form NO (Figure 1). The byproduct of this reaction is the aminoacid L-citrulline. The genes encoding for the three isoforms of NO synthase have been located to human chromosomes 7 (eNOS), 12 (nNOS) and 17(iNOS)[4–6], and the enzymes show sequence homology with each other and with cytochrome P450 reductase[7] (although NOS is not a member of the cP450 supergene family).

The chemistry of the five electron oxidation of L-arginine to form NO and citrulline is unusual and involves a series of co-factors including flavins, tetrahydrobiopterin, NADPH and calmodulin. Calmodulin appears to be important in activating electron transfer from flavins to the haem moiety of NOS; this determines the rate of flavin reduction, an important step in the biosynthetic pathway. Two of the isoforms (eNOS and nNOS) are calcium-calmodulin sensitive and the binding of the calcium-calmodulin to the enzyme complex determines the rate of NO generation. In contrast, iNOS binds calmodulin tightly, thus allowing maximum flavin reduction and rendering the enzyme effectively independent of the prevailing concentration of calcium/calmodulin.

NO, IMMUNITY AND INFLAMMATION

At a time when vascular pharmacologists were pursuing the identity of EDRF, immunologists were independently exploring the cytotoxic properties of macrophages that appeared to be dependent on generation of an oxide of nitrogen[8]. It is now clear that activated macrophages express high levels of NOS and produce large amounts of NO. Cytokines that induce the expression of iNOS in macrophages include interleukin-1 (IL-1), interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) and migration inhibitory factor. The NO generated contributes to the role of macrophages as highly effective killers of intracellular and extracellular pathogens—although NO itself is probably not the cytotoxic molecule; rather it may be peroxynitrite ($ONOO^{-}\cdot$) a product of the interaction between NO and superoxide (O_2^{-}).

It is also apparent that excessive NO can contribute to experimental immunopathology (diabetes, graft-versus-host disease, liver cirrhosis, rheumatoid arthritis) and expression of iNOS is thus necessarily under tight regulation. Several cytokines, including IL-4, IL-10[9,10] and transforming growth factor-beta, can down-regulate the induction of NO synthase in macrophages. In addition, macrophages exposed to endotoxin alone and then stimulated with a mix of IFN-γ and endotoxin express lower levels of NO synthase than cells stimulated without pre-exposure to endotoxin (i.e. they exhibit tolerance). NO itself can reduce the activity of NO synthase by feedback inhibition, and also inhibit the production of IFN-γ by Th1 cells (thus turning off its own synthesis from upstream in the inflammation cascade). iNOS expression is involved not only in macrophage biology, but also in the response of a wide variety of cells to inflammation and infection (see below).

NO IN THE CARDIOVASCULAR SYSTEM

All three isoforms of NOS have been implicated in cardiovascular control. There is a basal generation of NO in the arterial circulation of animals and human beings and this provides a continuous vasodilator tone (eNOS activity). Indeed, inhibition of NO synthesis causes substantial arteriolar vasoconstriction and increased blood pressure; genetically modified mice that lack the gene encoding for eNOS (eNOS knockout mice) are hypertensive[11]. The tonic NO-mediated dilatation of resistance vessels seems to be approximately equal in magnitude and opposite in effect to the tonic constriction produced by the sympathetic nervous system, suggesting that these two systems might work together to allow vascular tone to be adjusted rapidly in response to a variety of central and local stimuli; the sympathetic nervous system providing a mechanism for central control and endothelium-derived NO a system for local control.

The endothelium responds to chemical stimulation (e.g. agonists such as acetylcholine or bradykinin) and physical stimulation (e.g. shear stress). The precise mechanisms by which these different stimuli increase intracellular eNOS activity is not fully understood. Certainly, many stimuli cause an increase in intracellular calcium concentration and this will activate eNOS (see above). However, covalent modifications to the eNOS protein may also alter its activity

or intracellular localization and might provide a mechanism to target eNOS to particulate subcellular fractions or caveolae (specialized invaginations of the plasma membrane that serve as sites for the sequestration of signalling proteins including certain G-proteins, ion channels, protein kinases and receptors). Covalent modifications by which the enzyme is attached to a fatty acid or some other moiety of eNOS have all been described[12] (acylation, N-terminal myristoylation, reversible palmitoylation and phosphorylation).

