FUNCTIONAL AND STRUCTURAL ABNORMALITIES OF THE DERMAL MICROCIRCULATION IN HYPERTENSION AND HYPERCHOLESTEROLAEMIA

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DECLARATION

I declare that this thesis has been composed and written by myself alone. I have not presented the studies in this thesis in candidature for any other degree, diploma or qualification.

The study described in Chapter 2 was in collaboration with Dr Bill Haynes, University of Iowa, USA.

The study described in Chapter 3 was in collaboration with Dr Angela Shore, University of Exeter, UK, who was the 'second operator' in this study.

Signed:

Date: 27 November 1995

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ABSTRACT

Essential hypertension and hyperlipidaemia co-segregate in the population and act synergistically in increasing coronary heart disease. Using invasive procedures such as brachial artery infusion of drugs, functional abnormalities of the vascular endothelium have been observed in forearm resistance vessels in essential hypertension, and in hypercholesterolaemia. This 'endothelial dysfunction' has been attributed, at least in part, to impaired generation of nitric oxide. However, it is unclear whether this dysfunction is confined to nitric oxide in the forearm vascular bed, or whether other mediators and other microvessels are involved. Hypertension is characterised by increased peripheral vascular resistance, which may be due to functional or structural abnormalities in the microcirculation, particularly affecting pre-capillary vessels which make the greatest contribution to vascular resistance. However, from studies in established hypertension it is not possible to ascertain whether microvascular abnormalities are a cause or consequence of the rise in blood pressure (see below).

This thesis, therefore, aims (i) to examine the importance of nitric oxide in maintaining basal vascular tone and in the control of blood pressure, and to investigate the physiological role of nitric oxide in the human skin microcirculation; (ii) to establish the non-invasive technique of drug iontophoresis combined with laser Doppler fluximetry for assessing endothelial function in dermal microvessels in health, examining the principle mediators involved; (iii) to recruit patients with endothelial dysfunction, to determine whether microvascular abnormalities are present and measurable in the dermis using iontophoresis; (iv) to examine whether functional and/or structural abnormalities in the microcirculation are a cause or consequence of hypertension.

Inhibition of nitric oxide synthase with systemic administration of L-N^G-monomethyl-arginine (L-NMMA) increased blood pressure and substantially increased peripheral vascular resistance, emphasising the importance of nitric oxide in the maintenance of basal vascular tone. This led to an investigation of the role of nitric oxide at the microvascular level - the seat of greatest contribution to peripheral resistance. Using local brachial artery infusion of L-NMMA, tissue-specific differences in response suggested a selective role for nitric oxide in microvessels. To investigate further the role of nitric oxide and other mediators involved in microvascular function *in vivo*, the non-invasive technique of drug iontophoresis was used in combination with brachial artery infusion of L-NMMA and noradrenaline. Despite substantial reductions in forearm blood flow caused by these drugs, no differences were observed in dermal blood flow. However, aspirin, which blocks prostanoid synthesis via the cyclo-oxygenase pathway, inhibited the dermal response to acetylcholine (ACh). Thus, iontophoresis measured prostanoid-mediated cholinergic vasodilatation in human dermal vessels. With the pharmacological mechanisms of iontophoretic drug delivery emerging, the technique was extended to investigate endothelial function in 10 hypercholesterolaemic patients compared with 10 matched controls. In the patients, forearm vasodilatation was impaired with ACh but not with the endothelium-independent vasodilator, sodium nitroprusside (SNP). In contrast, dermal vasodilatation was impaired with SNP and not with ACh. In the presence of aspirin, in hypercholesterolaemic patients, forearm vasodilatation to ACh was partially restored, but dermal vasodilatation to ACh was impaired. Aspirin did not affect SNP responses. Therefore, impaired stimulated nitric oxide generation was confirmed in forearm resistance vessels in hypercholesterolaemic patients. In dermal microvessels in these patients, reduced smooth muscle sensitivity to nitric oxide was observed, and an enhanced, possibly compensatory, role for prostanoid vasodilators. To identify the likely importance of microvascular abnormalities in determining the familial risk of essential hypertension, subjects were recruited from an epidemiological model which identifies young people with contrasting predisposition to high blood pressure. In this model, subjects with a predisposition to high blood pressure which was familial were found to have structural rarefaction with a substantial increase in minimum vascular resistance in dermal microvessels. These structural abnormalities suggest that capillary rarefaction is a cause rather than a consequence of essential hypertension.

As hypertension and hypercholesterolaemia are cofactors which increase the risk of cardiovascular, cerebrovascular and renal diseases, an investigative method which would recognise individuals at risk would be a useful adjunct to their clinical management. Functional abnormalities are present in established essential hypertension, and in hypercholesterolaemia. The non-invasive technique of drug iontophoresis provides important new information about differences between dermal microvessels and forearm resistance vessels. However, further investigation is required if this technique is to become established in assessing endothelial function in large groups of patients.

Chapter 1

INTRODUCTION AND AIMS

1.1 BACKGROUND

Hypertension and hypercholesterolaemia, along with diabetes mellitus, obesity, smoking and left ventricular hypertrophy, are major risk factors associated with an increased risk of atherosclerotic vascular disease. Alterations in the function and structure of blood vessels, including impaired function of the vascular endothelium, are present in both essential hypertension and hypercholesterolaemia. In hypertension, functional abnormalities of blood vessels also include reduced capillary blood flow under resting physiological conditions (Duprez et al, 1992), and after vasodilatation produced by heating (Williams & Tooke, 1992). Structural abnormalities include reduced luminal diameter in arterioles (Folkow, 1990; Heagerty et al, 1993) and in capillaries (Lack et al, 1949; Landau & Davis, 1957; Harper et al, 1978). These abnormalities cause an increase in peripheral vascular resistance which characterises the disease, but it is unclear whether they are a cause or consequence of the rise in pressure. This thesis investigates methods which detect mechanisms of endothelial function in health, and of function and structure in hypertension and hypercholesterolaemia. An understanding of such mechanisms may target more appropriate intervention which would impede the complications of of these conditions.

1.2 VASCULAR FUNCTION

Functional abnormalities in essential hypertension may be explained by structural defects, as has been observed in studies showing reduced blood flow in vessels maximally dilated by physical stimuli such as heating (Williams et al, 1992). Blood velocity in finger nailfold capillaries has been measured using the videomicroscopy technique, and shown to be reduced (Duprez et al, 1992) or normal (Gasser & Bühler, 1992) in essential hypertension. Despite the findings of raised capillary pressures

(Williams et al, 1990) and normal venous pressures (Widgren et al, 1992) in essential hypertension suggesting that capillary or post-capillary vascular resistance is increased, it remains uncertain whether changes in larger arterioles or whether changes in capillaries are more relevant in human hypertension (Bohlen, 1989). Dysfuncton of the blood vessels, affecting the vascular endothelium, has been attributed to impaired nitric oxide generation, and has also been observed in hypertension (Calver et al, 1992; Panza et al, 1990 &1993) and hypercholesterolaemia (Chowienczyk et al, 1992; Casino et al, 1993).

1.2.1 Function of the vascular endothelium

The vascular endothelium forms the inner lining of all blood vessels, playing a major role in the regulation of vascular function rather than acting simply as a barrier between blood and vascular smooth muscle. It has become clear over the past 20 years that the endothelium produces a number of important relaxing and contracting factors. In 1980, Furchgott and Zawadski demonstrated that acetylcholine causes endothelium-dependent vasodilatation of rabbit aortic rings, via the release of a labile humoral factor, later known as endothelium-derived relaxing factor (EDRF) (Cherry et al, 1982). The humoral nature of EDRF was demonstrated in experiments in which it was released by endothelium-intact rabbit aortic strips and shown to cause the relaxation of adjacent endothelium-denuded vascular preparations (Furchgott, 1984; Griffith et al, 1984; Rubanyi et al, 1985). A variety of stimuli, such as adenine nucleotides, bradykinin (BK), the calcium ionophore, A23187, substance P, thrombin, hypoxia, increased flow, and electrical stimulation also induced endothelium-dependent relaxation of vascular tissue *in vitro,* through the release of EDRF (Furchgott, 1983). EDRF was shown to be released not only after stimulation by various agonists, but also under basal conditions (Griffith et al, 1984; Rubanyi et al, 1985; Martin et al, 1985).

The effects of EDRF on vascular smooth muscle and platelets are mediated through the action of soluble guanylate cyclase, leading to an increase in cyclic guanosine monophosphate (cGMP) (Ignarro et al, 1985; Rapoport et al, 1983; Waldman & Murad, 1987). Vascular relaxation to EDRF was inhibited by haemoglobin and methylene blue (Martin et al, 1985), and by dithiothreitol and hydroquinone (Griffith et al, 1984). Furthermore, the effects of EDRF were prolonged by superoxide dismutase (Gryglewski et al, 1986a; Rubanyi & Vanhoutte, 1986) and inhibited by Fe^{2+} (Gryglewski et al, 1986a). Superoxide anions were, therefore, suggested to contribute to the instability of EDRF, not only because superoxide dismutase prolonged the effects of EDRF, but also because inhibitors such as dithiothreitol, hydroquinone and pyrogallol were shown to generate superoxide anions as a result of their redox properties. In addition, cytochrome c, which removes superoxide anions, attenuated the action of redox compounds, with pyrogallol (a generator of superoxide anions) acting as an inhibitor of EDRF (Moncada et al, 1986).

Independently in 1986, Furchgott, and Ignarro and colleagues, suggested that EDRF might be nitric oxide, since both EDRF and authentic nitric oxide (generated by acidified nitrite; NO_2^-) activated guanylate cyclase, increased cGMP and caused smooth muscle relaxation. Both EDRF and nitric oxide were inhibited by haemoglobin, and by agents generating superoxide anions. Furthermore, the soluble guanylate cyclase inhibitor, methylene blue, antagonised arterial relaxations to nitric oxide, whereas inhibitors of cGMP-phosphodiesterase enhanced the response and caused an accumulation of cGMP (Ignarro et al, 1988).

In 1987, Palmer, Ferrige and Moncada demonstrated that EDRF was nitric oxide. Strips of endothelium-denuded rabbit aorta were perfused with effluent from porcine aortic endothelial cells in culture. Stimulation of the cultured endothelial cells resulted in release of EDRF and the relaxation of the bioassay tissues, a response that decayed

with time during passage down the cascade, and which was inhibited by haemoglobin and superoxide. When the generation of nitric oxide from cultured porcine vascular endothelial cells was stimulated by bradykinin, levels were measured directly using a chemiluminescent method. The resulting concentration-dependent release of nitric oxide accounted for the vasodilatation of endothelium-denuded rabbit aortic strips (Palmer et al, 1987), and the inhibition of platelet aggregation and adhesion (Radomski et al, 1987).

The mechanism whereby nitric oxide stimulates guanylate cyclase is unclear, but it has been proposed that nitric oxide reacts with the haem moiety, resulting in the formation of a nitrosyl-haem complex (Ignarro et al, 1988). The generation of this complex alters the configuration of the enzyme, converting it to its active form. The rise in cGMP then leads to the activation of cGMP-dependent protein kinases and smooth muscle relaxation by cytosolic calcium binding to calmodulin, resulting in the phosphorylation of myosin light chains, relaxation of myosin cross-link bridges, and smooth muscle cell relaxation (Rapoport et al, 1983).

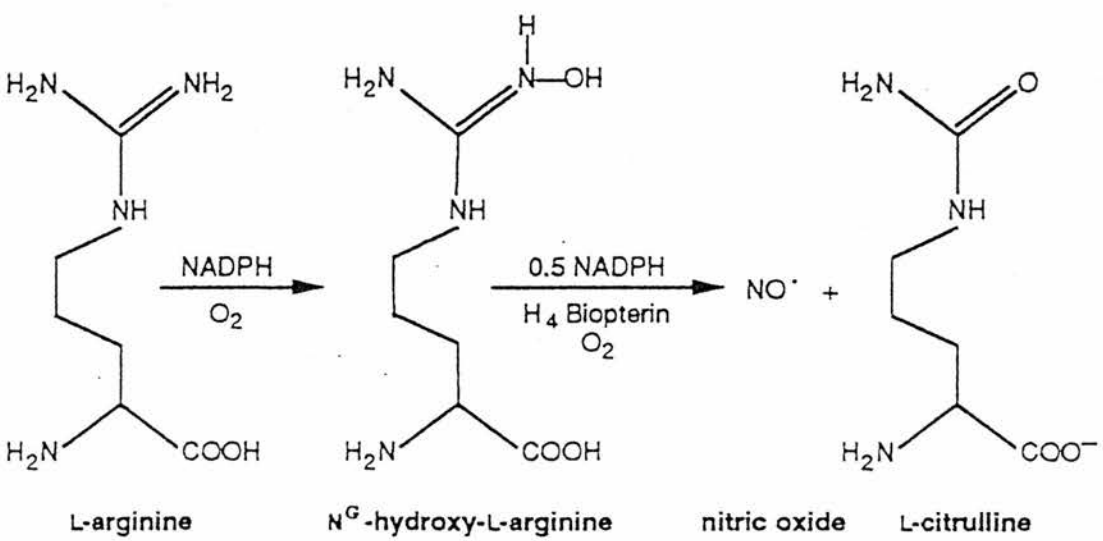
Shikano and colleagues (1988) have questioned whether EDRF is nitric oxide, demonstrating that nitric oxide, but not EDRF, relaxes non-vascular smooth muscle. However, other workers have shown that both EDRF, released from cultured endothelial cells or from rabbit aortic strips, and nitric oxide were equally effective in relaxing non-vascular smooth muscle (Gillespie & Sheng, 1988; Angus & Cocks, 1989). Shikano and colleagues (1988) also suggested that EDRF, but not nitric oxide, binds to anion exchange columns. In contrast, Furchgott and colleagues (1990) demonstrated that anion exchange resins inhibit the relaxing activity of authentic nitric oxide as well as EDRF from perfused rabbit aorta.

Myers and colleagues (1990) suggested that EDRF more closely resembled S-nitrocysteine (a nitrosothiol compound) which contains nitric oxide, than nitric oxide itself, since the amount of nitric oxide released from cultured endothelial cells under basal conditions or after stimulation by BK or the calcium ionophore A23187 (measured by the chemiluminescence method) was insufficient to account for the relaxing activity of EDRF. It is possible that there were discrepancies in the measurement of nitric oxide due to contamination with NO_2^-, which often occurs during preparation (Furchgott et al, 1990). The chemiluminescence method measures both nitric oxide and NO_2^-, and may explain why, for biologically equipotent solutions of EDRF and nitric oxide, the EDRF solution has been reported to have a lower concentration of nitric oxide (Rubanyi et al, 1990). Rubanyi and colleagues (1990) also found that, when equipotent solutions of EDRF and nitric oxide were passed through a reduced haemoglobin column, only nitric oxide gave the typical nitrosohaem electron paramagnetic resonance signal. However, NO_2^- can also react with haemoglobin to form paramagnetic nitrosyl-haemoglobin, thus giving the impression that the concentration of nitric oxide is greater than is actually the case.

Nitrosothiols are, however, unstable in a neutral aqueous environment and decompose rapidly, making it more likely that if EDRF was not nitric oxide, it would be a more complex compound, such as a nitrosyl non-haem iron complex with thiol-containing ligands (Vanin, 1991). Such complexes move within and between cells, and are readily oxidised to produce nitric oxide and iron, accounting for their vasodilator and platelet anti-aggregatory effects (Vanin, 1991).

In 1988, Palmer and colleagues demonstrated that L-arginine was the precursor for the synthesis of nitric oxide by vascular endothelial cells, since endothelial cells cultured in the absence of L-arginine for 24 h showed a decrease in the release of EDRF induced by bradykinin and A23187, an effect which could be restored by L- but not D-arginine

(Palmer et al, 1988). These findings also suggested the involvement of an enzyme (nitric oxide synthase) in the formation of nitric oxide. Using mass spectrometry, nitric oxide was shown to be formed from the terminal guanidino nitrogen atom(s) of L-arginine when cells were stimulated with bradykinin. N^{ω}Hydroxy-L-arginine is generated as an intermediate in the biosynthesis of nitric oxide and is coupled to the oxidation of 1 mol of nicotinamide adenine dinucleotide phosphate (NADP; or the reduced form: NADPH) by nitric oxide synthase (NOS). A further reaction converts the intermediate into nitric oxide and citrulline, in conjunction with the oxidation of 0.5 mol of NADPH and tetrahydrobiopterin (Fig. 1.1; Marletta, 1989; Stuehr et al, 1991). The biosynthesis of nitric oxide from L-arginine by NOS results in the formation of citrulline as a coproduct (Palmer et al, 1989) and the incorporation of molecular oxygen into both nitric oxide and citrulline (Leone et al, 1991).



(Marletta, 1989)

FIGURE 1.1:

Biosynthesis of nitric oxide and L-citrulline from L-arginine via the intermediate N^{ω}hydroxy-L-arginine.

1.2.2 Nitric oxide synthases

The L-arginine:nitric oxide pathway was, thus, established and two differentially regulated NOS isoforms were classified. Constitutive NOS was found to be calcium/calmodulin-dependent (Büsse & Mülsch, 1990; Mayer et al, 1990), and inducible NOS calcium/calmodulin-independent (Hauschildt et al, 1990), with both isoforms being NADPH- and tetrahydrobiopterin-dependent (Marletta et al, 1988; Kwon et al, 1989; Palmer & Moncada, 1989). However, the recent identification of a calmodulin binding sequence in mouse macrophage-inducible NOS (Cho et al, 1992), which tightly binds calmodulin, questioned the validity of this classification, and suggested that there might be three isoforms of NOS: two constitutive enzymes (one expressed in endothelium, the other in brain) and an inducible enzyme synthesised in macrophages.

This classification has been further updated, and NOS enzymes (now termed inducible, constitutive and neuronal) have been isolated from a range of tissues and species. Neuronal nitric oxide synthase (nNOS) generates nitric oxide which is a neurotransmitter and neuromodulator, released from non-adrenergic, non-cholinergic (NANC) nerves, and may be involved in nociception, penile erection and bladder sphincter control (Knowles & Moncada, 1994). Activation of constitutive nitric oxide synthase (cNOS) is calcium/calmodulin-dependent. Endothelial cNOS is sometimes referred to as eNOS. Activation of mouse macrophage NOS is not controlled by changes in intracellular calcium, since this form binds calmodulin tightly at basal intracellular levels of calcium (Cho, 1992). Induction of new protein is required to synthesise this NOS, hence it is called inducible (iNOS) (Xie et al, 1992). Recently, inducible NOS isolated from macrophages was shown to contain a haemoprotein which is directly involved in the oxidative conversion of L-arginine to nitric oxide and citrulline (White & Marletta, 1992).

In addition to its role in regulating vascular tone, nitric oxide inhibits platelet aggregation and adhesion (Radomski et al, 1987), and has a role in peripheral and central neurotransmission (Gillespie et al, 1989; Knowles et al, 1989). Immunohistochemical studies have localised the constitutive NOS in discrete neuronal populations within the brain (where nitric oxide mediates the actions of the excitory neurotransmitter, glutamate) and in cell bodies and nerve fibres in the mesenteric plexus of the intestine (where nitric oxide, released after neuronal stimulation, leads to relaxation of the intestine) (Bredt et al, 1990 & 1991). The synthesis of nitric oxide by this enzyme is rapid, short-lasting and not dependent on exogenous L-arginine.

Nitric oxide released from inducible NOS in macrophages contributes to their cytotoxic/cytotactic actions against tumour cells, bacteria and protozoa (Hibbs et al, 1990), whereas nitric oxide released after immunological stimulation of vascular endothelial cells contributes to pathological vasodilatation (Kilbourne et al, 1990; Rees et al, 1990a; Thiemermann & Vane, 1990) and tissue damage (Hutcheson et al, 1990; Palmer et al, 1992).

Following recent advances in molecular biology, the three isoforms of NOS have been cloned and sequenced: the constitutive enzyme from the rat (Bredt et al 1991) and bovine (Lamas et al, 1992) cerebellum, and rat (Sessa et al, 1992), bovine (Lamas et al, 1992) and human (Marsden et al, 1992) endothelium; and the inducible enzyme from the mouse macrophage (Xie et al, 1992). The three NOS isoforms show little sequence homology. The constitutive enzymes show only 60% homology (Lamas et al, 1992; Marsden et al, 1992) and are encoded by distinct genes (Lamas et al, 1992). The inducible isoform shows only 50-60% homology with the constitutive enzymes (Lamas et al, 1992; Sessa et al, 1992; Xie et al, 1992).

1.2.3 Nitric oxide in the regulation of vascular tone

In vitro

The release of nitric oxide from endothelial cells in culture (Palmer et al, 1988), the synthesis of citrulline by endothelial cell homogenates (Palmer & Moncada, 1989), and the endothelium-dependent relaxation induced by acetylcholine (Palmer et al, 1988) are inhibited by the L-arginine analogue and NOS inhibitor, L-N^G-monomethyl-arginine (L-NMMA). This inhibitor causes a significant endothelium-dependent contraction of rabbit and rat aortic rings (Palmer et al, 1988; Rees et al, 1990a). Both of these effects are reversible by L-, but not by D-arginine (Rees et al, 1989). These studies indicated that there is a continuous basal release of nitric oxide which is involved in maintenance of blood vessel tone, and that acetylcholine stimulates the synthesis of nitric oxide from L-arginine resulting in vasodilatation (Palmer et al, 1988).

There is evidence suggesting that basal and stimulated release of nitric oxide may be differentially modulated by changes in the concentration of extracellular calcium (Lopez-Jaramillo et al, 1990). Although, in the vascular endothelium, both mechanisms of nitric oxide release are calcium/calmodulin-dependent (Büsse & Mülsch, 1990), the release of nitric oxide by acetylcholine occurs over a wider range of calcium concentrations (0.5-2.0 mM), compared with those at which basal release occurs (0.75-1.50 mM) (Lopez-Jaramillo et al, 1990). There are several possible explanations for the differential requirements for calcium. First, there may be two different mechanisms by which calcium enters the cell to stimulate the synthesis of nitric oxide. For example, basal release may occur as a consequence of the opening of stretch-activated calcium channels by pulsatile flow (shear stress), with release by acetylcholine occurring through receptor-mediated calcium channels. Second, acetylcholine may utilise a greater amount of intracellular calcium.

In vivo

L-N^G-monomethyl-arginine causes a significant rise in blood pressure in anaesthetised rabbits (Rees et al, 1989), rats (Whittle et al, 1989; Tolins & Raij, 1990), and guinea pigs (Aisaka et al, 1989). This rise in pressure, accompanied by a decrease in renal, mesenteric, carotid and hindquarter vascular conductances in the conscious rat (Gardiner et al, 1990a), suggests a widespread role for nitric oxide in maintaining vascular tone throughout the cardiovascular system. This is supported further by the work of Vallance and colleagues (1989) in which brachial artery infusion of L-NMMA causes vasoconstriction, indicating that basal generation of nitric oxide maintains vascular tone by opposing vasoconstrictor mechanisms.

In anaesthetised rats, acetylcholine causes a fall in mean arterial blood pressure (Rees et al, 1990b) which is accompanied, in the conscious animal, by a hyperaemic vasodilatation in the renal vascular bed (Gardiner et al, 1990b). The hypotensive response to acetylcholine in anaesthetised rats is either partly (Rees et al, 1990b) or almost completely (Whittle et al, 1989) inhibited by L-NMMA. In man, the vasodilatation to acetylcholine in the dorsal hand vein and brachial artery is also partly inhibited by L-NMMA (Collier & Vallance, 1989; Vallance et al, 1989a & 1989b). The reason for the incomplete inhibition of the acetylcholine response is not clear, although a clue comes from work by Aisaka and colleagues (1989) in which they were unable to inhibit the acetylcholine-induced fall in blood pressure in anaesthetised guinea pigs, but were able to reduce the duration of hypotension. They suggested that the acetylcholine depressor response may have two phases, an initial phase in which the fall in blood pressure does not require increased synthesis of nitric oxide, and a succeeding phase in which longer-lasting dilatation can be inhibited by L-arginine analogues.

Invasive, intra-arterial infusion studies have shown that, in the forearm, cholinergic dilatation is mediated principally by nitric oxide, since the response is inhibited by the

nitric oxide synthase inhibitor, L-NMMA (Vallance et al, 1989), and reversed by L-arginine (Panza et al, 1993a). However, the relative contribution of the mediators of endothelium-dependent vasodilation varies between tissues. Thus, in renal arteries, prostacyclin is the principal mediator, accounting for the constrictor effect of cyclooxygenase inhibitors in this vascular bed (Breierwates et al, 1982; Ito et al, 1989). Vasodilatation by acetylcholine could also be due to presynaptic inhibition of noradrenaline release, but this is unlikely because the response is not affected by the non-selective α-blocker, phentolamine (Panza et al, 1990; Linder et al, 1990). Furthermore, vasodilatation is not inhibited by the cyclooxygenase inhibitor, aspirin (Linder et al, 1990). Another possibility is that the inhibition of acetylcholine-induced vasodilatation by L-arginine analogues is reduced under conditions of highly effective receptor-coupling (Giles et al, 1990), suggesting that the lack of inhibition by these analogues is not necessarily evidence for the lack of involvement of nitric oxide in endothelium-dependent relaxation responses. In addition, muscarinic receptor subtypes initiating vascular relaxation to acetylcholine *in vitro* are complex (Rubanyi et al, 1986c), and differential inhibition of different agonists by L-NMMA could result from actions on receptor subtypes linked with more than one effector mechanism, not all of which involve the L-arginine:nitric oxide pathway (Chowienczyk et al, 1993). Furthermore, vascular relaxations may be mediated through an endothelium-derived hyperpolarising factor (EDHF) (Feletou & Vanhoutte, 1988).