The functional significance of these modifications is not yet clear, but bradykinin (an endothelium-dependent dilator) promotes de-palmitoylation of eNOS and increases in shear stress promote myristoylation. The implications of these findings are that, even if the amount of enzyme stays constant and the level of stimulant stays constant, the amount of NO generation may fall or increase, or it might be localized to one particular area of the endothelial cell. NO generation within the endothelium might also be altered by changes in the amount of eNOS enzyme expressed, and studies in animals suggest that oestrogens can act in this way to cause vascular changes. This effect may contribute to the haemodynamic changes in pregnancy[13].

Platelets and white cells

NO generated by eNOS is not only released abluminally to relax vascular smooth muscle but also acts on the luminal side where it inhibits platelet aggregation[14] and helps prevent the adhesion of platelets and white cells to the vessel wall. Inhibition of NO generation does not appear to increase the adhesion of platelets to normal vessels, but enhances adhesion to damaged vessels. NO is also generated by activated platelets themselves, and activated platelets release mediators that stimulate eNOS activity in the endothelium. Together, endothelial and platelet generation of NO seems to provide a negative feedback mechanism to limit the extent of intravascular platelet activation. The antiplatelet effects of NO seem to be mediated by effects on cell surface adhesion molecules; NO inhibits the expression of P-selectin and/or the fibrinogen receptor (the IIb/IIIa receptor). The potential importance of the antiplatelet effects of NO is illustrated by studies of experimental vessel stenosis; in the presence of stenosis, vasoactive factors released by the adhering platelets appear to contribute to the generation of cyclical variations in flow. These effects are greatly enhanced if NO generation is blocked.

Atherogenesis

It seems that decreased activity of eNOS would be associated with increased vascular tone, enhanced vascular reactivity to constrictor agents and a predisposition to platelet and white cell adhesion and activation. Diminished endothelium-dependent relaxation has been described in vessels of laboratory animals or patients with overt atherosclerosis or various risk factors including hypercholesterolaemia[15], hypertension[16] and diabetes[17]. The possible consequences of diminished NO-mediated effects are becoming clearer. In particular, there is accumulating evidence that a diminution of NO generation, or increased destruction of NO, could promote atherogenesis and provide a mechanism to link apparently disparate risk factors to the single cardiovascular endpoint of atheroma formation. Indeed experiments in cholesterol-fed rabbits have demonstrated that inhibition of NO synthesis promotes the development of atheroma-like lesions, whereas supplementation with L-arginine prevents it[18]. The mechanisms might include effects on vascular reactivity and platelet function but also appear to involve infiltration of monocytes. Indeed the expression of monocyte chemotractant protein 1 (MCP-1) on endothelial cells is enhanced by cholesterol loading and is further enhanced if an inhibitor of NOS is administered.

How does NO act to inhibit cellular adhesion and prevent atherogenesis? One suggestion is that part of the protective effect of NO relates to its ability to affect generation or survival of other free radicals, in particular superoxide (O_2^-)[19], a radical that is produced in excess amounts during cholesterol loading. Activation of the nuclear transcription factor NFκB (a factor that promotes the transcription of a variety of mRNA encoding for inflammatory proteins) is redox sensitive. NFκB is normally bound in an inactive form together with inhibitory factor (IκBα). It is proposed that basal NO prevents generation of O_2^- or interacts with O_2^- to prevent dissociation of the IκBα/NFκB complex and subsequent activation of NFκB. Activation of NFκB initiates transcription of the genes encoding for MCP-1 and certain cell surface adhesion molecules including vascular cell adhesion molecule-1 (VCAM-1), and the net effect is to promote cellular adhesion and migration. Thus basal NO is seen as providing a continuous brake on the activation of chemoattractants and adhesion molecules through its interaction, either directly or indirectly, with the IκBα/NFκB complex.

Shear stress increases NO generation from eNOS and also makes cells resistant to the activation of NFκB by certain pro-inflammatory cytokines. This raises the intriguing possibility that the predilection of atheroma for sites of low shear stress in the arterial tree may be related to loss of the NO-mediated inhibition of NFκB activation. Activation of NFκB is also involved in iNOS induction (see below), and perhaps the constitutive production of NO by eNOS helps prevent the induction of the inflammatory isoform.

L-arginine supplementation

Supplementation with L-arginine seems to retard the development of atherosclerosis in the cholesterol-fed rabbit,

and restores the responses to endothelium-dependent dilator agents in animals and patients with hypercholesterolaemia. Although L-arginine is the substrate for NOS, the mechanism of this effect is far from clear. The amount of arginine required by the enzyme is in the order of 1 μmol/L and the circulating concentration is in the order of 100 μmol/L (i.e. the supply of arginine should not be rate-limiting for NO generation). How arginine exerts its effects remains to be determined. It is curious that arginine supplementation works when given *in vivo* but usually not when added to vessels from hypercholesterolaemic animals *in vitro*. One possibility is that the presence of an endogenous inhibitor of NOS (such as asymmetric dimethylarginine; ADMA[20]) competes with arginine for the active site. Studies are now underway to explore this and other possibilities.

nNOS in the vasculature

The role of nNOS in cardiovascular control is less clear cut. Nerves containing nNOS (nitrergic nerves) are found lying in the adventitia of certain vessels and provide a neurogenic vasodilator tone. This is well characterized in the corpus cavernosum[21], where activation of nitrergic nerves contributes to the process of penile erection. Nitrergic nerves are also thought to be important in the cerebral vasculature where they may help coordinate vasodilatation to ensure that blood flow increases to metabolically active brain tissue [see below, section on NO in central nervous system (CNS)]. Recently it has also been suggested that nNOS activity may regulate blood pressure (CNS section).

iNOS in the vasculature

In contrast to eNOS and nNOS, iNOS is not normally expressed in healthy vessels, but the enzyme is synthesized *de novo* in endothelial cells and smooth muscle cells exposed to bacterial endotoxin or certain inflammatory cytokines. Because iNOS activity is calcium-independent, the amounts of NO generated are large, and cause profound vascular relaxation and resistance to constrictor agents. *De novo* expression of iNOS has been implicated in the pathogenesis of septic hypotension[22], the local vasodilatation associated with chronic inflammation, and the pathogenesis of vascular leak. Experiments in patients confirm that NO is generated in excess quantities in sepsis and that inhibition of NOS with L-NMMA will restore blood pressure[23]. However, L-NMMA inhibits all three isoforms of NOS and the precise role of iNOS in human inflammatory conditions remains uncertain.

The heart

In addition to its effects on vascular behaviour, NO has been implicated as an endogenous regulator of myocardial contractility and heart rate. NO appears to mediate the negative chronotropic effects of carbachol, at least in neonatal rat myocytes. Moreover, NO seems to be negative-inotropic in many experimental systems and inhibition of NOS may increase contractility of myocytes[24] (the situation *in vivo* is complicated by the increase in afterload due to arteriolar vasoconstriction). The inotrope milrinone has been reported to decrease the expression of eNOS in the heart and this action might contribute to its effect.

iNOS may also contribute to the regulation of myocardial function. In conditions of inflammation, expression of iNOS in the heart leads to excessive suppression of cardiac function. This might explain the acute cardiac dysfunction that accompanies sepsis and also the more chronic changes that occur in certain types of heart failure. In animal models iNOS is expressed during rejection of transplanted hearts and during experimental viral myocarditis, and mRNA for iNOS has been found in all four cardiac chambers of patients with heart failure[25]. However, although iNOS expression appears to occur in failing human cardiac myocytes and has been implicated in the pathophysiology of dilated, ischaemic and valvular heart disease, the precise role and effects of iNOS expression remain to be determined. Indeed, it is possible that the NO is produced primarily as a host defence mechanism, and that the cardiac or vascular changes are secondary.

NO IN THE RESPIRATORY SYSTEM

NO may play an important part in regulating airway function and in the pathophysiology of inflammatory airway diseases. Endothelium-derived NO controls airway blood flow and indirectly regulates plasma exudation. Furthermore, just as NO relaxes vascular smooth muscle, so it relaxes the smooth muscle of the airway (albeit less potently)[26]. NO is a neurotransmitter of bronchodilator nerves in human airways and counteracts the bronchoconstriction due to cholinergic neural mechanisms. Inducible NOS has also been implicated in respiratory pathophysiology; iNOS is expressed in human epithelial cells in response to pro-inflammatory cytokines and oxidants, probably via activation of NFκB.

Expression of iNOS has been found in the epithelium of asthmatic patients[27] and in macrophages in the lungs of patients with bronchiectasis. The concentration of NO in the exhaled air of patients is increased in inflammatory airways disease, consistent with increased generation of endogenous NO in the lungs. Increased NO production in the airways may result in hyperaemia, plasma exudation, mucus secretion and (indirectly) increased proliferation of Th2 lymphocytes responsible for eosinophilic inflammation. Glucocorticoids inhibit the induction of iNOS in epithelial cells and reduce the exhaled NO to normal values. Selective

inhibitors of iNOS may therefore be useful in the treatment of inflammatory airway diseases, such as asthma (see below).