1.2.4 Inhibitors of nitric oxide synthesis

With the advent of other L-arginine analogues as inhibitors of NOS (e.g., L-N^G-nitro-arginine methyl ester (L-NAME) and L-N-iminoethyl-ornithine (L-NIO)), there is growing evidence suggesting that basal and stimulated NOS may be differentially regulated. This may explain the findings of Lopez-Jaramillo and colleagues (1990; see earlier) that changes in the concentration of extracellular calcium have a fundamental effect on stimulated and basal release of nitric oxide. The three

analogues, L-NMMA, L-NAME and L-NIO, have different potencies in inhibiting basal and stimulated nitric oxide synthesis in isolated rings of rat aorta, and in anaesthetised rats (Rees et al, 1990b), as well as in isolated perfused rabbit hearts (Smith et al, 1992). These inhibitors of NOS are discussed in the next chapter (Chapter 2), where the rationale is given for using L-NMMA as the inhibitor of choice throughout the studies described in this thesis.

Arachidonic acid, or one of its metabolites, may have a role in the differential regulation of basal and stimulated nitric oxide synthesis (Crack & Cocks, 1992). Thimerosal (an inhibitor of acetyl-CoA acetyltransferase) fully relaxed endothelium-intact rings of canine coronary artery. However, after the response to thimerosal had faded, these tissues no longer responded to acetylcholine, bradykinin or substance P, although they did respond to the nitric oxide donor, sodium nitroprusside. Endothelium-dependent contractions to L-NMMA and L-NAME were unaffected. Furthermore, L-NMMA and L-NAME blocked the relaxation to thimerosal. Since thimerosal induces the mobilisation of arachidonic acid, these authors suggested that arachidonic acid, or one of its non-cyclooxygenase metabolites, may play a role in the regulation of stimulated nitric oxide synthase, and that basal and stimulated release may be differentially regulated.

1.2.5 Vasoactive prostanoids

The endothelium produces other vasodilators, including prostacyclin (Moncada et al, 1991) and the putative hyperpolarising factor (Feletou & Vanhoutte, 1988; Kilpatrick & Cocks, 1994; Garland et al, 1995) (Figure 1.2). Stimulated endothelial cells (and circulating platelets) release free arachidonic acid from their membrane phospholipid pools and rapidly oxidise it to form the labile prostaglandin (PG) endoperoxides PGG_2 and PGH_2, via the cyclooxygenase enzyme which can be inhibited by aspirin (Flower, 1974; Schafer et al, 1984; Barrow et al, 1988). Platelets also oxidise arachidonic acid

to form hydroperoxy- and hydroxy-fatty acids via the lipoxygenase pathway which is not blocked by aspirin. The endoperoxides of endothelial cells and platelets serve as substrates for the enzymatic formation of two labile eicosanoids of potent but opposing biological effects, prostacyclin (PGI_2) and thromboxane A_2 (TXA_2). Endothelial PGI_2 synthase converts PGH_2 to PGI_2, which increases levels of cAMP in vascular smooth muscle to cause vasodilatation (Gorman et al, 1977).

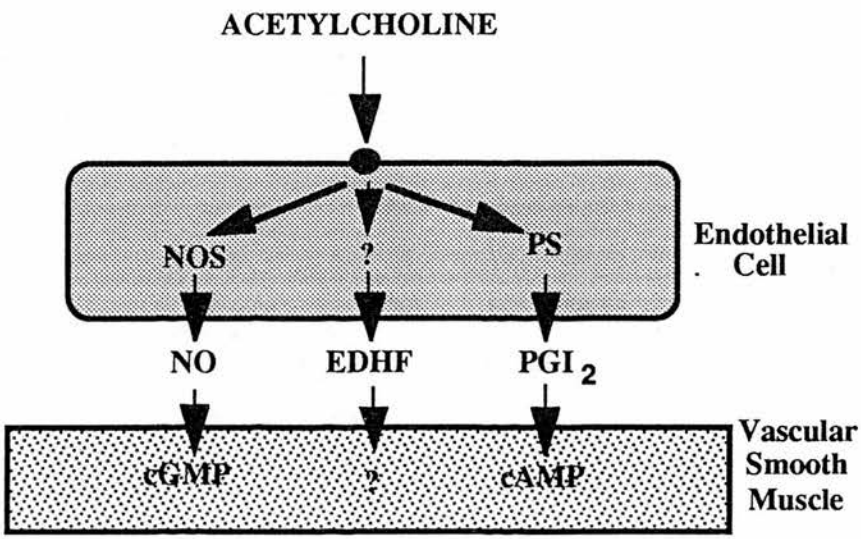


FIGURE 1.2:

Schematic diagram of acetylcholine stimulating an endothelial cell to generate nitric oxide (NO) through the action of nitric oxide synthase (NOS), and prostacyclin (PGI_2) through the action of prostacyclin synthase (PS), to cause relaxation of vascular smooth muscle by increasing cGMP and cAMP respectively. Endothelium-derived hyperpolarising factor (EDHF) may also be released.

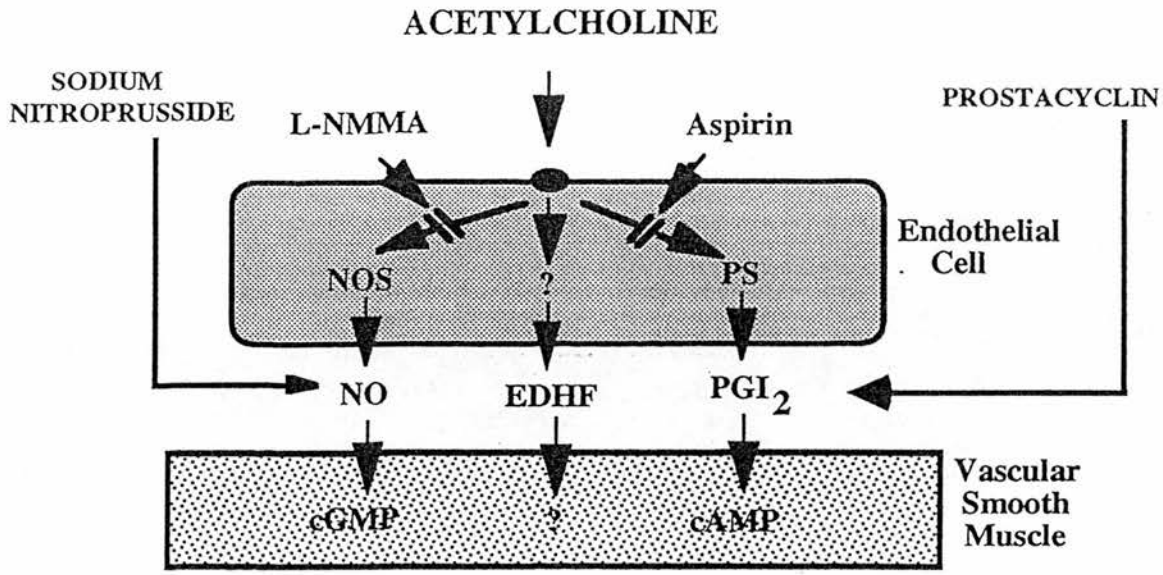


FIGURE 1.3:

Inhibition of endothelial generation of relaxing factors by the nitric oxide synthase inhibitor, L-N^G-monomethyl-arginine (L-NMMA) and the cyclooxygenase inhibitor, aspirin. Nitric oxide can be donated directly to the smooth muscle by nitrovasodilators such as sodium nitroprusside. Exogenous prostacyclin can also be donated. (Abbreviations as Fig. 1.2.)

Prostanoids may act synergistically with nitric oxide in some preparations, since vasodilator prostanoids augment endothelium-dependent relaxations (Shimokawa et al, 1988). However, inhibition of prostanoid generation by indomethacin does not influence the synthesis or action of nitric oxide released by endothelial cells (Gryglewski et al, 1986b), or the relaxation of rabbit mesenteric artery strips and arterioles of the hamster cheek pouch (Moncada et al, 1988; Rees et al, 1990c). Since prostacyclin and nitric oxide exert anti-atherosclerotic and anti-thrombotic effects (Vane

& Botting, 1994), these may have important implications in pathological conditions such as atherosclerosis.

Prostacyclin is a powerful inhibitor of the release of mitogens from platelets, endothelial cells and macrophages (Willis et al, 1987) and, thus, when formed by endothelial cells will suppress smooth muscle proliferation in atherosclerotic plaques. Prostacyclin inhibits accumulation of cholesterol in macrophages (Willis et al, 1986) and in vascular smooth muscle cells (Hajjar, 1985), and should, therefore, suppress the formation of foam cells which are involved in the early stages of atherosclerotic plaque formation (Chapter 5). This last effect is reinforced by the actions of prostaglandin (PGE_2), and high density lipoprotein (HDL). PGE_2 inhibits acetyl-CoA/cholesterol O-acetyltransferase (see above), and prostacyclin (PGI_2) stimulates acid cholesteryl ester hydrolase, whereas HDL serves as a cholesterol carrier (Hajjar, 1985). It may be, therefore, that the PGI_2 and PGE_2 generating system is responsible for clearing cholesteryl esters from the vascular wall, whereas inhibition of cyclooxygenase or prostacyclin synthase by lipid hydroperoxides (Lands, 1985) brings about an accumulation of cholesteryl esters in the vascular wall, formation of foam cells and atherogenesis.

This may be the major role for prostacyclin generated by endothelial cells. Blood pressure is not altered by administration of aspirin or indomethacin (Vane & Botting, 1994) which prevent both vasodilator and vasoconstrictor prostanoid production, thus, it is unlikely that prostacyclin is contributing to the maintenance of blood vessel tone in health. The role of prostacyclin in atherosclerotic vascular disease is discussed further in Chapter 5.

1.2.6 Constricting factors produced by the endothelium

The endothelium also produces vasoconstrictors, such as angiotensin II (Webb & Cockcroft, 1990) and endothelin-1 (ET-1). ET-1 is a 21-amino acid peptide isolated by Yanagisawa and colleagues (1988) from porcine aortic endothelial cells, and the most potent endogenous vasoconstrictor yet identified. ET-1 is the predominant peptide, but two other isoforms, ET-2 and ET-3, have also been isolated. ET-1 binds to at least two distinct receptor subtypes: ET_A which appears to be the major receptor mediating vasoconstriction in arteries; and ET_B which mediates release of endothelium-dependent vasodilating factors and is also present in some resistance and capacitance vessels where it contributes to vasoconstriction. ET-1 contributes to the maintenance of basal vascular tone (Haynes & Webb, 1994), which may be altered in diseases such as hypertension, heart failure, renal failure and Raynaud's disease (Haynes & Webb, 1993). There may be a balance, therefore, between endothelium-derived relaxing and contracting factors which is perturbed in some diseases. The role of the endothelins is not addressed in this thesis.

1.2.7 Dysfunction of the vascular endothelium

In the forearm of hypertensive humans, basal nitric oxide production is reduced since L-NMMA causes diminished vasoconstriction (Calver et al, 1992: Panza et al, 1993a) suggesting reduced basal nitric oxide production. Cholinergic dilatation and vasodilatation to an alternative stimulus to nitric oxide synthesis (substance P) may also be impaired (Panza et al, 1994). Both L-NMMA and L-arginine have no effect on cholinergic dilatation (Panza et al, 1993a and 1993b). In addition, some studies have shown that patients with essential hypertension also have impaired vasodilatation in coronary vessels following intracoronary infusion of acetylcholine (Treasure et al, 1993), and impaired cholinergic dilatation in excised gluteal resistance vessels (Falloon et al, 1992). In contrast, the findings of Cockcroft et al, (1994) suggest that impairment in stimulated vasodilatation in the forearm vasculature is not universal in

patients with essential hypertension. It may be that endothelial dysfunction causes selective impairment to acetylcholine in a subgroup of hypertensive patients, not represented in the latter study.

Indomethacin normalises impaired cholinergic dilatation in the forearm bed of hypertensive patients (Taddei et al, 1993). This normalisation of the acetylcholine response following cyclooxygenase inhibition has also been seen in spontaneously hypertensive rats (Iwama et al, 1992), and in stroke-prone spontaneously hypertensive rats (Diederich et al, 1990), suggesting that the release of relaxing factors is normal, but that the concomitant, possibly enhanced, release of a cyclooxygenase-dependent endothelium-derived contracting factor (probably prostaglandin H_2) (Taddei et al, 1993) accounts for the impaired relaxation. Further, defects in the metabolism of the contracting prostanoid, PGH_2 could cause an upregulation of its synthesis, which could inhibit prostacyclin synthase (Lin, 1994).

Patients with hypercholesterolaemia also have impaired basal (Casino et al, 1993) and stimulated (Chowienczyk et al, 1992; Casino et al, 1993) release of nitric oxide in the forearm vascular bed, and in the coronary circulation (Drexler & Zeiher, 1991). Hypercholesterolaemia leads to atherosclerosis, a process which is accelerated if hypertension is present. Thus, the capacity of the endothelium to maintain adequate tone and to prevent thrombus formation in hypercholesterolaemic patients may have important implications for atherogenesis. This is discussed in detail in Chapter 5.

It is unclear whether impaired nitric oxide generation in hypertension and hypercholesterolaemia is confined to skeletal muscle resistance vessels and coronary vessels, and whether the defect is specific to nitric oxide generation or whether, in humans, the impaired function also affects other mediators such as prostacyclin (see Chapter 5). Moreover, it seems that caution should be taken in assessing endothelial

dysfunction using the acetylcholine test, as some patients with hypertension (previously treated or untreated) have no difference in cholinergic vasodilatation in the forearm compared with normotensive controls (Cockcroft et al, 1994). Alternatively, the invasive methodology of forearm perfusion studies has not allowed sufficient numbers of patients to be examined to establish the epidemiology of endothelial dysfunction.

Central to this thesis, therefore, is the development of a simple, non-invasive technique for investigating endothelial function both in health and in diseases such as essential hypertension and hyperlipidaemia. The technique of transdermal delivery of drugs by iontophoresis combined with laser Doppler monitoring of blood flow (Chapters 4-6), will be used, not only to assess function, but also to explain the underlying pharmacological mechanisms of cholinergic vasodilatation in dermal microvessels.

1.3 VASCULAR STRUCTURE

1.3.1 Hypertrophy

In addition to functional abnormalities, defects in the structure of blood vessels may also explain abnormalities in hypertension. Resistance to blood flow can be altered by structural abnormalities of the vasculature, and in a single, straight blood vessel, resistance can be approximated using Poiseuille's equation, $R = 8lh/\pi r^4$, where R = resistance, l=the length of the vessel, h = blood viscosity, and r = the vessel radius, i.e.,

$$\text{Resistance} \propto \frac{\text{length of vessel x blood viscosity}}{(\text{vessel radius})^4}$$

Total resistance in a tissue, however, is influenced by many other structural, functional and rheological factors. The luminal sizes of resistance arteries brought to a state of complete relaxation are determined by their structural characteristics and distending pressure. In hypertensive patients, blood flow is reduced in vessels which are maximally dilated, thus, minimum resistance to flow is increased (Folkow et al, 1958 and 1990; Pedrinelli et al, 1990). These investigators induced maximum dilatation in forearm blood vessels in humans using physical or pharmacological means, and measured resulting blood flow plethysmographically, thus, providing a haemodynamic index for quantifying arteriolar structural changes in hypertension. To account for this combination of reduced flow and higher resistance, Folkow (1958) postulated the presence of structural abnormalities including a reduction in blood vessel luminal size together with an increase in wall thickness (i.e., increased vessel wall:lumen ratio). This theory for structural abnormalities has been the predominant explanation since Folkow's initial experiments. However, it may be possible that the increase in resistance could be due to a reduced number of vessels (rarefaction; see below), rather than increased wall:lumen ratio, and this will be investigated in Chapter 6.

It was previously thought that the increase in wall:lumen ratio was the result of growth of the media into the vessel lumen. Recently, however, Heagerty and colleagues (1993) have reported that, in patients with essential hypertension, myocyte number is normal, with no increase in growth rate. This may be explained by the 'remodelling' theory, i.e., a process of medial cells rearranged around a narrowed lumen (Baumbach & Heistad, 1989) (Fig. 1.4).

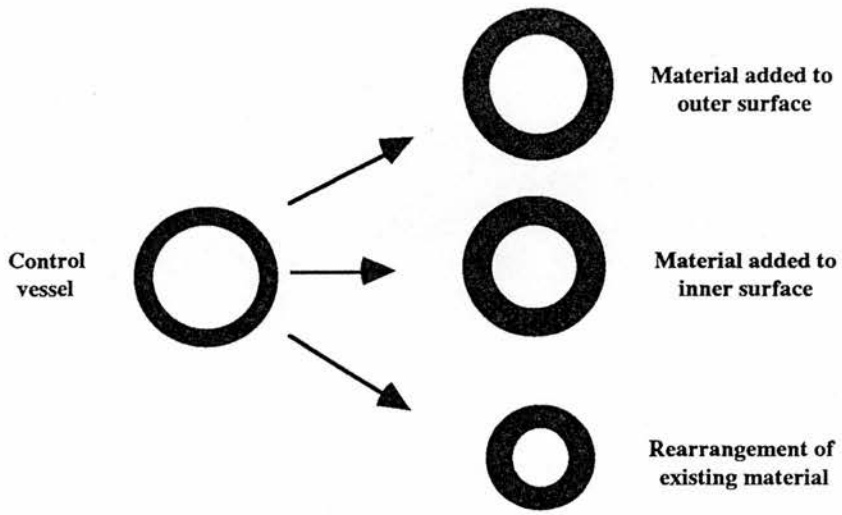


FIGURE 1.4:

Schematic, annotated drawing showing different modes of structural change of arteries. The lower artery shows remodelling as described by Baumbach & Heistad (1989), with consequent reduction in luminal diameter.

With sustained hypertension, vascular hypertrophy is correlated with left ventricular hypertrophy (Casale et al, 1986; Agabiti-Rosei et al, 1988 & 1991). Paradoxically, left ventricular hypertrophy correlates poorly with mean arterial pressure (Tarazi et al, 1982; Hartford et al, 1983) and is sometimes present before pressure starts to rise (Tarazi, 1985). Alternative mechanisms contributing to left ventricular hypertrophy include increased cardiac sympathetic nerve stimulation and the trophic effects of circulating catecholamines and angiotensin II (Hartford et al, 1983). Therefore, sympathetic nerves and circulating mediators may regulate growth and contraction of

the heart muscle (Simson, 1985) causing ventricular hypertrophy, as well as of blood vessel smooth muscle (Hart et al, 1980; Folkow, 1983) causing vascular hypertrophy.

Blood vessel compliance is also reduced in hypertension (Safar et al, 1992), and could be due either to structural changes in smooth muscle layer thickness or to alterations in function. If smooth muscle thickening causes encroachment into the lumen and the walls are distensible, it may be possible to compensate for reduced luminal diameter by increased stretching. However, in the common carotid artery of the anaesthetised rat *in vivo,* Levy and colleagues (1988 and 1990) observed that for any given pressure, luminal volume was significantly reduced in hypertensive rats indicating stiffer arterial walls. Following the application of potassium cyanide to poison the arterial smooth muscle, compliance increased significantly both in hypertensive and in normotensive rats, indicating the influence of vasomotor tone. However, compliance remained substantially lower in hypertensive rats, suggesting that structural factors were predominant in maintaining compliance.

As the microcirculation is area of the cardiovascular system making the greatest contribution to peripheral vascular resistance, alterations in microvascular structure and function effect such resistance. However, these alterations may or may not occur predominately in the microcirculation. Moreover, vessels affected by atherosclerosis are larger, and are important by their contribution to myocardial infarction and stroke. Microvessels with a luminal diameter of less than 150 μm have been classed as true resistance vessels (Prewitt & Wang, 1991; Struijker Boudier et al, 1992). However, it is often unclear from the literature whether or not reference to 'resistance vessels' includes these small microvessels. Schiffrin (1992) divides the arterial side of the circulation into two groups of vessels, depending on their internal (luminal) diameter, i.e., large blood vessels (>300 μm i.d.) and small blood vessels (<300 μm i.d.). The greatest resistance to flow occurs in the group of vessels with an internal diameter of

less than 300 μm, including the arterioles (<150 μm i.d.) which dominate the control of blood flow (Prewitt & Wang, 1991; Struijker Boudier et al, 1992). Peripheral vascular resistance, therefore, is dictated primarily by true resistance vessels of luminal diameter less than 150 μm. In essential hypertension, the characteristic increase in peripheral vascular resistance is therefore most likely to reflect structural or functional changes (see above) in these microvessels (Shore & Tooke, 1994).

The hypertrophy of cerebral vessels from stroke-prone spontaneously hypertensive rats is attenuated in vessels which have undergone sympathetic denervation (Hart et al, 1980). However, mechanisms other than sympathetic innervation also exert trophic effects on the vascular smooth muscle causing structural and functional changes. For example, catecholamines stimulate growth of vascular smooth muscle in culture (Blaes & Boissel, 1983) causing structural change, and hypertensive humans exposed to noradrenaline or serotonin show the functional abnormality of increased vascular reactivity (a greater force per unit area of muscle) compared with control subjects (Schiffrin, 1992). Evidence from humans (Campbell-Boswell & Robertson, 1981) and animals (Schelling et al, 1979) suggests that angiotensin II also has a trophic or mitogenic action on smooth muscle, and augments sympathetically-mediated arteriolar constriction in man (Seidelin et al, 1991). The effect of vasoconstrictors, therefore, appears to be amplified in hypertension, and other mediators, such as those produced by the vascular endothelium may also be involved in altering structure and function of blood vessels in this disease.

1.3.2 Rarefaction

As well as a reduction in luminal cross-sectional diameter, a reduction in the density of vessels per volume of tissue (rarefaction) will increase vascular resistance. Rarefaction may be functional when the tissues are temporarily not perfused or 'recruited', or structural, when the absolute number of vessels is reduced. Several studies have

confirmed capillary rarefaction in some but not all tissues from hypertensive animals (Hutchins & Darnell, 1974; Chen et al, 1981; Vicaut, 1992). In essential hypertension, structural differences in conjunctival capillaries were observed as long ago as the 1920s (Boas & Frant, 1922; Boas & Mufson, 1923), and more recently rarefaction has been demonstrated using conjunctival microphotography (Lack et al, 1949; Harper et al, 1978; Sullivan et al, 1983), and videomicroscopy of dermal vessels (Williams et al, 1986; Gasser & Bühler, 1992), when it was associated with luminal narrowing of capillaries.

1.4 HYPERTENSION AND ATHEROSCLEROSIS

Hypertension accelerates the atherosclerotic process (Ross et al, 1986) which is of particular importance in coronary arteries (Chobanian et al, 1989). Structural and functional alterations of blood vessels are not confined to hypertension, but may also play an important role in the pathophysiology of the complications of hypercholesterolaemia (Vanhoutte, 1991; Rabbani & Loscalza, 1991). Several experimental and clinical studies have shown that hypercholesterolaemia is associated with accelerated atherogenesis (Ross et al, 1986). However, before developing any anatomical evidence of atherosclerotic lesions, dysfunction of the endothelium may be present (Simionescu et al, 1990), particularly impaired endothelium-dependent relaxation (Shimokawa et al, 1989). High cholesterol, particularly low density lipoprotein (LDL) cholesterol, levels seem to impair the regulatory function of the endothelium long before the development of fatty streaks (Badimon et al, 1992). Thus, the potential for a non-invasive method of screening for dysfunction early in the development of the disease, would help in clinical management and prevention of complications, and this is the subject of Chapter 5.

1.5 FUNCTIONAL AND STRUCTURAL ABNORMALITIES: CAUSE OR CONSEQUENCE?

From studies in established hypertension it is not possible to ascertain whether vascular abnormalities are a cause or consequence of the rise in blood pressure. Circumstantial evidence suggests that they are causal, since they occur early in the development of hypertension (Sullivan et al, 1983; Lever & Harrap, 1992) and are not closely correlated with other consequences of hypertension such as increased left ventricular mass (Lucarini et al, 1991).

1.5.1 Blood pressure tracking

Blood pressure increases with age in children at all ages (Johnson et al, 1973; Katz et al, 1980; Hait et al, 1982), but is especially pronounced during the first year of life, a period of rapid growth (Tanner et al, 1966). Individuals maintain a consistent position over several years when ranked in terms of blood pressure (Rosner et al, 1977). Lever & Harrap (1992), therefore, propose a genetically determined imbalance in the endocrinological growth-promoting processes in children who become hypertensive. During maturation and ageing in adulthood, the rates of change in physiological and biochemical processes are different in essential hypertensive patients compared with normal adults, indicating a disordered biological clock in these patients (Birkenhager, 1972; Brown, 1983).