NO IN THE GASTROINTESTINAL SYSTEM

As in the respiratory system, NO generated by nNOS has been implicated in the control of smooth muscle tone. Indeed the abnormal function of the lower oesophageal sphincter in achalasia is likely to be due to impaired nitrergic inhibitory input[28] and lack of effective nitrergic innervation is associated with pyloric stenosis in animals[29] and man[30].

iNOS, too, has been implicated in gut pathology. Gastrointestinal complications commonly arise after administration of non-steroidal anti-inflammatory drugs, and recently the involvement of gut bacterial flora in the generation of non-steroidal-induced inflammation has been explored[31]. Indomethacin provoked a time-dependent increase in vascular leakage in the jejunum that was associated with the expression of iNOS activity, suggesting a role of the excessive local generation of NO in the intestinal inflammatory response. This suggestion is supported by the observation that the damage could be limited by giving an NO synthase inhibitor. Interestingly, treatment with the broad-spectrum antibiotic ampicillin was also shown to prevent indomethacin-provoked damage, suggesting a role for local toxins produced by the gut bacterial flora in the processes underlying induction of iNOS following indomethacin.

iNOS seems also to have a central role in the liver, and both parenchymal and non-parenchymal cells synthesize NO alter immune stimulation. Hepatic function is tightly regulated by the interaction of specific cell populations in the liver. Indeed all Kupffer cells, the fixed hepatic macrophages, and hepatocytes express iNOS in response to immune stimulation[32] and the NO generated has both autocrine and paracrine effects. Liver cells can express iNOS in response to a variety of stimuli including *Corynebacterium parvum* infection, endotoxin and certain cytokines. Within the liver, NO appears to modulate some fundamental intracellular functions such as protein synthesis, mitochondrial electron transport and components of the citric acid cycle.

NO IN THE CENTRAL NERVOUS SYSTEM

The brain possesses the richest capacity to generate NO. A common trigger for NO formation by neurons is activation of receptors for the major excitatory neurotransmitter, glutamate. Of particular significance is the NMDA class of glutamate receptor, whose associated ion channel permits substantial Ca^{2+} entry, but other mechanisms causing a rise in cytosolic Ca^{2+} in NOS-containing neurons, such as activation of AMPA-type glutamate receptors or metabotropic glutamate receptors (mGluRs) are also likely to be relevant. The main NO synthase isoform in the brain is nNOS although eNOS may be found in certain neurons. Glial cells, and possibly also neurons, can express iNOS after exposure to bacterial lipopolysaccharide and/or cytokines, at least when the cells are maintained in tissue culture.

In the CNS, NO generated by nNOS is unlike classical neurotransmitters in that it is relatively longlasting and behaves as a diffusible messenger. Because of the high rate of diffusion of NO in both lipid and aqueous environment, a point source is likely to influence neural function over a large tissue volume. A major physiological signal transduction pathway for NO in target structures is stimulation of the soluble form of guanylate cyclase leading to cyclic GMP (cGMP) accumulation.

NO has been implicated in a large number of physiological functions. At the cellular level, these include the acute modulation of neuronal firing behaviour as well as participation in several types of synaptic plasticity (i.e. a cellular basis of memory and learning). nNOS also appears to be important as a regulator of local cerebral blood flow, and behavioural experiments have suggested an involvement of NO in learning, pain, sleep, feeding and sexual function. Pathophysiologically, excessive NO generated by nNOS or iNOS has the capacity to destroy central neurons and is a suspected player in the acute loss of neurons taking place after focal or transient global ischaemia as well as in more chronic degenerative disorders such as Parkinson's disease, multiple sclerosis and motor neuron disease.

Responses to NOS blockade in mutant mice suggest that nNOS may also be involved in maintaining blood pressure. For example, mice lacking the eNOS gene are hypertensive but show a paradoxical fall in blood pressure in response to NOS inhibitors, suggesting that nNOS activity might contribute to increasing pressure. In addition NOS inhibitors cause a fall in cerebral blood flow in both normal mice and in eNOS knockout mice, indicating the possible importance of nNOS in mediating these changes in cerebrovascular reactivity[33].