Hence, alterations in the vasculature may have their origins in childhood, and may contribute to the aetiology of essential hypertension. Therefore, I have used a novel epidemiological model, the Ladywell 'four corners' model, to study causal relationships between vascular structure and function, and essential hypertension (Chapter 6).

1.6 SUMMARY

Functional abnormalities of microvessels observed in essential hypertension include reduced capillary blood flow both in the basal state (Duprez et al, 1992) and after vasodilatation induced by heating (Rayman et al, 1986; Williams et al, 1992). Using invasive procedures, functional abnormalities of the vascular endothelium have been observed in essential hypertension (Panza et al, 1990; Calver at al, 1992; Taddei et al, 1993) and also in hypercholesterolaemia (Chowienczyk et al, 1992; Casino et al, 1993). This 'endothelial dysfunction' has been attributed to impaired generation of nitric oxide.

Structural abnormalities in microvessels of hypertensive patients include: decreased luminal diameter occurring predominantly in larger arterioles (Folkow, 1990; Heagerty et al, 1993), and in capillaries (Lack et al, 1949; Landau & Davis, 1957; Harper et al, 1978); and a reduction in the density of vessels per volume of tissue (rarefaction) occurring predominantly in the smallest vessels (Lack et al, 1949; Landau & Davis, 1957; Harper et al, 1978; Sullivan et al, 1983; Henrich et al, 1988; Williams et al, 1986, 1990; Gasser & Bühler 1992; Prasad et al, 1995).

In human essential hypertension, it is unclear whether structural changes in capillaries or whether changes in larger arterioles are more relevant (Bohlen, 1989). Micropuncture studies show that capillary pressures are elevated in essential hypertension (Williams et al, 1990) while venous pressure is normal (Widgren et al, 1992), suggesting that capillary or post-capillary vascular resistance is increased. Therefore, microvessels are significantly involved in the pathophysiology of essential hypertension (Shore & Tooke, 1994).

1.7 AIMS

The aims of this thesis are:

- To examine the role of nitric oxide in the maintenance of basal vascular tone in health, and to investigate the importance of nitric oxide in the control of blood pressure and cardiovascular homeostasis (Chapter 2).

- To extend this investigation of nitric oxide to the skin microcirculation, examining its role in the control of microvascular blood flow in nutritive and arteriovenous anastomotic tissues of the hand skin (Chapter 3).

- To validate the non-invasive technique of transdermal delivery of drugs by iontophoresis combined with laser Doppler fluximetry (Chapter 4).

- To investigate the pharmacological mechanisms of cholinergic vasodilatation in dermal microvessels, using this iontophoresis technique, and to assess whether the cholinergic response is mediated by nitric oxide and/or prostacyclin (Chapter 4).

- To assess the ability of iontophoresis to measure endothelial function in hypercholesterolaemic patients (Chapters 5).

- To use the Ladywell 4 corners epidemiological model to study vascular structure and function in young people with contrasting predisposition to high blood pressure (Chapter 6).

Chapter 2

NITRIC OXIDE CONTRIBUTES TO BLOOD PRESSURE CONTROL IN MAN

2.1 INTRODUCTION

Nitric oxide is a short-lived free radical molecule with potent vasodilatory and anti-aggregatory effects (Palmer et al, 1987). It is generated by the vascular endothelium, through the action of the nitric oxide synthase enzyme on its substrate, the amino acid L-arginine (Moncada et al, 1991). Analogues of L-arginine, such as L-N^G-monomethyl-arginine (L-NMMA), and L-N^ω-nitro-arginine methyl ester (L-NAME) which circulate at low concentrations in blood (Vallance et al, 1992), are competitive inhibitors of nitric oxide synthase (Moncada et al, 1991). L-NMMA was used as the inhibitor of choice throughout the studies described in this thesis, because, firstly, it is a specific inhibitor of nitric oxide synthase (Moncada et al, 1990) without effects at muscarinic receptors (Buxton et al, 1993); secondly, it has been used in humans extensively (Vallance et al 1989; Calver et al, 1992a; Drexler et al, 1992; Chowienczyk et al, 1993; Panza et al, 1993b; Stamler et al, 1994). L-NAME was not used because it is an antagonist at muscarinic receptors (Buxton et al, 1993), and, by acting at histamine (H_2) receptors on vascular smooth muscle cells, L-NAME may block vascular relaxation independently of the endothelium (Kristic et al, 1991; Ortiz et al, 1992). By blocking generation of nitric oxide, L-NMMA constricts isolated blood vessels *in vitro* and is a pressor agent *in vivo* in animals (Moncada et al, 1991).

In healthy human subjects, *in vivo* evidence has previously been limited to brachial artery administration of L-NMMA, which causes around a maximum 50% reduction in forearm blood flow, suggesting that basal generation of nitric oxide may oppose vasoconstrictor mechanisms in forearm vessels (Vallance et al, 1989). The experiments described in this chapter provide evidence that nitric oxide generation influences blood pressure in healthy humans. The effects of systemic inhibition of nitric oxide synthesis in healthy human subjects were investigated using intravenous bolus administration of L-NMMA.

2.2 METHODS

2.2.1 SUBJECTS

Eight healthy male subjects (age range: 22-60 yr) gave written informed consent to this randomised, single-blind, two-phase crossover study, which was approved by the Lothian Ethics of Medical Research Committee. No subject received vasoactive or non-steroidal anti-inflammatory drugs during the week before each phase of the study, and all of the subjects abstained from alcohol for 24 hours, and from food, caffeine-containing drinks and cigarettes for at least 2 h before each phase. Subjects rested in the recumbent position during each phase, in a quiet room maintained at a constant temperature of 22-25°C. Intravenous cannulae were placed in the left and right antecubital veins under local anaesthesia (left for infusion; right for blood sampling).

2.2.2 EXPERIMENTAL PROTOCOL

(i) DOSE-RANGING PILOT STUDIES

There was an initial series of pilot dose-ranging studies in which two subjects received, on separate days, doses of L-NMMA in ascending order (0.03, 0.1, 0.3, 1.0 and 3.0 mg kg^{-1}) infused at 1 ml min^{-1} over 60 min. On a separate occasion, each subject received saline placebo, with the order of the infusions randomised. Further pilot studies were performed in five of the subjects, in whom 3.0 mg kg^{-1} L-NMMA was infused over 20 min. Further to the results of these pilot studies (Table 2.1), we examined the effects of 3 mg kg^{-1} L-NMMA administered over 5 min to eight subjects in a single-blind, placebo-controlled crossover study.

Dose (mg kg^{-1})	n	Change in blood pressure (mmHg) Systolic	Diastolic	Mean	Δ heart rate (beats min^{-1})	Δ cardiac index (l min^{-1} m^{-2})	Δ TPRI (dyn min cm^{-5} m^{-2})
Placebo	9	1.2±8.1	2.9±10.5	2.4±8.3	1.8±11.1	0.03±0.53	0.0±4.1
L-NMMA							
0.03 (over 60 min)	2	4.5±4.2	2.5±3.5	3.2±0.9	11.0±0.0	0.10±0.42	2.3±2.2
0.1 (over 60 min)	2	13.3±2.5	0.5±5.7	4.8±4.6	10.6±2.2	-0.20±0.14	2.5±2.3
0.3 (over 60 min)	2	2.5±19.8	6.3±16.6	5.0±17.7	12.0±8.5	0.25±0.21	1.7±5.3
1.0 (over 60 min)	2	1.5±14.1	-0.8±3.9	0.0±3.9	-10.8±2.5	-0.60±0.00	7.2±3.5
3.0 (over 60 min)	2	-4.5±7.8	0.3±9.6	-1.3±9.0	-10.5±1.4	-0.95±0.20	4.4±0.7
3.0 (over 20 min)	5	10.3±9.4	8.4±3.7	9.0±3.8	-9.9±4.0	-0.80±0.23	8.6±4.0
3.0 (over 5 min)	8	8.9±6.3	9.7±7.6	9.3±5.9	-12.5±3.9	-0.90±0.30	10.4±6.5

TABLE 2.1:

Maximal changes from baseline in haemodynamic parameters with ascending doses of L-NMMA. To show the range of changes that occur in normal circumstances in these parameters, the maximal changes that occurred with placebo in the definitive study are also shown. Δ = 'change in'; TPRI = total peripheral resistance index. Values are means ± SD.

(ii) DEFINITIVE STUDY

After the acclimatisation period (see above), subjects received infusion of 0.9% saline (1 ml min^{-1}) for 30 min followed, in random order, on separate occasions five days apart, by L-NMMA (3 mg kg^{-1}) or saline vehicle. Both L-NMMA and saline placebo were given for 5 min at 1.5 ml min^{-1}, followed by further saline infusion (1 ml min^{-1}) for 85 min.

2.2.3 Blood pressure and cardiac function

Blood pressure and heart rate (Takeda UA-751 semi-automated oscillometric sphygmomanometer) (Wiinberg et al, 1988), twelve lead electrocardiograms (ECGs) (Nihon-Kohden), and bioimpedance measures of cardiac function (stroke index, cardiac index; BoMed NCCOM3) (Northridge et al, 1990) were recorded at intervals throughout (Fig. 2.1). In brief, the bioimpedance method uses a constant sinusoidal alternating current (2 mA RMS, 70 kHz) which is applied between two self-adhesive Ag-Ag:Cl electrode pairs placed on the left and right lateral aspects of the neck and lower chest. The voltage associated with this is detected by two inner sensing electrode pairs placed 5 cm from the corresponding current applying electrodes and parallel to the current path. This voltage is relayed to an amplifier, and the ECG is signal is transmitted to a microprocessor within the apparatus. Stroke volume and cardiac index are calculated by the microprocessor which uses algorithms to produce on-line measurements of basal impedance, and peak rate of change of impedance which is dependent upon ventricular ejection time, derived from the ECG. The microprocessor uses the Sramek-Bernstein formula:

$$\text{Stroke volume} = \frac{L^3.dZ/dt[max].VET}{Zo}$$

Where L is the thoracic length estimated from the subject's weight and height using a nomogram, Z = impedance, dZ/dt [max] is the peak rate of change of impedance (Bernstein, 1986). Body mass index (BMI) was calculated by dividing the height in metres by the weight in kilograms squared. Mean arterial pressure was calculated as diastolic blood pressure + (pulse pressure)÷3. Total peripheral vascular resistance was calculated as mean arterial pressure/cardiac index, and expressed as dyn min/cm^5/m^2

2.2.4 Blood biochemistry and renal function

Each subject had safety tests (sodium, potassium, creatinine, urea, alkaline phosphatase, alanine transaminase, bilirubin, albumin, calcium and urate; and haemoglobin, white cell and platelet counts) performed before, and one month after finishing, the study. On each study day, venous blood and a two-hour urine sample, collected over the study period, were obtained for estimation of urine flow rate and creatinine clearance, as well as potassium, sodium and fractional sodium excretion rates.

2.2.5 DRUGS

Pharmaceutical grade (sterile and pyrogen-free) L-NMMA (Clinalfa AG, Laufelfingen, Switzerland) was dissolved in physiological saline and infused according to the above protocol.

2.2.6 STATISTICAL ANALYSIS

All data are expressed as means±SEM (except in the pilot study where means±SD are given). Data for stroke volume and cardiac output were corrected for body surface area, calculated to a standard nomogram to provide measures of stroke and cardiac index. Total peripheral resistance index was calculated as mean arterial pressure divided by cardiac index, and expressed as dyn min/cm^5/m^2.

Statistical analyses were performed on untransformed data. Basal values for blood pressure, heart rate, cardiac function and peripheral vascular resistance were compared using a one-factor measure analysis of variance. The effect of placebo and L-NMMA on each of the parameters measured was examined by 2-way repeated measures analysis of variance. Where analysis of variance showed a significant treatment effect ($P<0.05$), placebo and L-NMMA effects were compared at individual time points using

Student's paired *t*-test. P-values were corrected for the total number of comparisons by the method of Bonferroni. Statistical analyses were performed using StatView 512+ software (Brainpower Inc., Calabasas, California, USA) for the Apple Macintosh microcomputer.

2.3 RESULTS

(i) DOSE-RANGING PILOT STUDIES

At doses ≤1.0 mg kg^{-1}, there were no apparent trends in the effects for L-NMMA on haemodynamic parameters, particularly compared with the changes that ocurred with placebo (Table 2.1). At higher doses, there was an apparent decrease in heart rate and cardiac index, and an increase in total peripheral resistance, as assessed by the maximal changes occurring in these parameters. No effect on blood pressure was observed until a dose of 3.0 mg kg^{-1} was given over 20 min, when there were isolated increases in both systolic and diastolic blood pressure (Table 2.1), but these did not occur consistently at similar time points.

(ii) DEFINITIVE STUDY

2.3.1 Blood pressure and cardiac function

There were no significant differences between basal values for blood pressure, heart rate or cardiac function for the two study days. Compared with placebo, infusion of L-NMMA caused a modest, but significant, placebo-corrected increase in mean arterial pressure of 8±3%, from a value of 84±4 mmHg before dosing (P=0.04; Fig. 2.1).

There was a significant reduction in heart rate of 19±3%, from a predose value of 61±4 bpm (P=0.002). After an initial, non-significant, increase at 5 min, stroke volume also decreased after L-NMMA, and there was thus a large decrease in cardiac

index of 21±3%, from a predose value of 3.9±0.3 l min^{-1} m^{-2} (P=0.001). Calculated peripheral vascular resistance increased by 35±6%, from a predose value of 23±3 AU (P=0.02), i.e., the biggest effect observed. The haemodynamic changes returned towards basal values over 90 min (Fig. 2.1).

2.3.2 Blood biochemistry and renal function

Urine flow rate, potassium, sodium and fractional sodium excretion were higher with L-NMMA than with placebo (Table 2.2), approaching significance for fractional sodium excretion (P=0.06); creatinine clearance was similar on both days. L-NMMA did not cause adverse clinical events, alter the electrocardiogram or affect safety tests.

	Placebo	L-NMMA	P value
Urine flow rate (ml min^{-1})	0.74 ± 0.21	1.23 ± 0.43	0.12
Creatinine clearance (ml min^{-1})	112 ± 10	105 ± 10	0.30
Potassium excretion (μmol min^{-1})	48 ± 9	80 ± 8	0.13
Sodium excretion (μmol min^{-1})	87 ± 26	138 ± 21	0.09
Fractional sodium excretion (%)	0.60 ± 0.21	1.02 ± 0.25	0.06

TABLE 2.2:

Measurements of renal function.

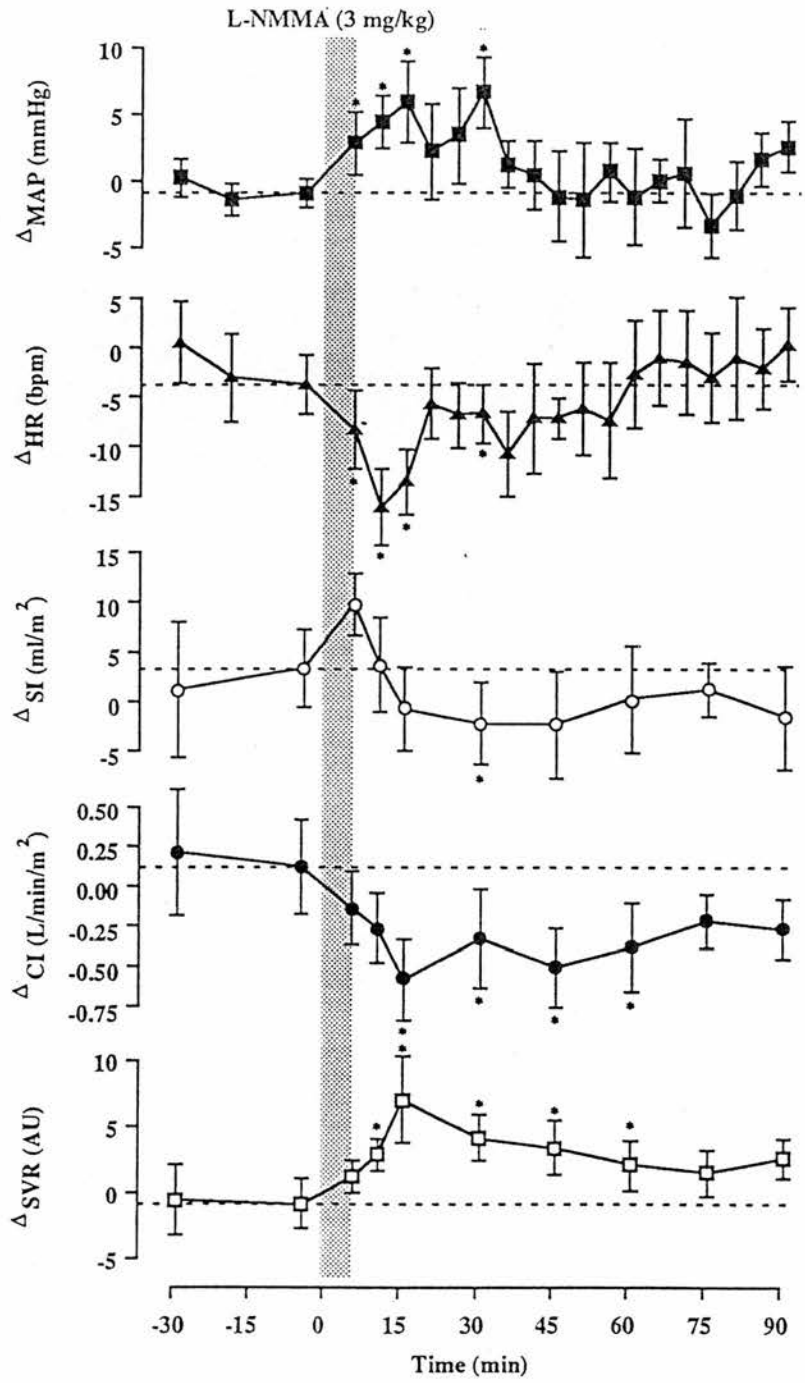


FIGURE 2.1:

Effects of 3 mg kg^{-1} L-NMMA given over 5 min (shaded area) on differences from placebo for mean arterial pressure (MAP; ■), heart rate (HR; ▲), stroke index (SI; ○), cardiac index (CI; ●) and systemic vascular resistance (SVR; □). The dashed line represents the last placebo-corrected pretreatment value for each parameter. * Significant ($p<0.05$) difference from predose placebo-corrected values.

2.4 DISCUSSION

These findings demonstrate that inhibition of nitric oxide synthesis with L-NMMA increases blood pressure in healthy human subjects. The 35% increase in calculated peripheral vascular resistance indicates that the net effect of basal generation of nitric oxide is to promote vasodilatation, and thereby oppose vasoconstriction in arterial resistance vessels. The pressor effect would have been substantially greater were it not for the associated reduction in heart rate, stroke index, and, consequently, cardiac index; probably mediated through a baroreceptor reflex. It is unlikely that inhibition of nitric oxide synthesis with L-NMMA reduced cardiac output directly, because nitric oxide itself reduces contractility of isolated cardiac myocytes (Brady et al, 1992). Interestingly, stroke volume did increase briefly 5 min after administration of L-NMMA (Fig. 2.1), suggesting that inhibition of cardiac nitric oxide generation may unmask a negatively inotropic effect of nitric oxide, before baroreflex mediated suppression of cardiac output develops. The increase in sodium excretion with L-NMMA, although non-significant, may have been due to a pressure-mediated natriuresis, or due to intrarenal actions of L-NMMA, and merits further investigation.

It has been suggested that the low systemic vascular resistance and hypotension of septic shock (Petros et al, 1991) and advanced liver failure (Midgley et al, 1991) may be due to excess generation of nitric oxide, possibly through endotoxin-mediated stimulation of the inducible form of nitric oxide synthase (Moncada et al, 1991). Petros and colleagues demonstrated an average increase of 22 mm Hg in mean arterial pressure in two patients with septic shock given a 1 mg kg^{-1} bolus of L-NMMA (Petros et al, 1991). However, in the absence of data from healthy subjects, they were unable to attribute a pathophysiological role to nitric oxide in this disease. The smaller pressor effect (8 mm Hg) of L-NMMA when given here at a higher dose (3 mg kg^{-1}) to

healthy subjects, confirms that nitric oxide does contribute to the decreased peripheral vascular resistance and hypotension of septic shock.

Nitric oxide is thought to have many other actions, including regulation of platelet, kidney and central nervous function (Moncada et al, 1991). Further specific investigations into the physiological actions of nitric oxide, and the therapeutic uses of nitric oxide synthase inhibitors, should be facilitated by this demonstration that L-NMMA is well tolerated in healthy human subjects.

Decreased generation of nitric oxide has been postulated to occur in essential hypertension (Calver et al, 1992) and chronic renal failure (Vallance et al, 1992). The large increase in calculated systemic vascular resistance in this study suggests that such decreased nitric oxide generation may have substantial effects on blood pressure and tissue blood flow. The likely site of this action, i.e., microvessels which contribute most to peripheral vascular resistance, is the subject of the next chapter.

Chapter 3

NITRIC OXIDE MEDIATES VASODILATATION IN SPECIFIC HUMAN SKIN MICROVESSELS

3.1 INTRODUCTION

As discussed in the Introduction (Chapter 1), the potent vasodilator nitric oxide is one of several mediators important in regulating vascular tone (Vallance et al, 1989; Haynes & Webb, 1994). In the previous chapter, systemic administration of L-NMMA increased arterial pressure, and markedly increased systemic vascular resistance, emphasising the importance of nitric oxide in the regulation of resistance vessel tone. Microvessels, particularly precapillary resistance vessels, make an important contribution to peripheral vascular resistance in established essential hypertension (Shore & Tooke, 1994). Thus, in this chapter, I shall concentrate on an investigation into the role of nitric oxide in the microvessels of the dermis of the hand.

The anatomy of the skin microcirculation is complex, and regulatory mechanisms which control blood flow may vary depending on the function of the tissue served. There appears, for example, to be a role for nitric oxide in the regulation of basal arteriolar tone in some animal tissues such as the skeletal muscle of the rat cremaster (Kaley et al, 1992; Sun et al, 1992), and the rabbit (Mügge et al, 1991) and cat hindlimb (Ross et al, 1991). However, in other tissues, such as the rat mesentery (Ebeigbe et al, 1990) and the second and third order arterioles of the hamster cremaster (Hester et al, 1993), nitric oxide appears only to be generated following stimulation by agonists such as acetylcholine. Moreover, in the hamster cremaster model, in which different sizes of arteriole were studied, nitric oxide appeared to contribute to basal tone in larger (65 μm) but not smaller vessels (30 - 45 μm) (Hester et al, 1993). In an investigation of the control of vascular smooth muscle in the hamster cremaster microcirculation, de Wit and colleagues (1994) suggest that vasodilators such as nitric oxide and prostacyclin act synergistically to increase cGMP and cAMP in smooth muscle, and to inhibit platelet activation. Therefore, these studies in animals suggest

that the role of nitric oxide in the microcirculation varies between tissues and between species, and that, even in the same tissue, this variation can depend upon vessel size.

In the human skin microcirculation, an impairment of endothelium-dependent relaxation has been suggested to cause skin ischaemia in excised skin flaps *in vitro* (Kreidstein et al, 1992), and nitric oxide does not appear to have a neurogenic role in the human skin microcirculation *in vivo* (Dietz et al, 1994). Although Coffman and colleagues (1994) have examined the contribution of nitric oxide to stimulated sympathetic vasoconstriction, no studies have examined whether basal generation of nitric oxide is important in the physiological regulation of human skin blood flow.

Arterioles and venules form two plexi in the dermis and it is from the superficial plexus that the capillary loops of the dermal papillae arise. In those skin areas serving a primarily thermoregulatory function, such as the finger pulp, arteriovenous anastomoses are frequently observed whilst other areas, such as the dorsum of the hand, have few arteriovenous anastomoses and perform a primarily nutritive role (Clark, 1938). The role of nitric oxide in regulation of blood flow may depend upon the function of the tissue being studied. I, therefore, investigated blood flow in both the nutritive tissue of the hand dorsum and finger nailfold, and in the thumb pulp, a tissue rich in arteriovenous anastomoses. The results suggest that endogenous nitric oxide production may be more important in regulating microvascular skin blood flow in regions rich in arteriovenous anastomoses than in areas containing mainly nutritive vessels.

3.2 METHODS

3.2.1 SUBJECTS

Six healthy male volunteers (aged 23-35 years), who had given their written, informed, witnessed consent, took part in this study which was approved by the

Lothian Ethics of Medical Research Committee. Subjects had not taken prescribed vasoactive medications in the month before the study, steroidal or non-steroidal anti-inflammatory drugs or aspirin in the week before the study; and all had abstained from food, caffeine-containing drinks, alcohol and smoking for at least 3 h before the study.

3.2.2 EXPERIMENTAL PROTOCOL

The experimental protocol is shown schematically in Figure 3.1. Subjects rested supine throughout the study with both arms at their sides, slightly bent and supported comfortably on padded benches. They acclimatised to an ambient temperature of 22-25°C for 30 min.

(i) Acclimatisation

During acclimatisation, a 27SW-gauge needle (Cooper's Needle Works, Birmingham, United Kingdom), connected to a 16G epidural catheter (Portex Ltd., Hythe, Kent, UK), was introduced into a brachial artery under local anaesthesia of 1% lignocaine (Astra Pharmaceuticals Ltd., Kings Langley, UK). Patency was maintained by infusion of physiological saline (0.9%; Baxter Healthcare Ltd., Thetford, Norfolk, UK) at a rate of 1.0 ml min^{-1} via a Welmed P1000 syringe pump (Welmed Clinical Care Systems, Bramley, Hampshire, UK. All instruments used to record forearm blood flow and microvascular measurements were positioned. The left brachial artery was cannulated in 5 subjects, and the right in 1 subject, due to a failed attempt at the left. Finger nailfold capillaries of both hands were examined by video microscopy, and on each hand the finger with the most clearly visible capillaries (usually the ring finger) was selected for subsequent measurement of capillary blood velocity as detailed below. To familiarise the subjects with the techniques, recordings of forearm blood flow, finger blood pressure, red cell flux in the hand dorsum and in the thumb pulp, capillary blood velocity, skin and ambient room temperature were all performed during this acclimatisation period, but were not used in the data analysis.

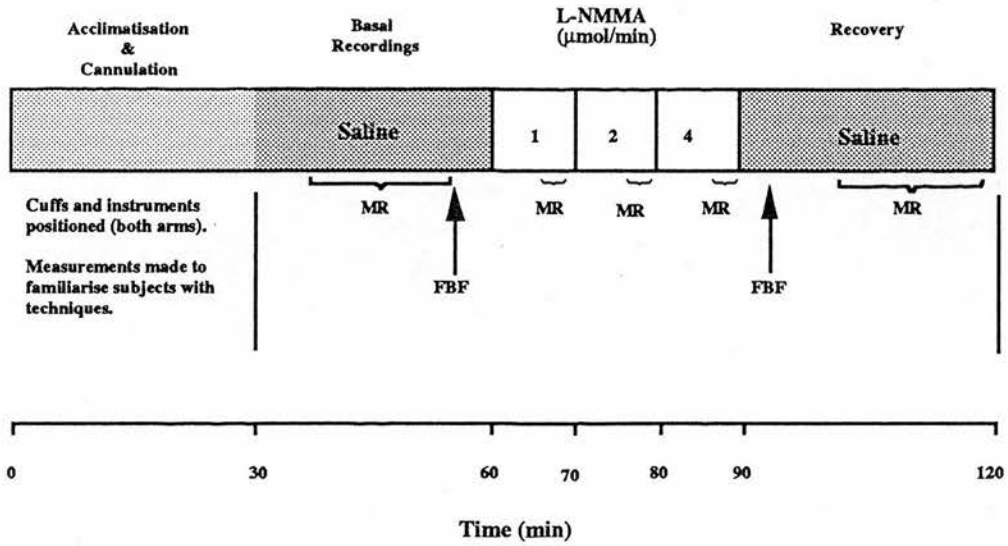


FIGURE 3.1:

Experimental design and protocol. Initial recordings made during the Acclimatisation & Cannulation phase served only to accustom subjects to the techniques, and data were not used in the analysis of results. Bilateral microvascular recordings (MR) of nailfold capillary blood velocity, hand dorsum and thumb pulp skin flux, skin temperature and finger blood pressure were made during each of the remaining phases as indicated. FBF = forearm blood flow.

(ii) Basal recordings

Once acclimatisation was complete, basal readings of microvascular red cell flux in the dorsum of the hand and in the pulp of the thumb, measured simultaneously on both hands, were obtained by laser Doppler fluximetry over a 5 min period. Capillary blood velocity (basal) was recorded, and the duration of this procedure was dependent upon

the ease with which high contrast, stable microscopy pictures were obtained and ranged from 20-30 min. Basal measurements of skin temperature, finger blood pressure, forearm blood flow, and laser Doppler biological zero, were also obtained, with forearm blood flow being measured at least 10 min before L-NMMA infusion and any subsequent microvascular measurement.

(iii) Recordings during drug infusion and recovery periods

L-NMMA was then infused intra-arterially, and microvascular blood flow, finger blood pressure and skin temperature were monitored during the final 5 min of each dose. Forearm blood flow and laser Doppler biological zero values (that is, the flux values obtained without arterial inflow at each of the 4 sites during wrist cuff arterial occlusion) were measured as soon as possible after the highest dose of L-NMMA (about 8 min after infusion had stopped). Microvascular blood flow, finger blood pressure and skin temperature were recorded over the final 20 min of the 30 min recovery period.

3.2.3 FOREARM BLOOD FLOW

The technique of measuring blood flow in limbs has been used for almost a century (Hewlett, 1909), but modern methods are based on Whitney's technique of venous occlusion, mercury-in-latex strain-gauge plethysmography (Whitney, 1953). These older strain-gauges have recently been superseded by indium/gallium-in-silastic versions which are easier to apply and more readily calibrated. Modern strain-gauge plethysmography is usually combined with brachial artery infusion of drugs which are given at local doses, affecting only the forearm vascular bed (Webb, 1995). The non-infused arm acts as a contemporaneous control throughout the experiment, and demonstrates that the local doses of drugs used do not produce systemic effects such as stimulation of neurohumoral reflexes and direct effects on other organs.

Subjects lay supine with their arms, resting on pillows, inclined at approximately 30 degrees to improve venous drainage. Wrist cuffs were applied and, during the recording period, were inflated to 220 mm Hg to exclude the hand circulation from the measurements. Upper-arm congesting cuffs were inflated to 40 mm Hg and blood flow was recorded for 10 seconds followed by a 5 second refilling period. Multiple 10 second measurements were made over a three minute period and the slopes of the final 5 recordings averaged to determine forearm blood flow. The apparent rate of arterial inflow to the forearm is disturbed during the first minute after inflation of the high pressure wrist cuff (Kerslake, 1949). Therefore, blood flow recordings made in the first minute after inflation were not used in the analysis. Calibration was achieved using the internal standard of the Vasculab strain gauge plethysmography unit (MedaSonics, Mountain View, California, USA).

3.2.4 MICROVASCULAR RECORDINGS

(i) Finger blood pressure

Cuffs were positioned on the first finger of each hand, and blood pressure measured simultaneously using a photoplethysmographic technique (Finapres, Ohmeda, Englewood, CO, USA). Finger blood pressure measurements by Finapres correlate with brachial and radial intra-arterial recordings, and acute changes in arterial pressure are accurately reflected by the device (Epstein et al, 1991). Two Finapres devices (one for each hand) were connected via a MacLab (AD Instruments, Castle Hill, NSW, Australia) digital converter to an Apple Macintosh microcomputer. Using Chart software (v 3.2.8; AD Instruments, Castle Hill, NSW, Australia), results were stored for subsequent analysis.

(ii) Skin temperature

Skin temperature was measured at various sites, including the dorsum of the hand, the volar surface of the forearm and the nailfold of the finger using adherent

thermocouples (Fluke 52, RS components, Corby UK). Skin temperature, together with room temperature, was recorded at 5-min intervals throughout the entire study.

(iii) Capillary blood velocity (CBV)

Finger nailfold capillaries were visualised in both hands using two independent, identical television video microscopy systems as described by Flynn et al (1989) (Fig. 3.2). Each television camera (Phillips LDH0703, KRP Power Source UK Ltd., Hambridge Lane, Newbury, UK) was connected to a microscope (Leitz CMM, Leica UK Ltd., Knowlhill, Milton Keynes, UK), and to a video cassette recorder (Panasonic AG-7350, KRP Power Source UK Ltd.) so that images could be recorded onto videotape for subsequent analysis. Finger nailfold capillary loops lie parallel to the surface of the skin and thus movement of blood cells within the capillaries can be clearly visualised. Six different microscopy fields, selected sequentially across the nailfold, were each recorded for 2.5 min.

Continuous measurement of capillary blood velocity (CBV) in the arterial limb of the capillaries was made by off-line analysis of the video recordings using computerised photometric temporal correlation (CapiFlow, CapiFlow AB, Kista S-16440 Sweden) (Fagrell et al, 1994). Two windows were positioned over the arterial limb of a capillary. Red cells moving up the capillary lumen appear 'black', and are not recognised by the computer. A photometric signal was generated when the computer recognised a plasma gap which appears 'white'. The signal detected at the second window is correlated with the original signal detected at the first. As the distance between the windows was known, the velocity could be computed. A cross-correlation limit of 0.4, time constant 2.0 s, and noise limit (delta) of 20 were used. Adequate cross-correlation is reliant upon a stable image with high contrast, recognisable peaks and troughs in the photometric signal, and a sufficient length of straight capillary limb over which to place the photometric windows. Although these criteria are readily met at

low velocities (<1.0 mm s^{-1}), recognisable fluctuations in the photometric signal at high flows may be less marked, due for example to changes in orientations or groupings of red blood cells as they ascend the capillary limb or to lack of plasma spaces. Thus, in such capillaries, the time during which the correlation reaches the cross-correlation limit may be low. To minimise variability, subjects were studied at the same time of day, and at the same environmental temperature, with the author as the sole investigator. At least 6 capillaries were analysed per subject. To minimise data selection from capillaries with low velocities, measurement of capillary blood velocity was attempted in all capillaries which were in focus in each video field, and a capillary was accepted when adequate correlation was achieved for at least one third of the recording period. In each capillary in which capillary blood velocity was measured, red cell column width was also measured at three sites on the arterial limb using a computerised window splitting technique (CapiFlow, CapiFlow AB, Kista S-16440 Sweden). This represents luminal diameter, assuming that the plasma column does not alter.

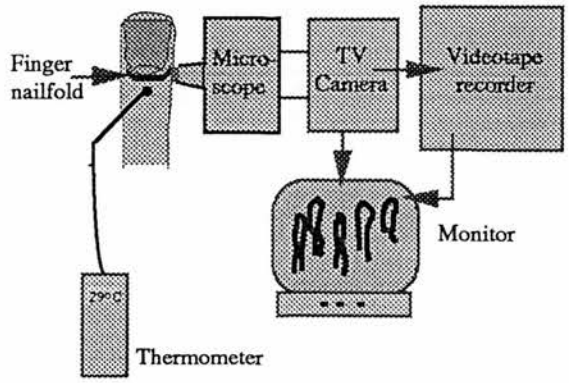


FIGURE 3.2:

Schematic presentation of the videomicroscopy equipment for recording capillary blood velocity.

(iv) Skin red blood cell flux: dorsal surface of the hand and thumb pulp

Laser Doppler blood flow measurement uses the Doppler shift of laser light. When the light is reflected off a moving object, its frequency is shifted, with the amount of shift being dependent on the speed of the moving object. Measurements of skin red cell flux and red cell concentration were made by laser Doppler fluximeter (MBF3D, Moor Instruments Ltd., Axminster, Devon, UK), which uses laser light to illuminate skin. Most of the light will be reflected by static tissue, but some will be reflected by moving red blood cells. The light reflected by the static tissue and the frequency-shifted light from moving red cells (Doppler broadening) is collected in a photodetector which mixes the waves scattered by different structures and detects the intensity of signals produced. The power spectral density of these signals is determined by the red cell concentration and velocity (Low et al, 1983). The MBF3D uses a low power laser (helium/neon; 1.5 mW) to generate one or two (if both channels are being used) non-injurious beams of infra-red light of wavelength 780-810 nm. Probes contain two glass fibres: one to transmit, the other to collect reflected light from a single point (1 mm^2) on the skin. The signal is then processed and amplified by a digital processor to display red cell flux, red cell concentration and mean red cell speed. The mean Doppler frequency has been predicted to be linearly related to blood flow (Bonner & Clem, 1981). *In vitro* studies of red cell blood flow in miniature polythene tubing by two separate groups have confirmed this linearity (Watkins & Holloway, 1981; Nilsson et al, 1980). In addition, *in vivo* studies in human skin (Watkins & Holloway, 1981; Stern et al, 1977), and in pig muscle using ^{133}Xe washout or radioactive microspheres (Öberg et al, 1979), have also confirmed this linear relationship. Laser Doppler probes were calibrated before every experiment (Barnett et al, 1990).

Skin red blood cell flux was simultaneously measured, bilaterally, in the dorsal skin of the hand adjacent to the base of the thumb, and in the thumb pulp. The probe holders

remained attached to the skin to ensure that flux was measured at exactly the same skin sites throughout the study. For each laser Doppler monitor, skin red blood cell flux was calculated using computerised analysis (Moorsoft, Moor Instruments Ltd.) as the average value over a 5-min period at each point during the protocol (basal, 1, 2, 4 μmol min^{-1} L-NMMA infusion, and recovery).

3.2.5 DRUGS

Pharmaceutical (sterile and pyrogen-free) grade L-NMMA was dissolved in physiological saline and infused via the brachial artery at a rate of 1.0 ml min^{-1} at three doses: 1, 2 and 4 μmol min^{-1} (each for 10 min) as an adaptation of a previous protocol (Vallance et al, 1989).

3.2.6 STATISTICAL ANALYSIS

All data are mean ± SEM. Results are given as percentage change from baseline; absolute values for all variables measured are given in Table 3.1. The values of the ratio infused/control were calculated for each subject, and the overall mean calculated and included in Table 3.1. Previous findings (Flynn et al, 1991) suggested that a gradual reduction in microvascular parameters occurs with acclimatisation time, particularly marked for dorsal skin red cell flux and capillary blood velocity. Therefore, statistical analysis was performed on the ratio infused:control to take into account such time-dependent changes in the control arm. The ratios of the microvascular variables were first analysed by repeated measures analysis of variance (ANOVA). If differences were significant, Student's paired *t*-tests were used to compare basal ratio values with values obtained during L-NMMA infusion and recovery.

3.3 RESULTS

3.3.1 FOREARM BLOOD FLOW

Forearm blood flow was reduced by 36.6 ± 3.1%; (95% confidence interval (CI) of 17.0 - 56.2; P=0.005; Table 3.1) following L-NMMA.

3.3.2 MICROVASCULAR RECORDINGS

(i) Finger blood pressure

Finger blood pressure increased during the experiment in both arms. In the control (P=0.04) and infused (P=0.003) arm, this increase with time was significant. However, the difference between the two arms was not significant (P=0.87; Table 3.1).

Variable	Arm	Basal	1 μmol min^{-1}	2 μmol min^{-1}	4 μmol min^{-1}	Recovery	ANOVA
Forearm blood flow	I	2.52±0.35				1.69±0.49	
(ml (100 ml)$^{-1}$ min^{-1})	C	3.10±0.70				3.59±1.51	
Ratio		0.88±0.07				0.58±0.31	P=0.005
Finger blood pressure	I	96±5	100±5	103±6	106±6	97±4	
(mean arterial pressure/mm Hg)	C	92±5	96±5	96±5	100±4	99±4	
Ratio		1.06±0.06	1.05±0.06	1.08±0.06	1.07±0.08	0.99±0.06	P=0.873
Pulp red cell flux	I	210±51	199±57	156±54	158±54	163±50	
(AU)	C	208±56	216±50	200±44	199±45	209±51	
Ratio		1.26±0.25	1.09±0.22*	0.89±0.21**	0.84±0.16*	0.95±0.23*	P=0.0001
Dorsum red cell flux	I	41±13	32±9	28±7	25±6	30±7	
(AU)	C	32±9	26±8	26±7	25±7	28±7	
Ratio		1.62±0.52	1.89±0.68	1.57±0.57	1.44±0.46	1.47±0.41	P=0.92
Capillary blood velocity	I	1.11±0.17	1.11±0.20	0.93±0.13	0.93±0.23	0.90±0.17	
(mm s^{-1})	C	1.09±0.19	1.07±0.22	0.98±0.21	0.92±0.22	0.87±0.20	
Ratio		1.08±0.12	1.08±0.08	1.03±0.09	1.10±0.11	1.14±0.11	P=0.69
Red cell column width	I	11.1±0.6	11.0±0.5	11.2±0.3	11.0±0.4	10.6±0.3	
(μm)	C	10.5±0.4	10.3±0.5	10.4±0.6	10.5±0.3	11.0±0.5	
Ratio		1.03±0.07	1.09±0.06	1.02±0.05	1.03±0.05	0.96±0.05	P=0.44
Skin temperature	I	32.1±1.0	30.8±1.6	30.5±1.6	29.9±1.4	28.7±1.6	
(°C)	C	31.5±1.3	30.1±1.4	31.0±1.4	30.4±1.2	29.5±1.1	
Ratio		1.02±0.02	1.00±0.01	0.99±0.01	0.98±0.01	0.96±0.03	P=0.25

TABLE 3.1:

Results for forearm blood flow, finger blood pressure, and for microvascular measurements, including values for skin temperature in the infused (I) and the control (C) arm. Results from each subject were averaged to give mean ± SEM. Ratios for each subject were calculated individually and expressed as mean in the table.

(ii) Skin temperature, capillary blood velocity, red cell column width, hand dorsal skin red blood cell flux, thumb pulp red cell flux

As expected (see above), dorsal skin temperature, red cell flux and capillary blood velocity fell gradually over the experimental period (Table 3.1). However, these time-related effects did not differ in the control arm compared to the arm infused with L-NMMA for dorsal red cell flux and capillary blood velocity (Fig. 3.3), or for red cell column width and skin temperature. In contrast, L-NMMA markedly reduced thumb pulp red cell flux (P=0.0001: Fig. 3.3; Table 3.1).

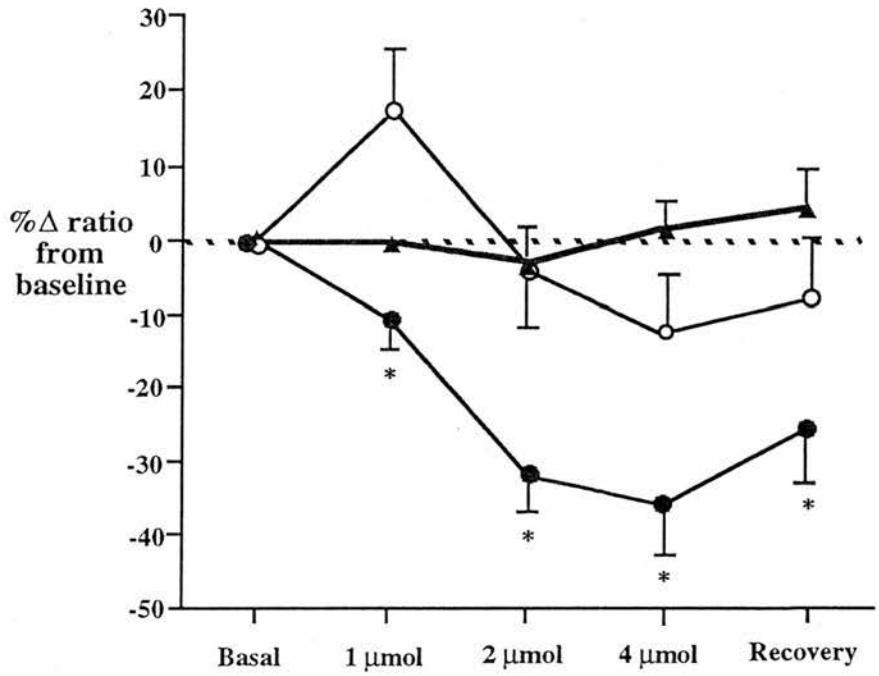


FIGURE 3.3:

Capillary blood velocity (▲), red blood cell flux in the dorsum of the hand (○) and finger pulp (●), are shown as % Δ of ratio infused:control arm from baseline. The difference between the effects of the 3 doses of L-NMMA (shown on the horizontal axis) on red cell flux in the dorsum and the pulp (ANOVA: P=0.037) is shown by asterisks.

3.3.3 Laser Doppler biological zero values

Biological zero, on either the pulp or dorsal site, was similar between individuals and was not changed by the L-NMMA infusion (pulp 12.8 ± 2.6 arbitrary units (AU) basal, 12.6 ± 2.5 AU 2-5 min post infusion; dorsum 5.4 ± 1.6 AU basal, 4.9 ± 1.6 AU 2-5 min post infusion), nor did biological zero differ in the two hands either before or after the infusion, (basal: thumb pulp 12.3 ± 2.2 v 13.2 ± 3.2 AU; dorsum: 5.5 ± 1.7 v 5.3 ± 1.7 AU; post infusion: thumb pulp: 11.9 ± 2.2 v 13.2 ± 2.9 AU; dorsum: 4.8 ± 1.6 v 5.0 ± 1.8 AU). The contribution of biological zero to the flux signal was relatively small, 7.3 ± 1.4% and 15.4 ± 1.2% of pulp and dorsum red cell flux respectively during basal measurements. Therefore, since the effects of biological zero are small in these circumstances (the maximum effect of subtracting biological zero would be a change in ratio by 0.2, or an alteration of percentage change by 6%), and biological zero cannot be measured at each time point throughout the experimental protocol, flux data are expressed without subtraction of biological zero.

3.4 DISCUSSION

These data show that doses of L-NMMA producing substantial reduction in forearm blood flow had no effect on red blood cell flux in the dorsal skin of the hand or on red blood cell velocity in nutritive nailfold capillaries. In contrast, red cell flux in the finger pulp, an area rich in arteriovenous anastomoses, was substantially reduced. This suggests that in the human skin microcirculation, endogenous nitric oxide plays a greater role in regulation of blood flow in areas rich in vessels with a mainly thermoregulatory function than in areas rich in vessels serving a primarily nutritive role.

All measurements were made in both the infused and contralateral arm; the latter acting as a contemporaneous control throughout the experiment so that physiological fluctuations in all parameters measured could be taken into account. This is based on evidence that micromolar doses of L-NMMA infused via the brachial artery exert a local effect only (Vallance et al, 1989). In addition, these doses are sufficient to substantially block the vasodilator response to acetylcholine (Chowienczyk et al, 1993), and that doses required for systemic effects are much higher (Chapter 2; Section 2.2.2; Stamler et al, 1994). The small increase in finger blood pressure in both arms was probably due to mild sympathetic activation associated with this slightly stressful study. Due to the hand ischaemia caused by the measurements of forearm blood flow, it was not considered possible to obtain measurements of forearm blood flow during L-NMMA infusion without jeopardising the measurements of microvascular flow which this study was primarily designed to examine. Therefore, forearm blood flow was measured shortly after L-NMMA infusion was stopped. When this difference in timing is taken into account, the effects of L-NMMA are similar to those reported by other workers using the same dose (Vallance et al, 1989; Calver et al, 1992).

Skin blood flow is variable from site to site and to a considerably lesser degree from moment to moment (Braverman & Schechner, 1991). The laser Doppler technique can also detect reductions in nutritive flux; for example, during finger cooling or changes in posture (Hassan & Tooke, 1988). However, there was no such reduction in the skin of the hand dorsum following L-NMMA infusion. In the present study, variability was minimised by using the same skin site to monitor red blood cell flux throughout the study, by obtaining an average flux value over at least a 5 min period, and, in the case of capillary blood velocity, by studying the same capillary for as many of the experimental periods as possible and for as long as possible (Fagrell et al, 1994).

The skin microcirculation has a complex architecture. The finger pulps and nailbeds are areas rich in arteriovenous anastomoses, conspicuous by their thick muscular walls and tortuosity (Grant & Bland, 1931); these are highly innervated and serve a thermoregulatory function (Clark, 1938). These vessels provide an alternative pathway from the arterial to the venous circulation, bypassing the terminal capillary bed. In contrast, arteriovenous anastomoses are infrequent on the dorsum of the hand or finger nailfold. Thus, as a result of this difference in skin architecture, the two skin sites we investigated may differ, firstly in the degree of neurogenic tone of the vessels, and secondly in the size of the blood vessels from which the laser Doppler signal is derived. In areas lacking arteriovenous anastomoses, the laser Doppler signal is derived from capillaries, arterioles and venules. However, as capillary flow accounts for around 10% of blood flow to the skin in these areas, the majority of the laser Doppler signal is derived from other vessels.

Studies in animals suggest that there may be interactions between endothelial release of nitric oxide and the perivascular nerves (Tesfamariam & Cohen, 1988). The overflow of noradrenaline from perivascular nerves was reduced in the presence of an intact endothelium in both the rabbit carotid and the canine pulmonary artery studied *in vitro,* suggesting that the endothelium may influence neurotransmission by a prejunctional mechanism (Tesfamariam et al, 1989). In addition, noradrenaline-stimulated release of endothelial derived relaxing factors may attenuate the mediated neurogenic vasoconstriction at the level of the smooth muscle cell. If basal release of nitric oxide opposes the tonic adrenergic vasoconstriction in human hand skin microcirculation, inhibition of nitric oxide synthase by L-NMMA would be expected to reduce skin blood flow particularly in areas of high basal flow or resting tone, including the finger pulp, in keeping with the results of the present study.