Early work on the different stroke and cerebral ischaemia models with the NOS-knockout mice has shed some light on the possible role of NO or its reactant products in mediating toxicity in brain[33]. However, controversy continues—partly because of the use of non-selective agents that block NO formation in neuronal, glial, and vascular compartments. For example, in mutant mice deficient in neuronal NOS, infarct volumes at 24 and 72 h after middle cerebral artery occlusion, and the overall neurological deficits, were less than those in normal mice. This result could not be accounted for by differences in blood flow or vascular anatomy. However, infarct size in the mutant became larger after endothelial NOS inhibition by nitro-L-arginine administration. Hence, neuronal NO production appears to exacerbate acute ischaemic injury, whereas vascular NO

protects after middle cerebral artery occlusion. These studies demonstrate how disruption of genes by homologous recombination in mice often clarifies the roles of individual genes. However, they also emphasize that many such knock-out mice display little or no change in phenotype, suggesting a redundancy of physiological pathways.

THERAPEUTICS

The L-arginine:NO pathway is widespread and involved in a large number of physiological and pathophysiological processes. By analogy with other biological mediators with multiple functions (e.g. serotonin) it might be anticipated that discovery of the pathway will lead to many new therapies. Drugs that donate NO have been in clinical use for well over 100 years: NO is the active constituent of glyceryl trinitrate and is liberated from the parent drug within the body. The existing donors of NO exert their major effect in the cardiovascular system and preferentially dilate veins (possibly because the endothelium of veins does not generate NO basally and so the smooth muscle is more sensitive to small amounts of NO). The challenge now is to design drugs that differ substantially from existing nitrovasodilators and show specificity for certain tissues, e.g. platelets, nerves, gut, lung. The feasibility of tissue-selective targeting of NO is supported by the observation that certain nitrosothiols have potent antiplatelet effects at doses that do not cause significant vasodilatation. There is considerable interest in NO donors that might specifically target the uterus to relax uterine smooth muscle, inhibit uterine contractions and thereby prevent premature labour. A small open label study of glyceryl trinitrate patches in women with premature labour suggests that donation of NO might be beneficial in premature labour, but well-controlled studies are needed.

Inhibitors of NOS might also have therapeutic utility. Indeed the prototype NOS inhibitor L-NMMA is already in clinical trials for the treatment of septic shock, and the results of a multi-centre study are expected in the near future. No doubt selective inhibitors of individual isoforms of NOS are in development and these should allow clearer definition of the roles of each enzyme in disease states. There is considerable interest in the possibility that selective inhibitors of iNOS will have widespread anti-inflammatory effects (sepsis, asthma, gut injury, rheumatoid arthritis). However, although iNOS is readily expressed in rodent cells and tissues, this does not seem to be the case for human beings and the precise role of iNOS in inflammatory conditions and host defence in man remains to be determined.

NO research is now firmly in the clinical arena, and as new drugs are developed and enter clinical trial some putative roles for NO will fall by the wayside and other unexpected effects will be revealed. There is no sign yet that the rate of publications on NO has begun to reach a plateau.

Acknowledgments Main speakers at the meeting were:
Professor K E Anderson (Lund University, Sweden): *Peripheral Nervous System*
Professor P J Barnes (National Heart & Lung Institute, UK): *NO in Lung Physiology and Pathophysiology*
Dr T R Billiar (University of Pittsburg, USA): *Regulation of Inducible NO Synthase*
Professor J P Cooke (Stanford University, USA): *L-arginine, NO and Atherosclerosis*
Dr M Feelisch (Schwarz Pharma, Germany): *Donors of Nitrogen Oxides*
Professor R F Furchgott (SUNY Health Science Center, Brooklyn, USA): *Summary*
Professor J Garthwaite (University College, London, UK): *Central Nervous System: Overview*
Dr C Lees (King's College School of Medicine and Dentistry, UK): *NO in Obstetrics and Gynaecology*
Professor F Y Liew (University of Glasgow, UK): *Role of NO in Specific Immunity*
Professor M A Marletta (University of Michigan, USA): *NO Synthase and Guanylate Cyclase*
Professor T Michel (Brigham and Women's Hospital, Boston, USA): *NO and the Heart*
Professor S Moncada (University College London, UK): *NO in Evolution*
Professor A Moskowitz (Massachusetts General Hospital, Boston, USA): *Cerebral Blood Flow Regulation and Ischaemic Neurodegeneration*
Professor M Radomski (University of Alberta, Canada): *NO, Platelets and White Cells*
Professor P Vallance (University College London, UK): *Cardiovascular Overview*
Professor Sir John Vane (William Harvey Research Institute, London, UK)
Dr B Whittle (Beckenham, UK): *NO: Inflammation, Cytostasis and Cytotoxicity*

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