Alternatively, these findings may reflect differences in vessel size, wall thickness, or function in the two skin areas. In animals, the role of nitric oxide as a regulator of basal tone is more marked in the larger arterioles of the hamster skeletal muscle microcirculation than in the smaller vessels (Hester et al, 1993) and similar findings have been reported for the rabbit ear branching arteries (Griffith et al, 1987). Whether such a size-determined differential control occurs in the human skin microcirculation, and whether it contributes to our findings, is unclear. Computerised models suggest that alterations in wall:lumen ratio may alter the change in resistance for a given degree of vascular smooth muscle shortening or lengthening (Egan et al, 1988). Such a mechanism may contribute to the observed greater fall in blood flow in the area rich in arteriovenous anastomoses in the present study, since the thicker walled arteriovenous anastomoses might be expected to have a larger increase in vascular resistance as smooth muscle shortens.

Finger nailfold capillary red cell velocity was unaltered by L-NMMA infusion. As red cell column width was also unchanged during the infusion, it is likely that capillary blood velocity reflected capillary blood flow. Increases in vascular resistance proximally may be compensated for by a reduction in resistance nearer the capillary level in an attempt to maintain nutritive capillary flow. These results confirm the more indirect laser Doppler assessments of dorsal hand skin, and suggest that either nitric oxide is not involved in the regulation of basal capillary nutritive flow, or that other mechanisms become operative and serve to compensate for the loss of endogenous nitric oxide.

Possible alternative explanations for these findings might relate to the differences in blood flow between the two skin areas. Blood flow to the finger pulp is considerably greater (about fivefold) than that to the dorsal regions of the hand. Thus, it is likely that L-NMMA would reach the pulp areas before reaching the nutritive areas, and that a

greater total amount of L-NMMA would be delivered to the pulp areas during the 30-min infusion. The exact relationships between rate of delivery, total dose of L-NMMA delivered to an area, basal levels of nitric oxide and the vasodilatory response to L-NMMA are unknown, but may provide a potential explanation for my findings. Alternatively, the higher flow rates to the pulp may result in a greater flow-dependent release of nitric oxide under basal conditions, and thus a greater inhibition by L-NMMA. Although there is some evidence that flow-dependent vasodilatation may occur in the forearm vascular bed, it has not been well described in human skin. In animals, it appears that the mediators involved in such flow-dependent vasodilatation may vary according to vessel size and vascular preparation. Whether or not this is the case in man is unknown. Because of the demanding nature of this study, and the requirement for duplicate apparatus and a second operator (AC Shore) so that the infused and non-infused arm could be studied simultaneously, I did not include a phase using a control vasoconstrictor such as noradrenaline, although this is a future possibility.

In the previous chapter, the global haemodynamic effects of systemically administered L-NMMA, indicated the relevance of nitric oxide to the maintenance of vascular tone and the control of blood pressure. The microcirculation in resistance beds makes the greatest contribution to peripheral vascular resistance. In the present chapter, therefore, I have focused on skin microvessels, investigating mechanisms underlying these systemic effects. Using pharmacological, microscopic and laser Doppler techniques, the results of the present study suggest that vascular resistance in the skin arteriovenous anastomotic pathway is increased by L-NMMA, although changes in resistance sufficient to reduce flow are not apparent in areas of mainly nutritive flow. Basal absolute flow was higher in the pulp than in the dorsum, and it may be that L-NMMA inhibited flow-induced release of nitric oxide (Rubanyi et al, 1986b). Thus, the investigations in this chapter have focused on nitric oxide as a mediator of

endothelium-dependent vasodilatation. The possible contribution from other mediators will be discussed in the chapters to follow.

Chapter 4

NON-INVASIVE ASSESSMENT OF ENDOTHELIAL FUNCTION IN HUMAN SKIN MICROVESSELS *IN VIVO*

4.1 INTRODUCTION

In the studies discussed so far, I have concentrated on investigating the physiological role for nitric oxide in healthy individuals. In this chapter, the pharmacology of endothelial function is investigated in blood vessels of the skin microcirculation, using the non-invasive, *in vivo* technique of drug iontophoresis. In subsequent chapters, this investigation will be extended to include alterations in endothelial function in hypercholesterolaemia and hypertension.

Previous investigations of endothelial function have relied on the invasive technique of arterial infusion of drugs, limiting the numbers of subjects that could be easily studied.

4.1.1 Iontophoresis

The principle of drug iontophoresis is that an electrical potential difference will actively cause ions in solution to migrate according to their electrical charge (Figure 4.1). As well as charge, the quantity and distribution of a drug delivered by iontophoresis is dependent upon molecular weight of the drug, magnitude and duration of the current applied, electrode composition (Yoshida & Roberts, 1992), and the site of iontophoresis.

The main barrier to topical application of drugs is the stratum corneum. When this is stripped from the skin, negligible resistance to ion flow is achieved (Wearley et al, 1989). However, when a potential gradient is established across skin with an intact stratum corneum, negatively charged fluorescein ions are readily transported (Burnette & Ongpipattanakul, 1987).

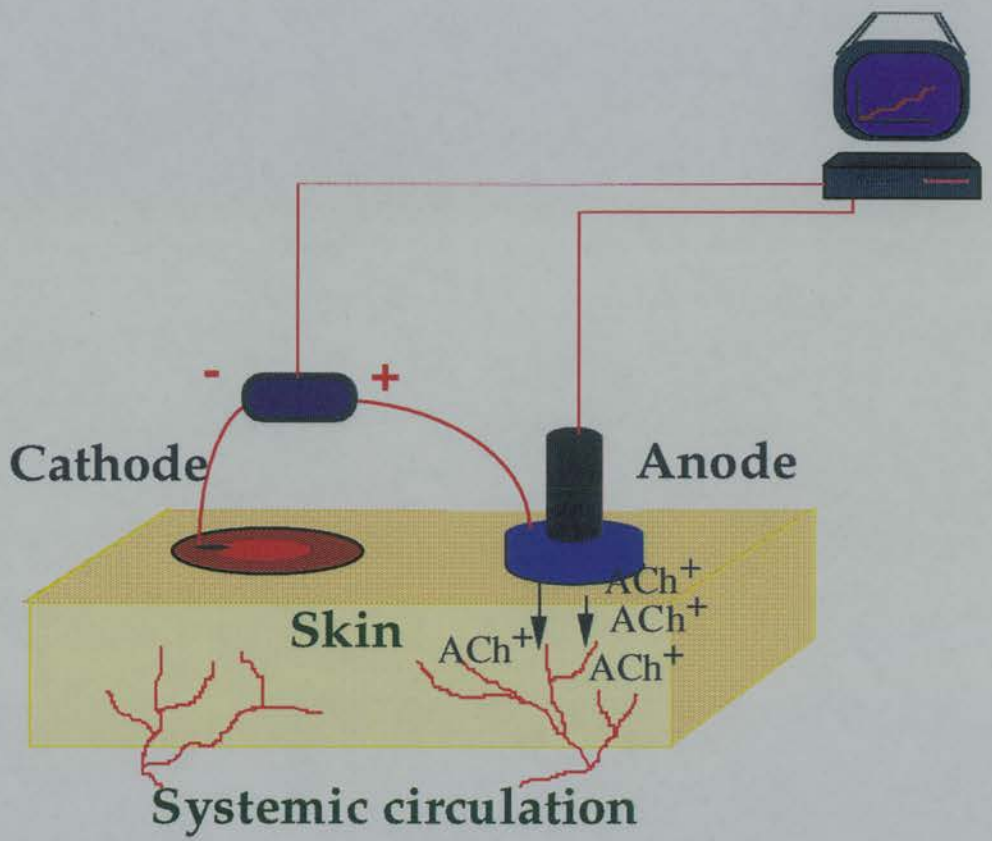


FIGURE 4.1:

Schematic diagram of the iontophoretic delivery of drug ions from the chamber (the anode in this case). Current is delivered by an iontophoretic device which is connected to and computer from where it is controlled. The computer is also used to perform the analysis.

In his classic experiment, Leduc (1900) connected two rabbits in series to an electrical generator (Fig. 4.2). The first rabbit was connected to the machine via an electrode containing strychnine sulphate (positive electrode). The electrode connecting the second rabbit to the machine contained potassium cyanide (negative electrode). To complete the circuit, the rabbits were connected to each other by a water electrode.

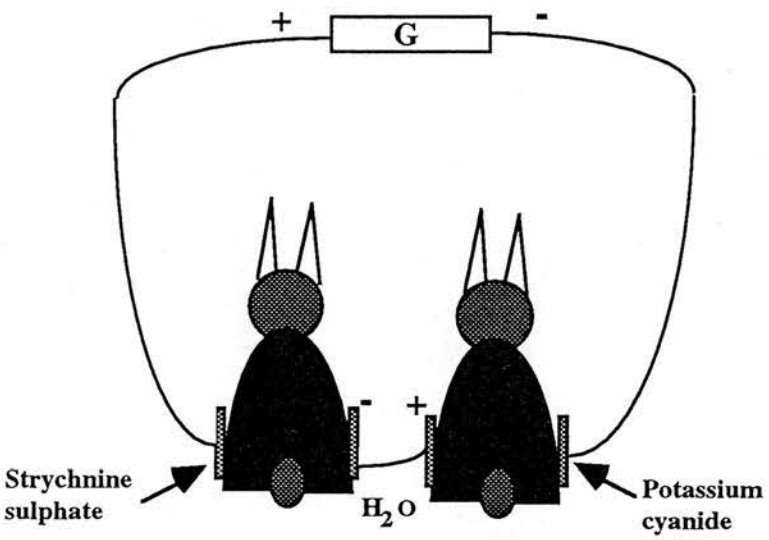


FIGURE 4.2:

Leduc's classic experiment. Two rabbits are placed in series with the same direct current circuit, with strychinine sulphate as the positive electrode and potassium cyanide as the negative electrode. Dramatic results are explained in the text. G = generator.

When a current of 40 - 50 mA was delivered the first rabbit was seized by tetanic convulsions due to the strychnine, while the second rabbit died from cyanide poisoning. However, when the two rabbits were replaced, and the direction of current reversed, neither animal was harmed.

Due to a technological inability to provide controlled iontophoretic delivery of drugs, iontophoresis went out of favour. More recently, many animal models have been used *in vitro* for iontophoretic drug delivery studies, including permeability studies in the hairless mouse (Burnette & Ongpipattanakul, 1987; Pikal & Shah, 1990), hairless guinea pig (Behl et al, 1989), and the hairless rat (Wearley et al, 1989 and 1990). From their initial investigation of skin permeability, ion transport mechanisms, and quantification of ions transferred across the skin, these studies aimed to establish iontophoretic delivery as an alternative route for therapeutic drug administration (see below). *In vivo* experiments have been performed to quantify delivery of simple inorganic ions in the pig (Phipps et al, 1889), to deliver a dobutamine analogue in the dog (Sanderson et al, 1987), radiolabelled dexamethasone in the monkey (Glass et al, 1980), and insulin in the rabbit (Meyer et al, 1989),

In humans, use of the technique as a means of actively delivering therapeutic agents locally has been of interest to dermatologists (for reviews, see Glass et al, 1980; Singh & Maibach, 1993 & 1994). Its success in delivering anticholinergic agents such as poldine methyl sulphate (Hill, 1976), glycopyrronium bromide (Abell & Morgan, 1974), and atropine (Gibinski et al, 1973) in treating hyperhidrosis (for review, see Sloan et al, 1986) has made it well-established in clinical practice.

Iontophoresis of drugs for their local effects has also been combined, for investigative purposes, with transcutaneous electrical nerve stimulation (TENS) to examine neuropeptide release from specific stimulation of polymodal C-nociceptors (in sensory

C-fibres) which terminate just below the stratum corneum (Garnsworthy et al, 1988; Westerman et al, 1988). Neuropeptides released include substance P and calcitonin gene-related peptide (CGRP), which were studied in relation to the development of erythematous wheals (Brain & Edwardson, 1989). The TENS technique has also been combined with iontophoresis in an investigation of neurovascular function in diabetes mellitus (Westerman et al, 1988). Although these investigative studies may be useful in neurophysiology, they do not examine the effects of drugs on microvascular blood flow, or the pharmacological mechanisms involved.

In addition, recent studies have investigated iontophoresis as a method for delivering drugs systemically, e.g., verapamil (Wearley et al, 1988), propranolol (Padmanabhan, 1986), dexamethasone (Glass et al, 1980), and peptides such as insulin (Chien, 1987; Meyer et al, 1989), arginine-vasopressin (Waud, 1967; Benarjee & Ritschel, 1989; Craane-van Hinsberg, 1994), and gonadotropin-releasing hormone (Miller et al, 1990). However, the duration of iontophoresis is often several hours before drug concentrations become measurable in plasma (Singh et al, 1995). Further, in administering drugs systemically using this method, benefits include circumventing hepatic first-pass metabolism through controlled delivery, for example, to reduce toxic effects of drugs such as azidothymidine (AZT) used in the treatment of AIDS (Wearley & Chien, 1990).

4.1.2 Iontophoresis in assessing endothelial function

Despite these extensive studies, no studies have examined the pharmacological mechanisms underlying the vasodilatation of dermal vessels in response to vasoactive agents such as acetylcholine and sodium nitroprusside. The previous two chapters have focused on the importance of nitric oxide in vascular homeostasis. However, as discussed in Chapter 1, cholinergic vasodilatation is dependent on a functioning vascular endothelium but may be mediated by generation of mediators other than nitric

oxide, such as prostacyclin or EDHF. In the present studies, the aims were: (i) to investigate whether iontophoresis of vasodilators acetylcholine and sodium nitroprusside would produce increases in blood flow in skin microvessels and whether these could be assessed, quantitatively, by laser Doppler fluximetry; (ii) to use nitric oxide synthase and cyclooxygenase inhibitors to investigate the pharmacological mechanisms of cholinergic vasodilatation in the dermal vessels; (iii) to establish whether the non-invasive technique of transdermal iontophoresis combined with laser Doppler fluximetry can be used to assess cholinergic vasodilatation in large numbers of patients.

4.2 METHODS

4.2.1 SUBJECTS

Ten healthy men (aged 23-39 yr) took part in the pilot studies; 6 (aged 23-35 yr) in the later studies with L-NMMA and noradrenaline; and a different group of 6 (aged 25-36 yr) in the aspirin study. All participants gave their written, witnessed, informed consent to participate in these studies which were approved by the Lothian Ethics of Medical Research Committee. They abstained from food, caffeine-containing drinks, alcohol and smoking for at least 4 h before each study.

4.2.2 EXPERIMENTAL PROTOCOL I: IONTOPHORESIS PILOT STUDIES

Subjects acclimatised to an ambient temperature of 22-25°C for 30 min, and rested supine throughout the recordings. The skin was cleansed with an isopropyl alcohol swab at the beginning of acclimatisation, and left undisturbed for at least 10 min before attaching a perspex iontophoresis chamber (Moor Instruments Ltd., Millwey, Axminster, Devon, UK), using a double-sided adhesive ring, to the volar aspect of the

right arm. A thermocouple was taped to an adjacent site, so that skin temperature could be monitored throughout iontophoresis experiments and maintained above 32°C (Westerman et al, 1988; and personal communication). Using a 2 ml syringe without a needle, 0.25 ml vehicle or drug was injected into the iontophoresis chamber. A laser Doppler probe was then inserted, to measure flux continuously during each sequence (see below) by laser Doppler fluximetry (described in detail in Chapter 3; Section 3.2.4; p 48). Data were downloaded onto a personal computer for subsequent computerised analysis (Moorsoft V4.241, Moor Instruments Ltd.). The chamber was then removed and cleaned before the procedure was repeated on a neighbouring site with the next solution.

(i) Iontophoretic charge

In delivering drugs across the skin, iontophoresis is based on the principle that the magnitude of an electrical charge (Q) is dependent on the length of time (t) a current (I) is passed ($Q = It$). To ensure minimal discomfort to subjects, small currents (100 - 200 μA; see below) were applied for 10 - 80 s. After agents for iontophoresis were injected into the chamber, and the probe inserted, iontophoresis was started (Moor Iontophoresis Controller 1, Moor Instruments Ltd.) with currents of 100 or 200 μA, of increasing duration (100 μA for 10 s; 200 μA for 10 s; 200 μA for 20 s; 200 μA for 40 s; and 200 μA for 80 s) such that charges of 1, 2, 4, 8 and 16 mC were applied. Response periods were allowed after each charge, before the start of the next: 60 s for 1 and 2 mC, 90s for 4 mC, 120 s for 8 and 16 mC, as these were sufficient to record maximal values before a return toward baseline (Fig. 4.5).

(ii) Vehicle pilot study

Before iontophoresing drugs, an inert vehicle had to be developed and validated. Using the protocol for iontophoretic charge described above, saline (0.9%) was iontophoresed (Fig. 4.3). As vasodilator responses to saline alone were elicited from

the lowest iontophoretic charge upwards, saline was replaced by tap water and the protocol repeated. Tap water also caused vasodilatation, albeit at higher iontophoretic charges, and was replaced by distilled, de-ionised, HPLC-grade water (Rathburn Chemicals Ltd., Walkerburn, Scotland, UK). This still caused some effect at the higher charges and, in addition, induced slight pain at the site of iontophoresis. No burns occurred, but a painless erythematous weal was produced, subsiding after 1-2 hours. Next, methylcellulose gel was reconstituted using the same distilled, de-ionised water to give a 4% concentration. The gel was left to set in a refrigerator overnight before use, and stock solutions subsequently stored at 4°C. This vehicle did not cause responses at 1, 2, 4 and 8 mC (Fig. 4.3) irrespective of the direction of the current, and was adopted into the protocol. In addition to being iontophoretically inert, the gel provided a stable medium for drug handling which enabled controlled transdermal delivery, and did not cause pain or weals.

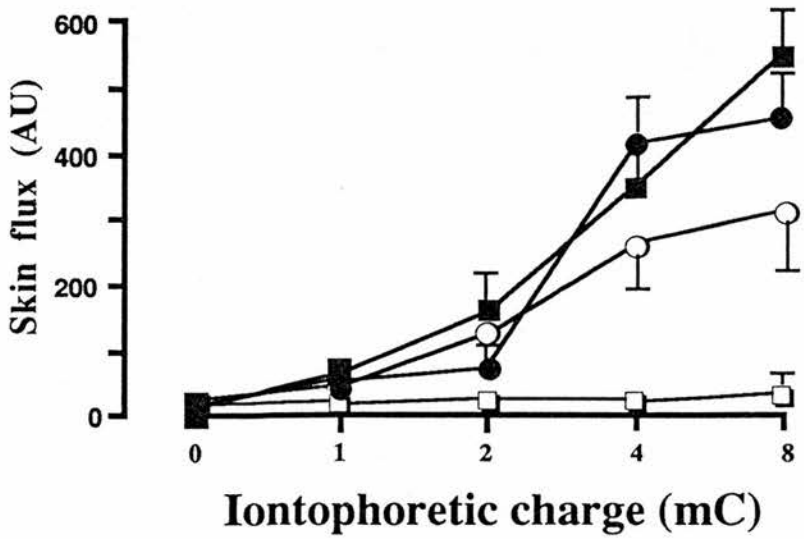


FIGURE 4.3:

Development of an inert vehicle for drug iontophoresis. Responses to saline (■), tap water (●), distilled, de-ionised, HPLC-grade water (○), and methylcellulose gel (2%) reconstituted in distilled, de-ionised, HPLC-grade water (❑).

(ii) Drug concentrations

Acetylcholine was used as an endothelium-dependent, and sodium nitroprusside as an endothelium-independent, vasodilator. For iontophoresis, acetylcholine (Sigma Chemicals Ltd., Poole, Dorset, UK) was weighed (50 mg) and dissolved in de-

ionised, distilled water (1.25 ml) to give a 4% solution. Immediately before each study, this was mixed 1:1 in the de-ionised vehicle (above) to give a final 2% working solution. Sodium nitroprusside (50 mg) (Nipride, Roche Pharmaceutical Products Ltd.) was reconstituted in exactly the same way immediately before each study, to give a 4% solution. This was mixed 1:1 in the de-ionised vehicle (above) to give a final 2% working solution. In the initial studies, these drugs were iontophoresed at even lower concentrations (1%) by doubling the volume of water and gel at the time of reconstitution. Maximal responses were achieved with 2% concentrations using the 1-8 mC charge range, whereas 1% concentrations required an additional charge (16 mC) to acheive the maximum response (Fig. 4.4). At 16 mC, vehicle alone began to elicit responses. Therefore, 2% concentrations delivered from 1-8 mC were used in all later studies. This protocol gave intra-individual and inter-individual coefficients of variation of 34±4 and 41±5% for acetylcholine, and 34±4 and 29±3% for sodium nitroprusside respectively for iontophoretic drug delivery. Acetylcholine, sodium nitroprusside and vehicle were applied in this way in random order in all experiments. Mean flux, measured at the plateau of the response for each drug charge, was expressed in arbitrary units (AU). A typical trace for acetylcholine 2% is shown in Fig. 4.5.

4.2.3 LASER DOPPLER BIOLOGICAL ZERO

Laser Doppler biological zero values were recorded at each iontophoretic site by recording flux during inflation of an upper arm cuff to 220 mm Hg. However, the range of biological zero values obtained was low (between 4.5 and 13.1; mean 7.48±0.32 AU). These values are negligible in relation to the vasodilatation to iontophoresed drugs (Table 4.1; Figs. 4.3 and 4.4), thus, they were not taken into account in analysis of results

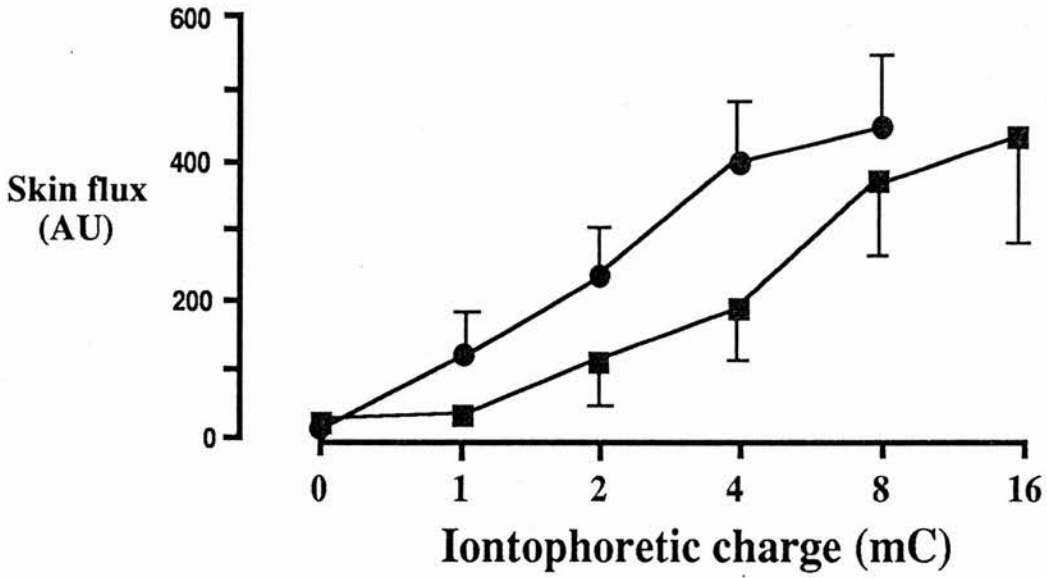


FIGURE 4.4:

Using 2% (●) concentrations, there is a leftward shift in the iontophoretic response curve for acetylcholine compared with 1% (■).

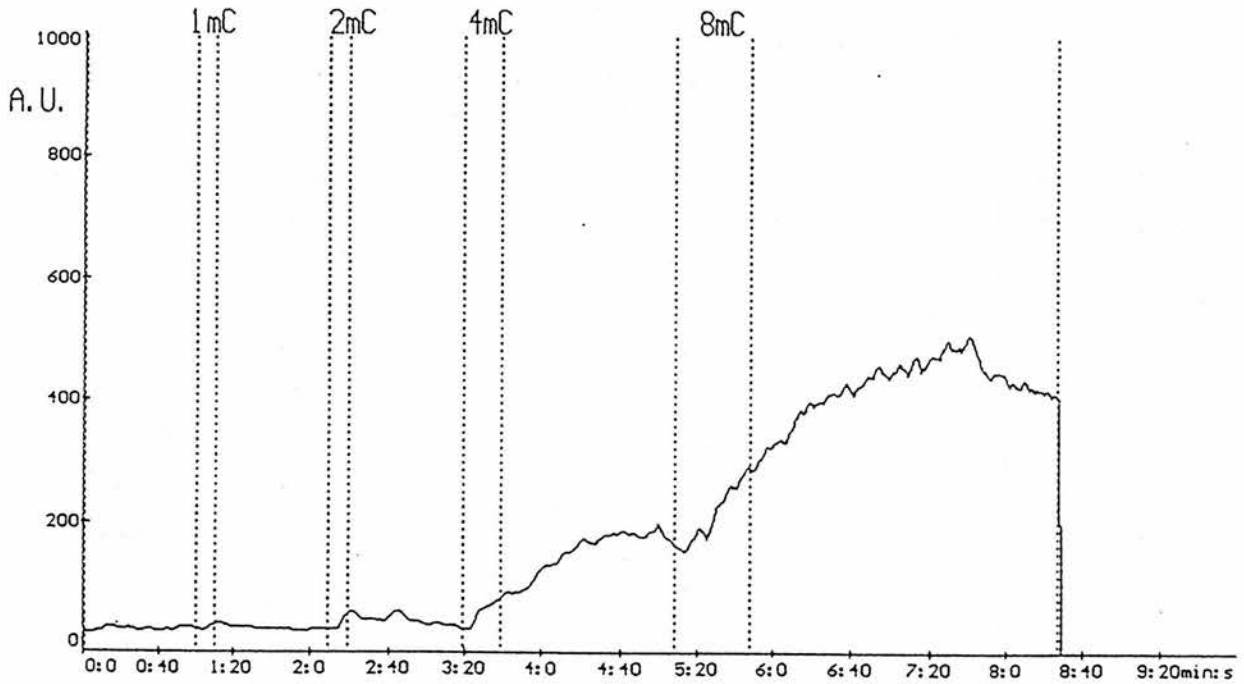


FIGURE 4.5:

Laser Doppler blood flow trace during the iontophoresis of acetylcholine 2% in a healthy individual. The sequence shows flux measured in arbitrary units (vertical axis), against time in minutes (horizontal axis). Iontophoretic charges in millicoulombs (mC) are shown above the sequences.

4.2.4 EXPERIMENTAL PROTOCOL II: DEFINITIVE STUDIES

Effect of L-NMMA and noradrenaline

This was a two phase, randomised, single-blind crossover study, separated by 14 days, in 6 healthy male subjects. Following cannulation of the left brachial artery (Chapter 3; Section 3.2.3; pp 44-45), basal forearm blood flow was recorded in both arms during 0.9% saline infusion (at 1 ml min^{-1}). Acetylcholine and sodium nitroprusside were prepared and iontophoresed according to the protocol described above, with the modification that each drug (order randomised) was iontophoresed in both arms simultaneously using two identical iontophoresis controllers. In one phase, L-NMMA (Chapter 3; Section 3.2.5; p 49) dissolved in physiological saline, was infused via the brachial artery at a rate of 1.0 ml min^{-1} at 4 μmol min^{-1} (for 30 min) while iontophoresis was performed and then forearm blood flow measurements repeated in the final 5 min. In the other phase, noradrenaline (Levophed, Sanofi Winthrop Ltd., Guildford, Surrey, UK), prepared in 0.9% saline, with ascorbic acid (10 μg ml^{-1}) as an anti-oxidant, was infused at increasing doses (60-480 pg ml^{-1}) (Walker et al, 1992) until 40% vasoconstriction was achieved to match the L-NMMA effect. Noradrenaline is a catecholamine derived from the amino acid L-tyrosine, released from sympathetic post-ganglionic nerve terminals. It is a potent agonist at α-adrenoceptors, causing constriction of vascular smooth muscle (Weiner, 1985) independently of the endothelium. The infusion was then continued for 20 min while iontophoresis was repeated, at least 10 min after the last forearm blood flow recording.

Effect of aspirin

This was a two phase, randomised, single-blind crossover study in 6 healthy male subjects. In one phase, iontophoresis of acetylcholine and sodium nitroprusside (order randomised) was performed in the right arm, before and after bolus intravenous injection of aspirin (Aspégic; Laboratories Synthelabo, Paris, France) (600 mg in 5 ml water for injection), via a vein in the antecubital fossa. Aspirin is the acetyl ester of

salicylic acid, and was used because it is a potent, irreversible inhibitor of cyclo-oxygenase, the enzyme responsible for the production of prostaglandins and thromboxanes (Vane, 1971; Roth, 1975; Barrow, 1988), achieving almost total inhibition 30 min after administration (Heavey, 1985). In the other phase, iontophoresis of the same agents was performed before and after 0.9% saline placebo injection. Phases were separated by 14 days.

4.2.5 FOREARM BLOOD FLOW AND DRUG INFUSION

Basal forearm blood flow was measured in both arms simultaneously by venous occlusion plethysmography as previously described (Chapter 3; Section 3.2.3; pp 44-45). In the L-NMMA phase, forearm blood flow was then measured about 30 min after the start of infusion, i.e., as soon as iontophoresis was finished. In the noradrenaline phase, once a 40% reduction in flow was achieved, a period of 10 min was allowed before starting iontophoresis. Final forearm blood flow measurements were then made, before stopping the infusion. Forearm blood flow is given as the ratio of flow in the infused arm to that in the control arm, expressed as percentage change from baseline.

4.2.6 STATISTICAL ANALYSIS

Data are represented as mean±SEM. Analysis of iontophoretic response curves was by repeated measures analysis of variance.

4.3 RESULTS

4.3.1 EFFECT OF L-NMMA AND NORADRENALINE

L-NMMA caused a reduction of forearm blood flow in the infused arm (4.4±1.3 to 2.4±0.8 ml 100 ml^{-1} min^{-1}) but did not affect flow in the control arm (3.6±0.8 to

3.6±0.5 ml 100 ml^{-1} min^{-1}). The ratio of flow in the infused:control arm was 1.2±0.2 before and 0.7±0.2 ml 100 ml^{-1} min^{-1} during L-NMMA infusion, giving a percentage fall from baseline of 43±2% (*P*=0.001). The effect of noradrenaline was well-matched since it caused a reduction in forearm blood flow in the infused arm (3.6±0.7 to 2.0±0.5 ml 100 ml^{-1} min^{-1}) but not in the control arm (2.6±0.4 to 2.7±0.6 ml 100 ml^{-1} min^{-1}). The ratio of flow in the infused:control arm was 1.4±0.2 before and 0.8±0.2 during noradrenaline infusion, i.e., a reduction of 44±2% (P=0.001).

Brachial artery infusion of L-NMMA or noradrenaline did not alter the response to iontophoresis of acetylcholine and sodium nitroprusside in the infused arm (Table 4.1 and Fig. 4.6), and responses were similar to those recorded in the control arm.

	acetylcholine					sodium nitroprusside				
	Basal	1 mC	2 mC	4 mC	8mC	Basal	1 mC	2 mC	4 mC	8mC
L-NMMA										
Flux infused arm (before)	26±5	126±42	232±54	324±72	360±80	52±10	94±19	240±63	399±93	494±119
Flux infused arm (during)	39±11	131±43	239±61	332±76	395±102	29±6	73±31	140±48	257±60	366±79
Flux control arm (before)	40±15	141±55	213±58	310±59	337±48	63±21	112±45	202±84	308±82	365±75
Flux control arm (during)	43±7	245±58	378±84	451±99	497±99	46±14	111±54	180±73	324±105	438±115
Ratio (infused/control)	1.3±0.2	1.0±0.5	1.3±0.7	1.0±0.3	0.9±0.2	0.7±0.1	0.8±0.3	0.8±0.3	0.8±0.2	0.7±0.2
Noradrenaline										
Flux infused arm (before)	32±8	120±36	218±39	296±40	342±40	64±22	95±41	149±51	276±51	342±58
Flux infused arm (during)	57±13	117±17	252±48	300±55	366±69	51±15	79±30	151±35	236±37	335±54
Flux control arm (before)	39±8	226±27	346±54	449±93	469±99	46±9	134±81	210±108	335±97	394±95
Flux control arm (during)	46±13	171±69	322±110	395±115	433±112	41±9	91±53	203±78	339±97	424±103
Ratio (infused/control)	2.1±0.6	0.7±0.5	2.2±0.8	1.5±0.4	1.3±0.4	1.2±0.4	2.4±1.1	1.7±0.7	1.1±0.3	1.1±0.2
Aspirin										
Flux before infusion	32±2	211±72	347±69	408±76	473±81	31±6	80±30	142±46	260±62	302±59
Flux after infusion	35±10	119±25	177±34*	204±37*	222±43*	40±6	117±26	203±38	306±55	355±58
Ratio (Post aspirin/pre aspirin)	1.1±0.3	0.8±0.2	0.5±0.1	0.5±0.1	0.4±0.1	1.6±0.4	3.0±1.0	3.0±1.7	1.5±0.4	1.3±0.3

TABLE 4.1:

Iontophoretic responses to acetylcholine and sodium nitroprusside before and after brachial artery infusion of L-NMMA or noradrenaline, and before and after bolus intravenous injection of aspirin. Results for skin flux from each subject were averaged to give means ± SEM in arbitrary units of flux (AU). Ratios for each subject were calculated individually and expressed as means in the table. For the systemic aspirin study, the ratios post-aspirin:pre-aspirin are also given.

*P<0.01 by ANOVA.

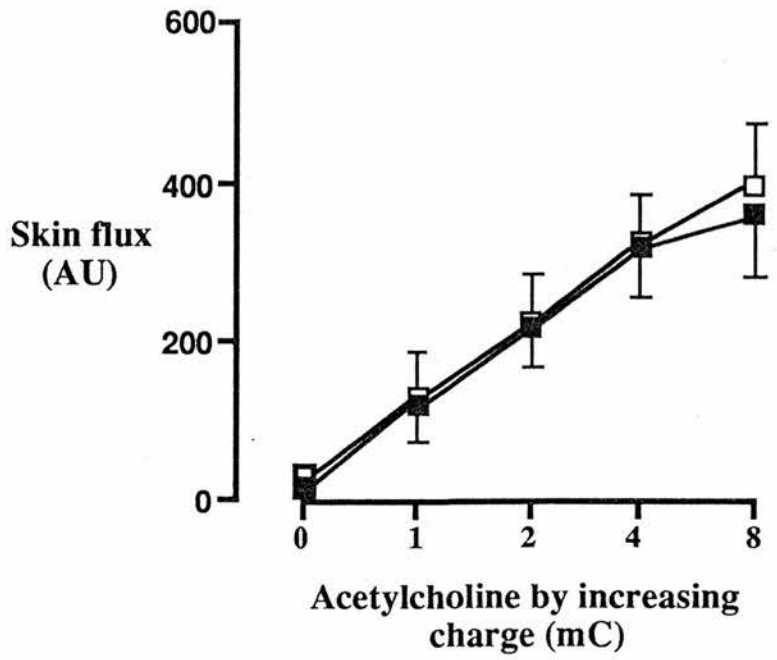


FIGURE 4.6:

Vasodilatation during iontophoresis of acetylcholine before (■) and during (❑) brachial artery infusion of L-NMMA. Units of flux are given as arbitrary units (AU).

4.3.2 EFFECTS OF ASPIRIN

Aspirin inhibited the vasodilator response to acetylcholine (Fig. 4.7), but did not affect the response to sodium nitroprusside (Table 4.1). Placebo injection had no effect on the response to either drug (Table 4.1).

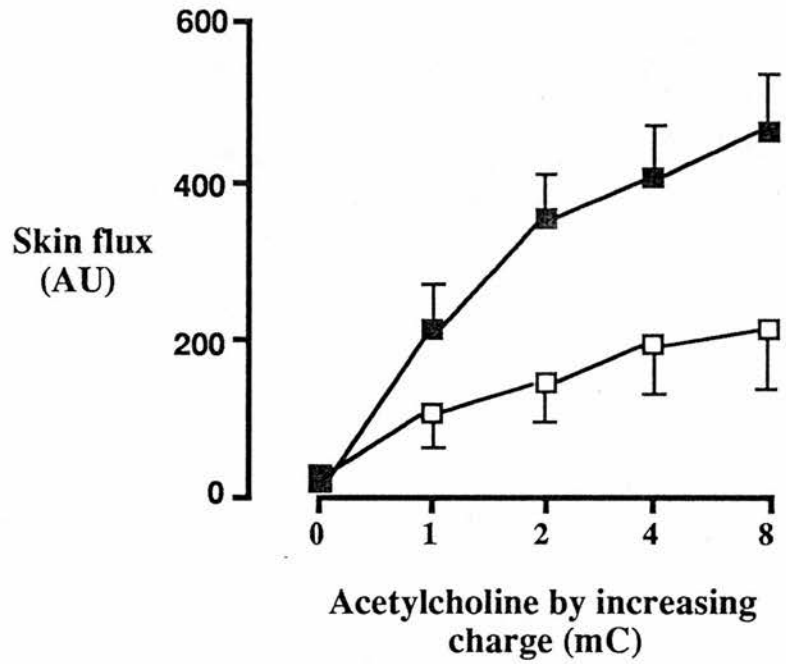


FIGURE 4.7:

Vasodilatation during iontophoresis of acetylcholine before (■), and 30 min after (❐), intravenous injection of aspirin (600 mg). Reduction in flow by aspirin was significant ($P<0.01$). Units of flux are given as arbitrary units (AU).

4.4 DISCUSSION

Acetylcholine and sodium nitroprusside produced dose-related increases in blood flow in skin microvessels measured, quantitatively, by laser Doppler fluximetry. Neither the nitric oxide synthase inhibitor, L-NMMA, nor the non-selective α-adrenoceptor agonist, noradrenaline, infused via the brachial artery, had any effect on responses to iontophoresed acetylcholine or sodium nitroprusside in forearm dermal vessels. Aspirin, but not placebo, markedly reduced cholinergic vasodilatation in dermal vessels. This non-invasive protocol, therefore, measures cholinergic vasodilatation in human dermal vessels, and includes the most favourable electrical charges for drug delivery without discomfort to subjects. No non-specific response to iontophoresis was observed with the charges used, combined with a non-ionic vehicle. By keeping currents low while increasing the duration of iontophoresis, thermally-induced axon reflexes were avoided. Thus, by preventing substance P release, and the inflammatory response to pain, mediated by vasoactive agents such as bradykinin and histamine (Magerl et al, 1990; Westerman et al, 1990), vessels were not induced to become more permeable (Low & Westerman, 1989).

This technique was used to investigate the mechanisms of cholinergic vasodilatation in dermal vessels. Responses to sodium nitroprusside in dermal vessels were unchanged following brachial artery infusion of L-NMMA and noradrenaline, or systemic administration of aspirin. In contrast, vasodilatation in response to acetylcholine was inhibited by aspirin but not by L-NMMA, suggesting that it is, at least in part, mediated by vasodilator prostanoids. There remained an element of cholinergic vasodilatation which is not inhibited by aspirin (approximately 44%). This may be because nitric oxide or EDHF generation may contribute more importantly to cholinergic vasodilatation when prostacyclin generation is inhibited. These results with iontophoresis are analogous to findings in renal arteries where prostacyclin is an important mediator of vasodilatation (Breierwates et al, 1982; Ito et al, 1989), and to

findings in the perfused rat superior mesenteric arcade *in vitro,* where cholinergic dilatation is inhibited by inhibitors of prostanoid synthesis, dexamethasone and indomethacin, but not by the nitric oxide synthase inhibitor, L-NAME (Walker et al, 1995).

Previous studies which have used intra-arterial infusion of drugs combined with blood flow measurements in the forearm, have equated endothelial dysfunction with an impairment in basal (Calver et al, 1992a; Panza et al, 1993a) and stimulated (Panza et al, 1990; Linder et al, 1990) nitric oxide generation. Although iontophoresis provides a non-invasive method of investigating endothelial function, it would appear to be assessing a different pathway. This is consistent with findings from the previous chapter, which suggested that mechanisms of vasodilatation may be dependent on the size and rate of blood flow through the vessels, and on the particular tissue being studied. Further investigation into the pharmacological mechanisms of vasodilatation in skin microvessels, using this non-invasive technique, is required before iontophoresis may be useful, in the long term, in studying large groups of patients in epidemiological and clinic-based research.

The contribution from EDHF to iontophoretic vasodilatation has not been investigated, and its importance could be investigated by iontophoresis in the combined presence of aspirin and L-NMMA. If an element of vasodilatation were to remain in the presence of these inhibitors, and could be abolished with a potassium channel blocker such as tolbutamide, the contribution from EDHF could be confirmed.

With the proviso that the mechanisms underpinning the technique require further investigation, iontophoresis may have potential in studying patients. Patients who have impaired nitric oxide generation as assessed by the 'gold standard' intra-arterial technique, may also have an impaired prostacyclin generation in skin microvessels. If a

correlation exists, iontophoresis may be useful as a marker of, for example, atherogenesis in hypercholesterolaemia, as endothelial dysfunction is known to exist in this condition long before any evidence of the presence of atheroma (Rabbani & Loscalzo, 1991; Arcaro et al, 1995). As the principal mechanism of vasodilatation in forearm dermal vessels would appear to be prostanoid-mediated, iontophoresis could complement the intra-arterial method of investigating endothelial dysfunction, by assessing a different pathway of cholinergic dilatation. Such an investigation, in patients with hypercholesterolaemia, is the subject of the next chapter.

Chapter 5

NON-INVASIVE ASSESSMENT OF ENDOTHELIAL FUNCTION IN HYPERCHOLESTEROLAEMIA

5.1 INTRODUCTION

Along with diabetes mellitus, obesity, smoking and left ventricular hypertrophy, hypertension and hypercholesterolaemia are major risk factors associated with atherosclerotic vascular disease (McMahon & MacDonald, 1986; Dollery, 1987). These factors co-segregate in the population and act synergistically in enhancing the risk of coronary artery disease. In particular, hypertension and hypercholesterolaemia interact closely in potentiating atherogenesis.

A balance exists among haemodynamic factors, serum lipids, platelets, and the blood vessel wall. Alterations in this balance may favour atherosclerosis. Endothelial cells act not only as a lining for blood vessels, but also have many functional roles (Chapter 1; Sections 1.2.1, 1.2.5 and 1.2.6) which, when perturbed, may participate in atherogenesis (Gimbrone, 1976). These functions include the provision of a non-adherent surface for leukocytes and platelets; a permeable barrier that controls the exchange of nutrients and fluid between the plasma and the arterial wall; maintenance of vascular tone by release of nitric oxide, prostacyclin, endothelin and angiotensin II; formation and secretion of a series of growth-regulatory molecules and cytokines; formation and maintenance of connective tissue matrix, including the basement membrane upon which it lies, collagen and elastic fibres, and proteoglycans; the capacity to modify substances from the plasma such as lipoproteins that endothelial cells can oxidise or otherwise modify as they are transported into the artery wall; provision of a nonthrombogenic surface by the formation of ectoADPase, PGI_2, and heparin sulphate; and provision of anti-coagulant and pro-coagulant properties (Raines & Ross, 1995).

Alterations in one or more of these functions of the endothelium are important during the early phase of atherogenesis. One of the earliest changes in the endothelium is seen

in its permeability, wherein increased amounts of lipoprotein (in hypercholesterolaemic patients) are transported by the endothelium to localise in the subendothelial space of the intima (Simionescu et al, 1990). During transcytosis, many of these lipoprotein particles may be modified by oxidation or glycation by the endothelium (Steinberg, 1991). The formation of oxidised LDL (oxLDL) may in turn have repercussions on the endothelium because oxLDL may induce expression of genes that cause it to produce chemotactic molecules, additional cell adhesion and growth regulatory molecules, and cytokines (Raines & Ross, 1995). In addition, macrophages become engorged with oxLDLs and lose their scavenging function to become foam cells which give rise to the characteristic fatty streak of early plaque formation. Endothelial cells have shear response elements which can also induce changes in gene expression at sites of change in blood flow (Nagel et al, 1994) and as a result of hypertension (Chobanian, 1989). The capacity of the endothelium to maintain normal arterial tone represents an important contribution from these cells via several molecules, including nitric oxide and prostacyclin. Ironically, the capacity of the endothelium to produce nitric oxide may also play a role in its capacity to further oxidise LDL to form oxLDL, which may also result from endothelial lipoxygenases (Chester et al, 1990). Nitric oxide is not only vasodilatory but also prevents platelet adhesion and aggregation, and leukocyte adhesion (Radomski et al, 1987). Endothelium-derived vasoconstrictors such as ET and angiotensin II are also involved in the maintenance of vascular tone. Therefore, the balance of these vasodilator and vasoconstrictor effects is important in determining the dimensions of the arterial luminal diameter, and essential in arteries affected by atherosclerosis in regulating flow to the heart muscle and brain (Shimokawa & Vanhoutte, 1990; Dzau, 1990).

Demonstrations of endothelial dysfunction in hypercholesterolaemic animals (Andrews et al, 1987; Cohen et al, 1988; Verbeuren et al, 1986; Shimokawa & Vanhoutte, 1989; Tomita et al, 1990) and humans (Ludmer et al, 1986; Chowienczyk et al, 1992; Casino

et al, 1993) have been attributed to attenuated endothelium-dependent vasodilatation, and taken to reflect impaired nitric oxide generation. However, this abnormality may be due to an increased degradation of nitric oxide by superoxide anion or other oxygen-derived free radicals (Gryglewski et al, 1986; Minor et al, 1990; Mügge et al, 1991), or altered activity of nitric oxide synthase (Mitchell et al, 1990).

Despite the above comments, the means by which hypertension induces lesions of atherosclerosis is poorly understood. Genetic studies of hypertension in animals have used a restriction fragment length polymorphism for renin in the Dahl/JR salt-sensitive rat, which co-segregates with existing hypertension, suggesting that a genetic locus linked with this polymorphism is somehow responsible for the hypertension (Dzau, 1990). Both smooth muscle hypertrophy and intimal hyperplasia occur in animal models of experimentally induced hypertension, as well as in arteries of humans with hypertension (Owens, 1989). In the rat, these hypertrophic events have been shown to be due, at least in part, to an increase in ploidy and in the size of the arterial smooth muscle cells. There seems to be an interrelationship between mechanisms which induce smooth muscle proliferation and smooth muscle contraction (Raines & Ross, 1995). Elements of the sympathetic nervous system which are activated in hypertension can induce vascular wall growth. Angiotensin II is elevated in many hypertensives and can directly mediate smooth muscle hyperplasia (Schelling et al, 1991; Baker et al, 1992). Other vasoactive substances such as serotonin, endothelin-1, and thrombin can also stimulate smooth muscle replication, whereas inhibitors of vasoconstriction, such as atrial natriuretic peptide may have an opposing effect (Dzau, 1987).

Similar to changes observed in experimentally induced hypercholesterolaemia, Chobanian (1990) has demonstrated endothelial dysfunction in experimental hypertension with monocyte adhesion and trasendothelial migration of the adherent cells. The monocytes become macrophages after they enter the intima even though little

or no lipid accumulated in them under these conditions. The combined effects of hyperlipidaemia and hypertension in rabbits can lead to a marked enhancement of the lesions of atheroslerosis (Ross, 1995). Thus, the common theme of endothelial dysfunction, inflammation, and a fibroproliferative response recurs in hypertension as well as hyperlipidaemia.

Patients with essential hypertension and hypercholesterolaemia have impaired vasodilatation in forearm and coronary vessels following intra-arterial infusion of acetylcholine (Chapter 1; Section 1.2.7 and 1.4). It is unclear, however, whether impaired nitric oxide generation in hypertension and hypercholesterolaemia is confined to skeletal muscle resistance vessels and coronary vessels, and whether the defect is specific to nitric oxide generation or also affects other mediators such as prostacyclin.

Having described, in the previous chapter, the pharmacological mechanisms of a non-invasive method for investigating endothelial function, this technique is now compared with the 'gold standard' intra-arterial method of assessing endothelial function in hypercholesterolaemic patients. The aim of this chapter is to establish, in hypercholesterolaemic patients, whether endothelial dysfunction is restricted the forearm skeletal muscle vessels, or also measurable in the dermal microcirculation.

5.2 METHODS

5.2.1 SUBJECTS

This study compared 10 hypercholesterolaemic patients who were recruited from the Cardiovascular Risk clinic at the Western General Hospital in Edinburgh, and 10 healthy control subjects recruited by advertisement (Table 5.1). No subject received vasoactive or non-steroidal anti-inflammatory drugs during the week before each phase

of the study, and all of the subjects abstained from alcohol for 24 hours, and from food, caffeine-containing drinks and cigarettes for at least 2 h before each phase. Subjects rested in the recumbent position during each phase, in a quiet room maintained at a constant temperature of 22-25°C. All participants gave their written, witnessed, informed consent to participate in these studies which were approved by the Lothian Area of Medical Ethics Committee.

5.2.2 ARTERIAL INFUSION AND FOREARM BLOOD FLOW STUDY

This part of the study took place on two separate occasions, separated by 14 days. On one study day, having acclimatised to an ambient temperature of 24-26°C for 30 min, basal measurements of forearm blood flow were obtained (methodology described in Chapter 3; Section 3.2.3; pp 44-45). A pharmaceutical-grade, sterile and pyrogen-free preparation of acetylcholine (Miochol, IOLAB, Bracknell, UK) was diluted in physiological (0.9%) saline to give 7.5 and 15 $\mu g\ ml^{-1}$ solutions which were each infused intra-arterially at 1 $ml\ min^{-1}$ (Chowienczyk, 1992). The same pharmaceutical brand of sodium nitroprusside described in Chapter 4 (Section 4.2.2; p 69) for iontophoresis was used for intra-arterial infusion in the present study. However, its preparation was different: sodium nitroprusside was reconstituted in physiological saline and diluted to give 3 and 10 $\mu g\ ml^{-1}$ solutions which were also infused intra-arterially at 1 $ml\ min^{-1}$ (Chowienczyk, 1992).

Acetylcholine and sodium nitroprusside were then infused, in random order, via the left brachial artery at 1 $ml\ min^{-1}$ for 6 min at each dose (Chowienczyk et al, 1992). Forearm blood flow measurements were obtained during the final 3 min of each dose. On the second study day, this protocol was repeated 30 min after intravenous bolus injection of aspirin 600 mg (as in Chapter 4).

5.2.3 IONTOPHORESIS STUDY

Iontophoresis with acetylcholine and sodium nitroprusside was performed, on the third day of the study, before and after administration of aspirin (as in Chapter 4).

5.2.4 STATISTICAL ANALYSIS

Data are represented as mean±SEM. Analysis of iontophoretic response curves was by repeated measures analysis of variance (StatView on Macintosh).

	Sample size (M/F)	Age range (yr)	MAP (mm Hg)	Total serum cholesterol (mmol l^{-1})	Weight (kg)
HC	7/3	38-63 (mean: 52±3)	97.8±3.6	7.9±0.2 (range: 7.2-9.2)	86.7±4.1
Controls	7/3	38-63 (mean: 49±4)	92.9±2.8	4.2±0.2 (range: 3.2-5.1)	73.3±3.1
ANOVA		P=0.07	P=0.32	P=0.0001	P=0.07

TABLE 5.1:

Demographic data from hypercholesterolaemic patients and from age- and sex-matched control subjects. HC = hypercholesterolaemic patients; MAP = mean arterial blood pressure. Data from both groups were analysed by repeated measures analysis of variance (ANOVA), and P-values are shown.

5.3 RESULTS

5.3.1 ARTERIAL INFUSION AND FOREARM BLOOD FLOW STUDY

The increase in forearm blood flow following intra-arterial infusion of acetylcholine was significantly blunted in hypercholesterolaemic patients compared with healthy controls (Fig. 5.1). Aspirin had no effect on controls, but restored the vasodilator response towards normal in hypercholesterolaemic patients (Fig. 5.1). Responses to sodium nitroprusside are shown in Fig. 5.2 and were not different between groups.

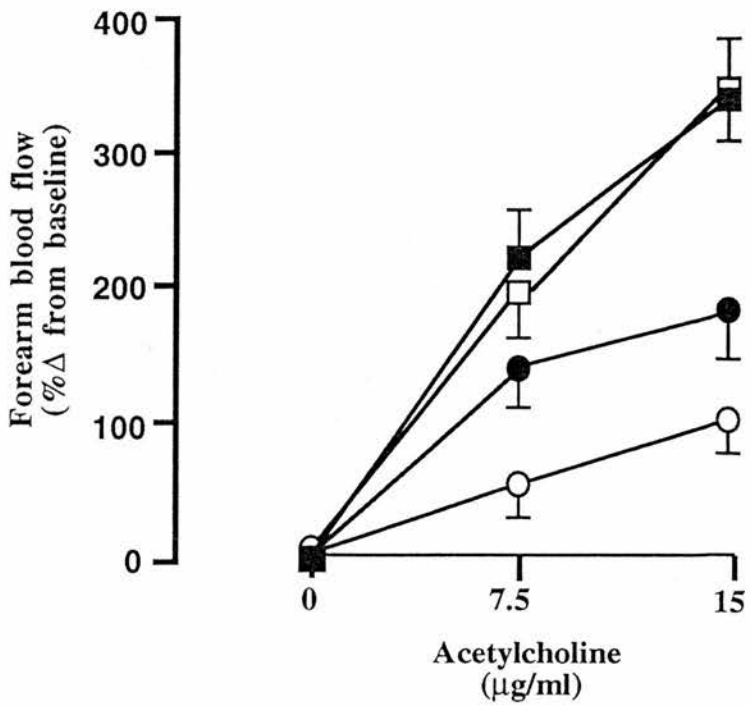


FIGURE 5.1:

Forearm vasodilatation in response to intra-arterial acetylcholine in hypercholesterolaemic patients (○), expressed as percentage change (%Δ) from baseline, was significantly lower than the vasodilatation in control subjects (■) (P=0.0002). Bolus intravenous injection of aspirin (600 mg) did not affect the control subjects (□), but potentiated the response in the patients (●) (P=0.001).

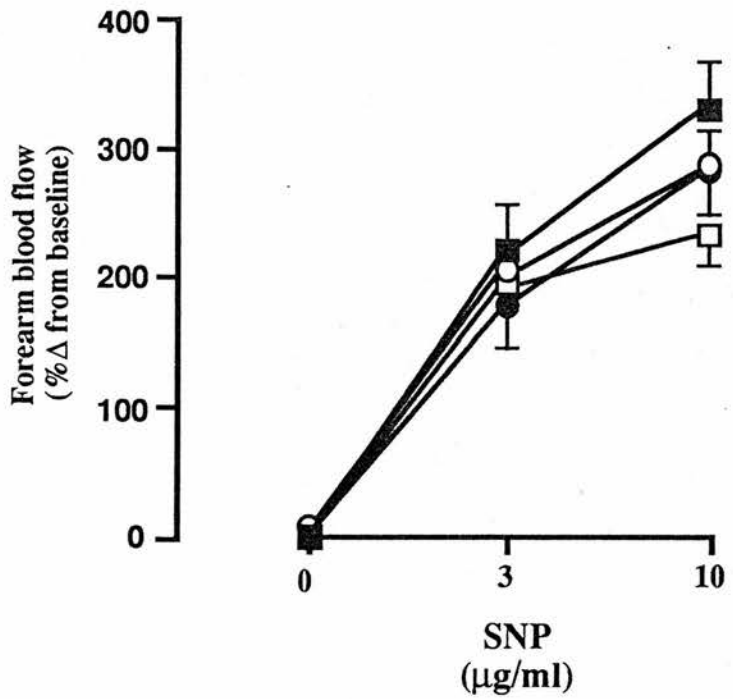


FIGURE 5.2:

Forearm vasodilatation in response to intra-arterial sodium nitroprusside in hypercholesterolaemic patients (●), expressed as percentage change (%Δ) from baseline, and in healthy controls (■) before intravenous injection of aspirin (600 mg). Responses after aspirin injection in patients (○) and in controls (□) are also shown.

5.3.2 IONTOPHORESIS

The increase in skin blood flow in response to the iontophoresis of acetylcholine was similar for both groups before aspirin. However, the inhibitory effect of aspirin on dermal cholinergic dilatation was greater in the hypercholesterolaemic group (Fig. 5.3) such that, in the presence of aspirin, cholinergic vasodilatation was markedly impaired (P=0.0001). Responses to sodium nitroprusside were lower in the patient group, both before and after aspirin (Fig. 5.4).

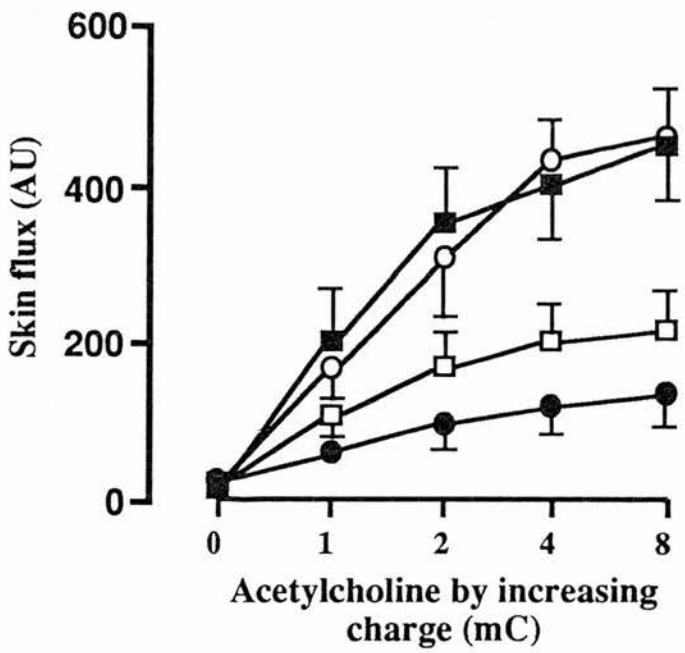


FIGURE 5.3:

Iontophoresis of acetylcholine caused a dose-related increase in skin red cell flux in hypercholesterolaemic patients (○) and in healthy control subjects (■). After bolus intravenous injection of aspirin (600 mg), this response was partially inhibited in the controls (❐) and more substantially in patients (●) (P=0.0001).

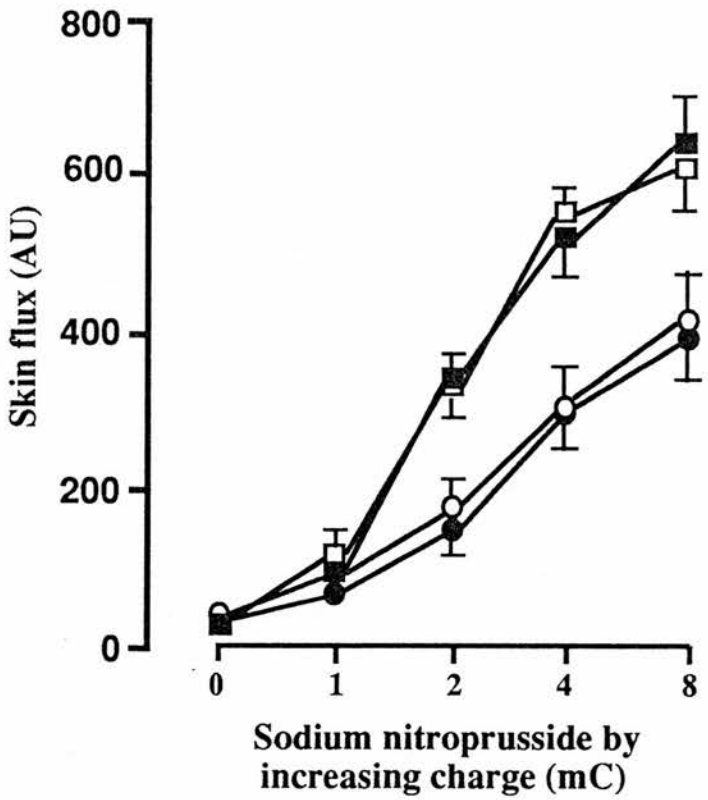


FIGURE 5.4:

Iontophoresis of sodium nitroprusside caused an increase in skin red cell flux in hypercholesterolaemic patients (○) and in healthy controls (■). Basal responses in hypercholesterolaemic patients were lower and unaffected by aspirin (●). sodium nitroprusside response in controls was also unaffected by aspirin (❑).

5.4 DISCUSSION

In keeping with other studies (Chowienczyk et al, 1992; Casino et al, 1993), the findings of the present study confirm that cholinergic vasodilatation is impaired in hypercholesterolaemic patients (Fig. 5.1). In the forearm, this defect is partially reversed by aspirin, suggesting either that a vasoconstrictor prostaglandin influences tone in this vascular bed in hypercholesterolaemia, or that in the presence of aspirin, nitric oxide release is improved in hypercholesterolaemia. This partial reversal is consistent with results in essential (but not secondary) hypertension where the vasodilator response to acetylcholine is increased in the presence of indomethacin (Taddei et al, 1993). In addition, this normalisation of the acetylcholine response following cyclooxygenase inhibition has been seen in spontaneously hypertensive rats (Iwama et al, 1992), and in stroke-prone spontaneously hypertensive rats (Diederich et al, 1990). This evidence suggests that the release of relaxing factors may be normal, but that the concomitant, possibly enhanced, release of a cyclooxygenase-dependent endothelium-derived contracting factor (probably prostaglandin H_2) accounts for the impaired relaxation. Furthermore, defects in the metabolism of the contracting prostanoid, PGH_2 could not only upregulate its synthesis, but could also inhibit prostacyclin synthase (Lin et al, 1994). However, there appeared to be no differences in forearm vasodilatation to sodium nitroprusside between the two groups. Both sodium nitroprusside and native nitric oxide act by stimulating soluable guanylate cyclase. Therefore, impaired vasodilation to acetylcholine may have nothing to do with the amount of nitric oxide generated or reduced smooth muscle responsiveness, and it may be that the explanation of impaired nitric oxide production usually given to account for endothelial dysfunction may need to be re-addressed.

Results in dermal vessels were different from those in the forearm. Hypercholesterolaemic patients did not have impaired cholinergic vasodilatation in

dermal vessels in the absence of aspirin, possibly because nitric oxide generation is normal, or is not an important mediator of vasodilatation in the dermis, consistent with results from the previous chapter. However, the inhibiting effect of aspirin was greater in these patients so that impaired cholinergic vasodilatation was evident in the presence of aspirin. This suggests that they have increased generation of dilator prostanoids by acetylcholine, but decreased generation, or sensitivity to, other mediators.

Responses to sodium nitroprusside in the dermis were also different between healthy subjects and hypercholesterolaemic subjects. In the forearm, previous investigators have used sodium nitroprusside as a control to show that changes in cholinergic vasodilatation are not mediated by altered vascular smooth muscle sensitivity to nitric oxide. In the present study, sodium nitroprusside sensitivity in the forearm was not different in hypercholesterolaemia and not affected by aspirin. However, in dermal vessels, the vasodilatator response to sodium nitroprusside was impaired in hypercholesterolaemia and unaffected by aspirin. As sodium nitroprusside acts by stimulating guanylate cyclase to increase cGMP levels, it may be that, in the dermis, there is a defect in this second messenger system, and therefore, a reduced sensitivity to nitric oxide, whether endothelium-derived or exogenously-donated. Vasodilatation mediated by prostanoids such as prostacyclin is cAMP-dependent, and, in dermal vessels, this pathway may be favoured in hypercholesterolaemia.

Thus, smooth muscle sensitivity to sodium nitroprusside appears only to be reduced in the dermis, while endothelial dysfunction affects the forearm and the dermis: vasodilatation to acetylcholine appears to depend on the local balance between vasoactive prostanoids in these tissues.

In hypertensive animal models (spontaneously hypertensive rats), the sodium nitroprusside response is enhanced compared with normotensive controls (Wistar-

Kyoto rats) (Tesfamariam et al, 1988; Diederich et al, 1990), a finding which is more pronounced after the endothelium has been removed. This may be due to hypertrophy of the media in spontaneously hypertensive rats leading to increased responsiveness to sodium nitroprusside, or due to removal of endothelium-derived contracting factors. Vascular hypertrophy may not be present in dermal microvessels of hypercholesterolaemic patients, and may explain why there was reduced, rather than enhanced sensitivity to sodium nitroprusside. In addition, the findings of an impaired sodium nitroprusside response could account for the impaired cholinergic dilatation in dermal vessels in hypercholesterolaemia in the presence of aspirin, since dilatation may then depend on nitric oxide.

The present findings in hypercholesterolaemia suggest that, in contrast with the forearm circulation, dermal vessels have increased dilator prostanoid generation, and the impairment of cholinergic vasodilatation in the presence of aspirin can be attributed to impaired vascular smooth muscle sensitivity to nitric oxide. The 'endothelial dysfunction' of hypercholesterolaemia may, therefore, be different between certain vascular beds and certain endothelial mediators. However, in addition to assessing endothelial function, iontophoresis also provides important information on smooth muscle function.

Despite the discussion in Section 5.1, the relationship between hypertension and atherosclerosis and its clinical complications is poorly understood. Further study is needed of the mechanisms by which hypertension itself and in combination with other risk factors affects the arterial wall. Whether structural or functional abnormalities of the vessel wall themselves contribute to the development of hypertension is the subject of the next chapter.

Chapter 6

MICROVASCULAR FUNCTIONAL AND STRUCTURAL ABNORMALITIES: CAUSE OR CONSEQUENCE OF ESSENTIAL HYPERTENSION?

6.1 INTRODUCTION

Established essential hypertension is characterised by increased peripheral vascular resistance, reflected predominately in vessels with an internal diameter of <100 μm (Struijker Boudier et al, 1992). This may be due to structural abnormalities, such as reduced luminal diameter, or a reduction in the density of vessels per unit volume of tissue (rarefaction), or functional abnormalities or a combination of both. However, from studies in established hypertension, it is not possible to ascertain whether vascular abnormalities are a cause or consequence of the rise in blood pressure.

Hypertension has been defined by the World Health Organisation criteria to be present if systolic blood pressure (SBP) is 140 mm Hg or more and/or diastolic blood pressure (DBP) is 95 mm Hg or more. As such, it has been shown to affect up to 24% of all individuals from populations within developed societies, and up to 50% of people over fifty years of age (Burt et al, 1995). The cardiovascular, cerebrovascular and renal complications of hypertension exert a significant impact on health care resources. A recent survey has shown that only 53% of all hypertensives are on treatment for hypertension in order to prevent secondary complications (Burt et al, 1995). Despite this, hypertension remains the commonest reason for initiating life-time medication (Rose, 1985). In 95% of cases, hypertension is a primary condition labelled as essential hypertension, on the basis that no underlying or secondary causes for the elevation in blood pressure (BP) such as renal artery stenosis, hyperaldosteronism or phaeochromocytoma have been diagnosed (Berglund, 1985). However, the causes of elevated BP in essential hypertension remain unclear.

Hypertension does not have a defined phenotype, but is simply the tail of a skewed frequency-distribution curve for blood pressure (Pickering, 1968). The distribution of measured BP among individuals from a population is a consequence of their biological

diversity, their adopted lifestyle and errors or variation in the measurement. Various arbitrary values have been used to define hypertension of differing severities in the clinical setting, but measured blood pressure has been shown both to increase with age and be influenced by situational effects at the time of measurement. Assessments of the distribution of blood pressure within populations have often been based on single measurements thereby potentially over-estimating the true prevalence of essential hypertension (Pickering, 1961). In early studies, complications such as heart failure, impaired renal function or retinopathy were often labelled as a complex of symptoms equating with 'hypertension' and subsequently included in the analysis of some populations, leading to falsely high estimates (Pickering, 1961; Murphy et al, 1967).

Familial co-segregation of high blood pressure and its complications is well-documented (Miall et al, 1963; Ward, 1990), and could be a consequence of common environmental, genetic or combined factors. In addition, it could be due to the influences of age, sex and other confounding genetic or environmental factors such as obesity. These might not be randomly distributed through affected families, potentially introducing bias. Many early studies of 'essential' hypertension should also be interpreted with caution. The medical technology of today was not available to early investigators, and reports of quite severe hypertension in children (Ayman, 1934) suggests that secondary causes may not have been adequately excluded from the groups studied.

Between populations, blood pressure differences appear to be influenced more by environmental and socio-cultural factors, in particular, dietary sodium intake, physical activity, obesity and psychosocial stress (Ward, 1990). Though genetic factors may play a role, for example by influencing sodium handling (Oshima et al, 1994) or the degree of obesity, 'Westernisation' of lifestyle is likely to be a major factor. However, within groups from diverse ethnic and genetic backgrounds, the presence of familial

co-segregation has prompted investigators to look at genetic causes. Platt (1947) found an excess of hypertension in parents of hypertensive probands, compared to the parents of normotensives, but relied on histories suggestive of hypertension-related complications (Platt, 1947). Reported transmission of high blood pressure through three generations (Ayman, 1934), together with an apparent partition in the frequency-distribution curve for blood pressure measurements from siblings of hypertensive patients giving a bimodal curve with approximately equivalent portions, prompted him to suggest that blood pressure was inherited in a single autosomal dominant gene (Platt, 1947 and 1959). However, Murphy (1964) showed that apparent bimodality or polymodality could arise from a number of errors, including observer differences, digit preference in blood pressure recording (i.e., selecting against intermediate values), the class intervals used in presenting the data, and small sample sizes, even when taken from a population with a unimodal distribution.

From a larger number of subjects in the *Precursors of Hypertension and Coronary Disease Study,* coordinated by John Hopkins University, both SBP and DBP were shown to have distributions with a positive skew which could be restored to a binomial distribution following logarithmic transformation (Murphy et al, 1967). Hamilton and colleagues (1954) recognised the need for adjustment of BP for age and sex, and found no natural dividing line between hypertensives and normotensives (Hamilton et al, 1954a, and 1954b). They did, however, find a consistent familial co-segregation, with regression coefficient for SBP and DBP of 0.22 and 0.18 respectively, irrespective of whether the probands were normotensive or hypertensive (Hamilton et al, 1954c). In a larger population-based study which took a random sample from the general population, in Rhondda Fach, UK, it was estimated that 33% of SBP and 20% of DBP was genetically determined. No suggestion of bimodality was seen for BP, but it was accepted by the authors that single gene inheritance could account for a very small number of cases (Miall, 1963).Other population-based studies including the

Tecumseh (Deutscher et al, 1966) and Framingham (Castelli et al, 1986) studies in the USA have found variable but significant regression coefficients for first degree relatives of hypertensive probands.

If genetic factors are important in the aetiology of essential hypertension, they potentially could be operating before the clinical diagnosis is made, hence it has been of interest to ascertain whether familial co-segregation of BP existed from childhood. A study performed in children aged 2-14 years from 192 families demonstrated familial co-segregation of BP (Zinner et al, 1971). This was achieved by observing that the distribution of mean familial scores (in standard deviation units (SDU) where one SDU is equal to the individual's BP minus the mean for their specific age- and sex-group divided by the SD of mean BP in that group) differed significantly from the expected normal distribution (which would have an SD of one, and a mean of zero). Stronger regression coefficients existed between child propositi and their siblings compared with their mothers. A weak correlation between infant and maternal BP has been found at birth, but a significant relationship with paternal BP was not found until one month old (Zinner et al, 1985). At birth, infant BP could be affected by the intrauterine environment which in turn may be influenced by both maternal genes and environment. A significant contribution to any expected correlation in BP between parents and offspring and between offspring could be a consequence of shared environment. In the case of DBP, 67% of the expected correlation was calculated to be due to environment, and 33% due to shared genes (Annest et al, 1979).

Pickering and Platt became engaged in a highly publicised debate over the inheritance of essential hypertension, but Pickering has gained more support for his proposal of a polygenic inheritance together with an environmental influence as the basis for the disease (Pickering, 1961 and 1968; Platt, 1947 and 1959; Miall, 1963; Williams et al,

1992). It has now been estimated that between 20-60% of the population variability in BP is genetically determined (Ward, 1990; Soubrier, 1993).

Recently, clear examples of autosomal dominant inheritance of hypertension mediated by single gene defects have been described. Glucocorticoid-remediable hyperaldosteronism (GRA) does not reliably have hypokalaemia but it always presents early in life with a positive family history of low renin to allow distinction from essential hypertension. With this in mind, diagnosis of GRA rests on finding elevated urinary excretion of steroid metabolites, 18-hydroxycortisol and 18-oxycortisol. Lifton and colleagues identified linkage of GRA with a restriction fragment length polymorphism arising from a fusion event occurring between the ACTH responsive regulatory sequences of the 11ß-hydroxylase gene and the coding sequences of the aldosterone synthase gene. This has resulted in ectopic expression of aldosterone synthase in the adrenal zona fasciculata which responds to stimulation by ACTH rather than angiotensin II or potassium (Lifton et al, 1992).

Liddle's Syndrome (pseudoaldosteronism) has long been recognised as having an autosomal dominant mode of inheritance with constitutive activation of the Na^+-H^+ exchange channel in the distal tubule (Botero-Velez et al, 1994). Recently, polymorphisms for the gene coding for the ß-subunit of the epithelial Na^+-channel have been found to be linked with hypertension in five kindreds with the syndrome (Shimkets et al, 1994). It is, therefore, quite possible that other single gene defects may be identified causing hypertension in certain kindred (Kurtz, 1993).

Other candidate genes have included one for electrolyte metabolism, e.g., for sodium-lithium countertransport (Hasstedt et al, 1988) and urinary kallikrein (Berry et al, 1989), and the glucocorticoid receptor gene (Watt et al, 1992). However, polygenic

and environmental causes are also considered important, and it is likely that hypertension results from an overlap of all of these factors.

High blood pressure in humans may result from the interaction of a number of genes - with each other and with the environment (Lindpainter, 1993). The genetic heterogeneity of the human race and the difficulty in controlling environmental factors makes genetic investigation problematic. Recent work by Barker (1989, 1990, 1992 and 1995), approaches the 'initiation' of hypertension from a different perspective, suggesting a fetal origin for this and other cardiovascular diseases.

Barker and colleagues suggest that the origins of adult hypertension are initiated before childhood, i.e., during fetal growth (Barker et al, 1990; Barker, 1992). The hypothesis states that fetal undernutrition in middle to late gestation 'programmes' later development of coronary heart disease, hypertension and diabetes (Barker, 1992). Low birth weight and a disproportionate placental weight, head size, and length are markers of lack of nutrients or oxygen at critical stages of fetal development. This programming causes permanent alterations in structure, physiology and metabolism of affected individuals. Earlier this century, geographical patterns of poor maternal health and physique, poor fetal growth, and high death rates from cardiovascular disease have been derived from maternal and infant mortality data (HMSO, 1910; Campbell et al, 1932). The first evidence of an association between birth weight and adult cardiovascular disease came from the 'Hertfordshire' retrospective study, in which 5654 men, born between 1911-30, were studied. Those who had the lowest weights at birth and at year 1, subsequently had the highest death rates from ischaemic heart disease (Barker et al, 1989). In following up adults who were born between 1935-43 in a hospital in Preston which kept detailed, standardised records of births, Barker gained the evidence which confirmed his 'programming' hypothesis (Barker et al, 1990). These observations support Folkow's proposal that adult hypertension is

determined by initiating and amplification mechanisms (Folkow, 1978). Furthermore, the theories of Barker are supported by increasing evidence from other workers (Edwards et al, 1993; McKeigue et al, 1994; Fall et al, 1995), and have prompted a part of the study described later in this chapter.

6.1.1 Vascular abnormalities in essential hypertension: cause or consequence?

Ascertaining whether vascular functional and/or structural abnormalities are a cause or consequence of essential hypertension does not require the use of molecular genetics, but of a novel epidemiological model. In epidemiological studies of high blood pressure, it has often been difficult to identify familial predisposition in groups of young people with and without a family history, because of a lack of parental blood pressure data (Watt, 1986; Ward, 1990). In some studies, a family history has been determined on the basis of information supplied by subjects at interview rather than on the basis of data from both parents (Orchard et al, 1982; Sigurdsson et al, 1983). The Tecumseh study (Deutscher et al, 1966), however, showed that the proportion of offspring in the top 20% of their age- and sex-specific blood pressure distribution is greatest when both parents are in the top 20% and least when both parents are in the bottom 20%. The same pattern was observed in relation to offspring in the lowest 20% of their blood pressure distribution. This high/high; low/low approach was used in a study in South Wales (Watt et al, 1983), but comparison of individuals with and without a family history provides a minimal contrast in predisposition (Watt, 1986). Thus, even in the Tecumseh and South Wales studies, subjects from both the high/high and the low/low groups showed a wide range of blood pressure levels, with only a general tendency towards high and low blood pressure respectively (Watt et al, 1992). This led Watt et al (1992) to devise a novel epidemiological model (described below) providing contrasting predisposition to high blood pressure.

In 1977, during the screening programme for the MRC Mild Hypertension Trial, blood pressure was measured in 603 couples at the Ladywell Medical Centre. In 1985, blood pressure was measured in 864 of their offspring (aged 16-24 years). The object was to define groups with contrasting predisposition to high [H] or low [L] blood pressure, and to define further whether the predisposition was familial [F] or non-familial [NF]. Offspring blood pressure was plotted against mean parental blood pressure using age-adjusted Z-scores (Figure 6.1). To get at least 50 in each group, parental and offspring Z-scores both outwith 0.3 SD of the mean were chosen to define the 'four corners'. Offspring, therefore, had either a predisposition to high blood pressure which was non-familial [HNF]; a predisposition to high blood pressure which was familial [HF]; a predisposition to low blood pressure which was familial [LF]; or a predisposition to low blood pressure which was non-familial [LNF]. In this model, therefore, subjects are identified as having either a high or low risk of developing hypertension on the basis of their blood pressure in early adulthood, and are further classified as to whether the risk is familial or sporadic on the basis of both their parents' blood pressure. Factors which are associated with higher blood pressure in the offspring irrespective of parental blood pressure are likely to be secondary to high blood pressure and would be seen in HNF and HF offspring vs. LNF and LF offspring. Factors which are associated with high blood pressure would be seen only in those offspring whose parents' blood pressure is high, and are themselves likely to explain the familial predisposition to hypertension (Fig. 6,1).

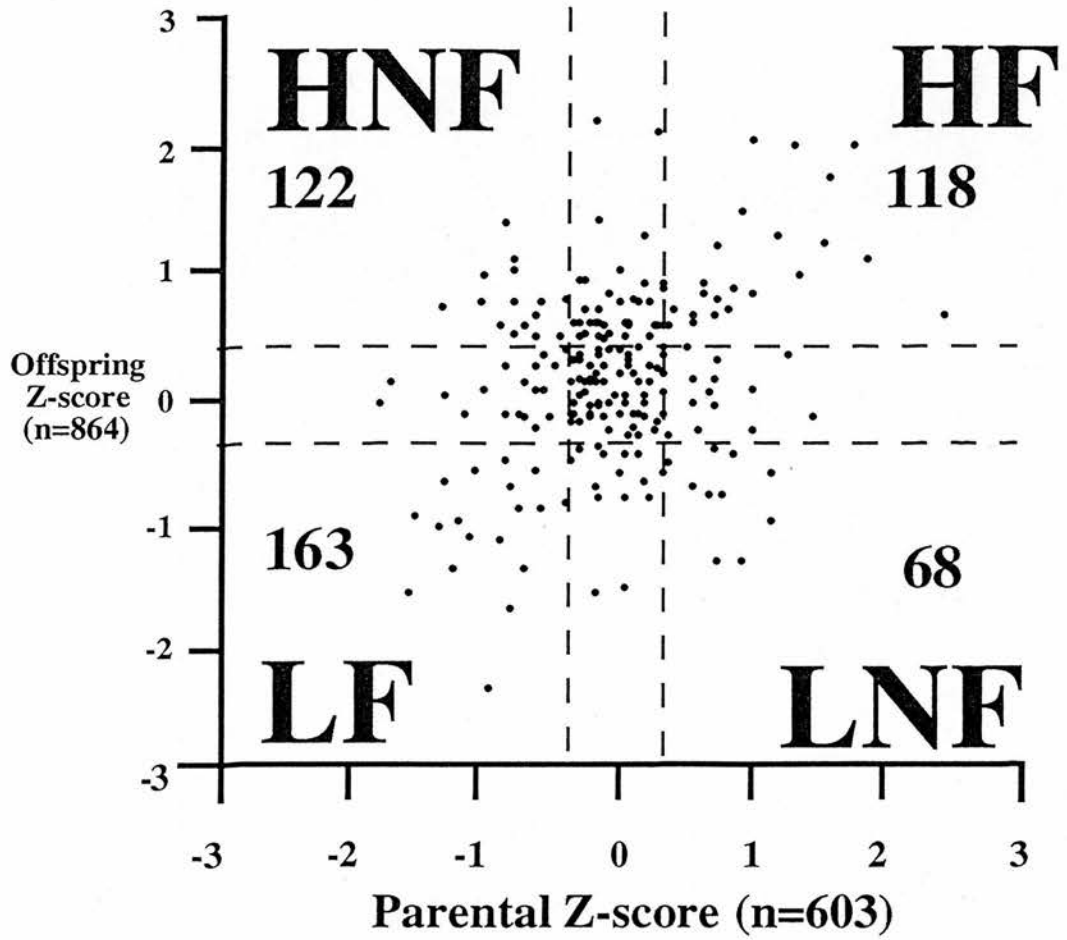


FIGURE 6.1:

The Ladywell 4 corners model of contrasting predisposition to high blood pressure (see text). For clarity, only a sample (approximately 25%) of the 864 data-points are shown.

This model has previously been used to investigate abnormalities of glucocorticoid metabolism and the renin-angiotensin system (Watt et al, 1992). Offspring with a

predisposition to high blood pressure were found to have elevated plasma angiotensinogen, angiotensin converting enzyme and angiotensin II levels, and increased circulating levels of cortisol and 18-OH corticosterone, only when the predisposition was familial [HF]. This group also had raised atrial natriuretic peptide levels, but this was not statistically significant. In addition, 27% of this group were homozygous for the larger allele of the restriction fragment length polymorphism identified by the glucocorticoid receptor gene probe, compared with 9% of offspring who had a familial predisposition to low blood pressure [LF].

Despite findings of linkage between the angiotensin converting enzyme gene in spontaneously hypertensive rats (Iwai & Inagami, 1992), no such linkage was found in humans in another previous phase of this study (Harrap et al, 1993). Also, the S_A gene (Harrap et al, 1995) was found not to influence the renal characteristics (variations in electrolyte metabolism and the renin-angiotensin system) that may contribute to the development of hypertension.

6.1.2 The present study

In Chapter 1, the concept of vascular functional and structural defects contributing to hypertension was introduced. Chapters 2-4 were concerned mainly with the functional importance of blood vessels in healthy subjects, particularly in relation to the vascular endothelium which produces mediators influencing blood vessel tone. In the previous chapter, the investigation included patients with established hypercholesterolaemia and differences between forearm resistance vessels and dermal microvessels were observed. In this chapter, attention is focused on whether functional or structural abnormalities are a cause or consequence of essential hypertension. Iontophoresis of acetylcholine and sodium nitroprusside, as described previously, was performed as a method of assessing endothelial function in the group. In addition, urinary and plasma nitrate levels were estimated as an indication of nitric oxide production (Green et al,

1982). In essential hypertension, the characteristic increase in peripheral vascular resistance may reflect structural as well as functional changes in these blood vessels (Chapter 1), thus, the structure of microvessels was also investigated.

6.2 METHODS

6.2.1 SUBJECTS

The study described in this chapter was conducted between March 1993 and December 1994. 105 male offspring drawn at random from each of the 4 corners were recruited. Details of the subjects are shown in Table 6.1. The author did not know from which corner subjects were drawn until data collection and analysis was complete (the 'code' was held by Professor Graham Watt, Department of General Practice, University of Glasgow). No subject was taking relevant regular medication. Subjects gave written, witnessed, informed consent. Approval was obtained from the Lothian Ethics of Medical Research Committee.

6.2.2 EXPERIMENTAL PROTOCOL

After an overnight fast from 2200 h, abstention from alcohol and caffeine for 12 hours and from proprietary drugs including aspirin for 10 days, subjects attended the clinical laboratory at 0900 h, bringing with them a 24-hour urine collection, which they had started the morning before the study. Height and weight were recorded before subjects lay supine and acclimatised to a controlled environmental temperature of 22-25°C for ≥40 minutes, during which baseline recordings of blood pressure and cardiac function were made. Blood was then taken for biochemical and nitrate level determination. The following measurements were then made in sequence. In the right arm, maximum vasodilatation of skin microvessels in response to local heating to 42°C followed by a period of ischaemia was measured by laser Doppler fluximetry. In the left arm, nailfold

capillary blood velocity, red cell column width and capillary numbers on the dorsum of the ring finger were measured by intravital videomicroscopy (as discussed in Chapter 3 Section 3.2.4; pp 46-47). Iontophoresis of acetylcholine and sodium nitroprusside was performed in the volar surface of the right arm (Chapter 4; Section 4.2.2; pp 65-67 and 68-69). Blood flow was measured in the right forearm before and after ischaemia, using strain gauge plethysmography (Chapter 3; Section 3.2.3; pp 44-45). The protocol was completed by 1130 h.

6.2.3 BLOOD PRESSURE AND CARDIAC FUNCTION

Blood pressure was recorded 4 times at 5-minute intervals using the Hawksley random-zero sphygmomanometer (Wright & Dore, 1970) as in earlier phases of the study. Because of the recent controversy over this device (Conroy et al, 1993), for calculations of vascular resistance these measurements were repeated using a semi-automated machine (Takeda UA 751 sphygmomanometer) which has been independently validated (Wiinberg et al, 1988; Evans et al, 1989). The mean of the last three recordings was used in subsequent analyses. Mean arterial pressure was calculated using the formula given in Chapter 2 (Section 2.2.3; p 32). Measurements of cardiac output and stroke volume were made during the final 20 min of the acclimatisation period, using a non-invasive bioimpedance method (BoMed NCCOM3; Chapter 2; Section 2.2.3; p 32). Results were divided by body surface area to give cardiac index and stroke index respectively. Peripheral vascular resistance was calculated as (mean arterial pressure with Takeda instrument)÷(cardiac index).

6.2.4 IONTOPHORESIS

Iontophoresis of acetylcholine and sodium nitroprusside was performed as described in Chapter 4 (Section 4.2.2). In the present study, acetylcholine, sodium nitroprusside and vehicle were iontophoresed in the right arm in random order.

6.2.5 PLASMA AND URINARY NITRATE LEVELS

A fasting blood sample was taken from each subject at the end of the acclimatisation period. 10 ml was collected in a lithium-heparin tube and centrifuged at 3000 G for 10 min. Plasma was then pipetted into a plain tube and stored at -80°C until all samples were available for analysis. The urine volume was measured, and the aliquoted into smaller containers for future analysis.

Using the method of Green et al (1982), plasma and urine samples (each of 2.5 ml) were defrosted at room temperature for 2 hours. In brief, 0.5 ml from each plasma sample, was pipetted into an Eppendorf microcentrifugation tube, to which 0.1 ml of 35% sulphosalacylic acid (Sigma-Aldrich Company Ltd., Poole, Dorset, UK) was added to deproteinise the sample. Each mixture was vortexed for 10 s every 5 min for 30 min at room temperature, using a bench-top agitator (Fison's WhirliMixer; Fison's Ltd., Loughborough, UK), and centrifuged in a bench-top centrifuge (Camlab Microcentrifuge 154; Camlab Ltd., Cambridge, UK) at 13 000 G for 5 min. The supernatant (0.2 ml) was transferred to a fresh Eppendorf tube and neutralised by adding 0.06 ml 5% sodium hydroxide in 0.3 ml 5% NH_4Cl mobile phase buffer. Urine samples did not require deproteinisation, and were diluted 10 fold in buffer before being injected into the spectrophotometer.

Each sample of 50 µl was injected, using a glass syringe (SGE Series II, Crawford Scientific Ltd.), via an infusion pump (P100 Isocratic Pump; Spectra Physics, Thomson Scientific Ltd., Oxford, UK), into a u.v./visible spectrophotometer (Spectra Series UV100; Spectra Physics). The nitrate in each sample was reduced to nitrite by cadmium in the column. Nitrite reacts with Griess reagent in the column to form a dye, and its absorbance was detected at 546 nm. The spectrophotometer was connected to an Apple Macintosh microcomputer via a digital converter (MacLab; Chapter 3; Section

3.2.4; p 45). Data were stored in a Chart program (Chapter 3; Section 3.2.4; p 45) for subsequent analysis.

6.2.6 DERMAL VASODILATATION

Increases in dermal blood flow were estimated by laser Doppler fluximetry (Chapter 3; Section 3.2.4; p 48) in response to two stimuli. One of these local heating of the skin; the other, ischaemia induced by inflation of an upper arm cuff to 220 mmHg.

(i) Local heating of skin

The right forearm was pre-heated to 36°C using a hairdryer, and a brass skin warmer 1 cm in diameter (Skin Temperature Controller, Moor Instruments Ltd., Axminster, Devon, UK) was attached to the volar surface using a double-sided adhesive disc (Rayman et al, 1986). A thermocouple wire was positioned through a fine channel offset from the centre of the skin warmer and temperature was recorded using a Fluke 52 K/J digital thermometer. The skin was heated to 42°C for 30 min, after which the thermocouple was removed and replaced with a laser Doppler probe so that flux could be recorded for 30 s. The channel could be rotated so that flux was averaged from 8 different sites.

(ii) Post-ischaemic hyperaemia

Immediately after heating, an upper arm cuff was inflated to 220 mm Hg for 5 min and flux was measured at the same site for 30 s after releasing the cuff. The mean flux during these 30 s was used for further analysis.

6.2.7 CAPILLAROSCOPY

(i) Capillary blood velocity

Capillaries were visualised using a single television videomicroscopy system as described in Chapter 3 (Section 3.2.4; pp 46-47). The television camera was connected

both to a microscope and to a video cassette recorder so that images could be recorded for subsequent analysis.

To analyse at least six capillary loops, 4-6 different microscopy fields selected from across the nailfold were each recorded for 2.5 minutes. In some subjects it is impossible to visualise as many as six capillary loops due to damage or injury to the skin (e.g., in manual workers or nail-biters). These subjects were excluded from further analysis, so that studies were completed for 20 HNF; 22 HF; 21 LF; and 20 LNF subjects. For these subjects, capillary blood velocity was measured in the arterial limb of six capillaries by off-line analysis of the video recordings using computerised photometric temporal correlation (Chapter 3; Section 3.2.4). Capillary blood velocity is variable from one capillary to the next within the same individual and, in the present study, gave an intra-subject coefficient of variation (CV) of 31±2%. However, as the mean of six capillaries was taken from each individual, the *inter*-subject variability was only 12.3±1%.

(ii) Red cell column width

Red cell column width was measured at 3 sites on the arteriolar limb of each of the six capillaries using computerised window splitting (Fagrell et al, 1994). Red cell column width is a surrogate measure of luminal diameter if it is assumed that the width of the plasma layer at the circumference of the red cell column remains constant between groups. The CV for red cell column width was 14±1%

(iii) Capillary numbers

To measure capillary numbers, the summits of capillary loops were visualised 'end-on' in the dermis of the dorsum of the 2nd phalanx of the left ring finger. Perfused capillaries (i.e., those in which moving erythrocytes were seen) were counted in 60 second recordings from each of 6 fields of 0.5 x 0.5 mm (calibrated with a graticule

micrometer) both before and during venous occlusion with a digital cuff connected to a mercury sphygmomanometer and inflated to 40 mm Hg for 10 min. This technique was successful in all study participants. Intra-subject coefficents of variation between the six recordings were 8±1% for basal capillary number and 7±1% for capillary number during venous occlusion.

6.2.8 FOREARM BLOOD FLOW AND VASCULAR RESISTANCE

Forearm blood flow was measured using venous occlusion plethysmography with temperature-compensated indium/gallium in silastic strain gauges calibrated on the limb, as previously described (Chapter 3; Section 3.2.3; pp 44-45). In brief, during 3 min recording periods a wrist cuff was inflated to 220 mmHg to exclude the hand circulation and flows were measured for 10 s in every 15 s by repeated inflation of an upper arm congesting cuff to 40 mmHg. After basal recordings, the upper arm cuff was inflated to 220 mmHg for 12 min and recordings were made for the first 60 s after release of the cuff, i.e., during the maximum reactive hyperaemic period (Patterson, 1955; Pedrinelli et al, 1990). The slopes of the final 5 recordings from each period were averaged to determine flow, and minimum vascular resistance was calculated as (mean arterial pressure with Takeda instrument)÷(forearm blood flow).

6.2.9 BIRTH WEIGHT

Subjects were asked to establish their mothers' recall of their birth weight and to bring details when attending the study.

6.2.10 STATISTICAL ANALYSIS

Results are mean ± SEM. Comparisons across the 4 corners were by factorial analysis of variance, followed by comparison of corners by Fisher's probability of least squares difference test when P values for the analysis of variance were <0.05.

When a difference was observed between HNF and HF offspring, there was a possibility that it related to the small difference in blood pressure between these two groups, which has developed since the study was established. Therefore, multiple regression analyses were performed with controlling variables of mean arterial pressure (using the Hawksley) and corner allocation (either HNF or HF assigned values of 0 and 1).

When no significant difference was observed in the analysis of variance, 95% confidence intervals of the observed differences between HF and LF offspring were calculated from an unpaired two-tail Student's *t* test. These corners were chosen since they contain subjects with the greatest contrast in their predisposition to high blood pressure.

6.3 RESULTS

6.3.1 BLOOD PRESSURE AND CARDIAC FUNCTION

See Table 6.1. Blood pressures were higher, as expected, in HNF and HF than in LF and LNF offspring. In addition, blood pressures were significantly higher in HF than in HNF offspring. Pulse rate was higher in corners HNF and HF than in LF and LNF. Cardiac index, stroke index, and total peripheral vascular resistance were not significantly different between corners. However, the 95% confidence intervals of the differences were wide for cardiac index, for which there was a trend towards higher values in HNF and HF offspring.

Corner	HNF	HF	LF	LNF	ANOVA†
Offspring blood pressure	High	High	Low	Low	
Parental blood pressure	Low	High	Low	High	
Number of subjects	26	25	30	24	
Age range (mean in years)	23-32 (28)	23-33 (29)	23-32 (29)	25-32 (28)	P=0.47
Body mass index	24.4±0.5	25.4±0.7	24.1±0.6	23.4±0.6	P=0.11
Birth weight (g)	3599.4 ±82.5	3487.9 ±122.2	3712.4 ±82.5	3526.6 ±110.1	P=0.4
Systolic blood pressure (mmHg by Hawksley)	118±1	123±2	111±1	111±2	P<0.0001 A vs. B,C,D B vs. C,D
Diastolic blood pressure (mmHg by Hawksley)	78±1	82±2	72±1	73±1	P<0.0001 A vs. B,C,D B vs. C,D
Mean arterial Pressure (mmHg)	91±1	96±2	85±1	86±1	P<0.0001 A vs. B,C,D B vs. C,D
Pulse rate (beats per minute)	65±2	65±2	58±2	58±1	P<0.004 A vs. C,D B vs. C,D
Cardiac index (dyn min m^{-5})	4.3±0.2	4.5±0.2	4.0±0.12	3.9±0.2	P=0.10
Stroke index (l min^{-1} m^{-2})	65±3	65±3	67±2	64±3	P=0.87
Peripheral vascular resistance index (dyn min cm^{-5} m^{-2})	21.5±1.1	21.8±1.2	20.8±0.6	22.2±1.0	P=0.77
Basal forearm vascular resistance (mmHg ml^{-1} min 100 ml)	43.6±3.5	39.8±2.3	38.8±2.6	44.2±3.2	P=0.40
Minimal forearm vascular resistance (mmHg ml^{-1} min 100 ml)	2.5±0.2	2.8±0.1	2.4±0.2	2.8±0.3	P=0.30
Capillary blood velocity (mm sec^{-1})	1.4±0.2 (n=20)	1.0±0.1 (n=22)	1.2±0.1 (n=21)	1.3±0.2 (n=20)	P=0.50
Red cell column width (μm)	9.2±0.3 (n=20)	9.0±0.3 (n=22)	8.9±0.3 (n=21)	9.1±0.2 (n=20)	P=0.80

TABLE 6.1:
Demographic, haemodynamic, and capillaroscopy data for subjects from the four corners of the study. † when P<0.05 for analysis of variance, subsequent comparisons between corners refer to P<0.05 for Fisher's two-group probability of least squares difference test.

6.3.2 IONTOPHORESIS

Responses to sodium nitroprusside were higher in HF and LNF offspring than in HNF and LF offspring (Fig. 6.2). Responses to acetylcholine, however, were not significantly different between the groups (Fig. 6.3).

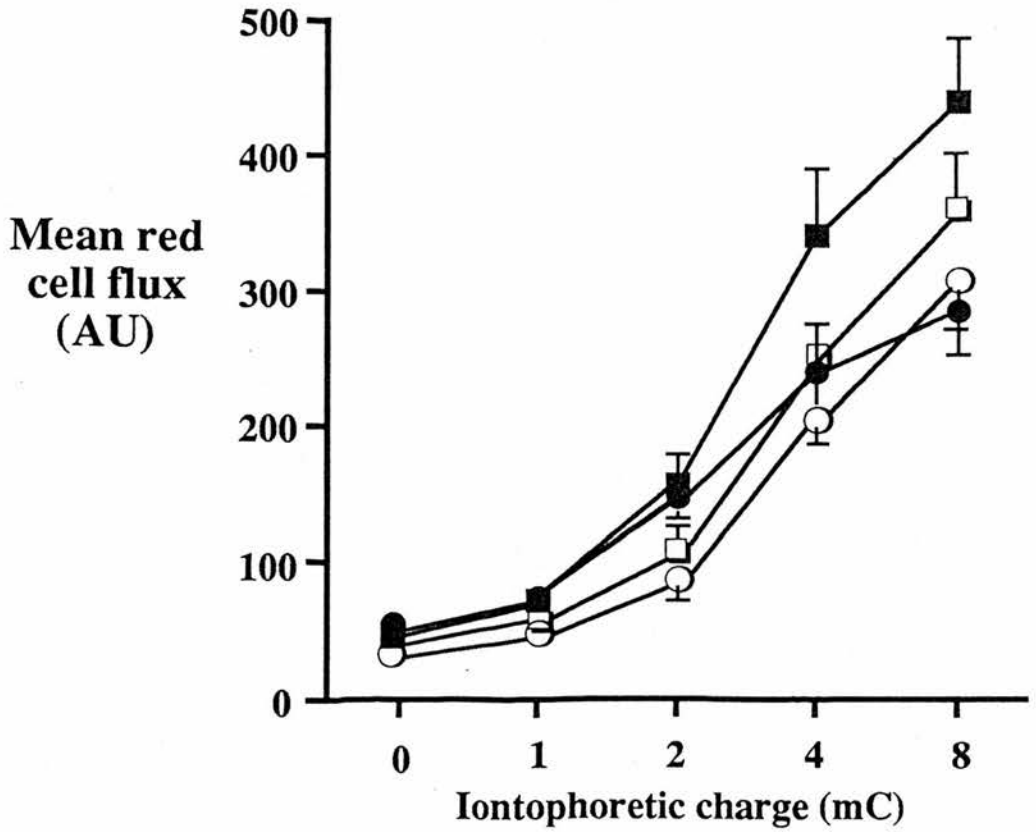


FIGURE 6.2:

Dose response to transdermal iontophoresis of sodium nitroprusside across the 'four corners': HNF: high offspring/low parents (●); HF: high offspring/high parents (❐); LF: low offspring/low parents (○); and LNF: low offspring/high parents (■).

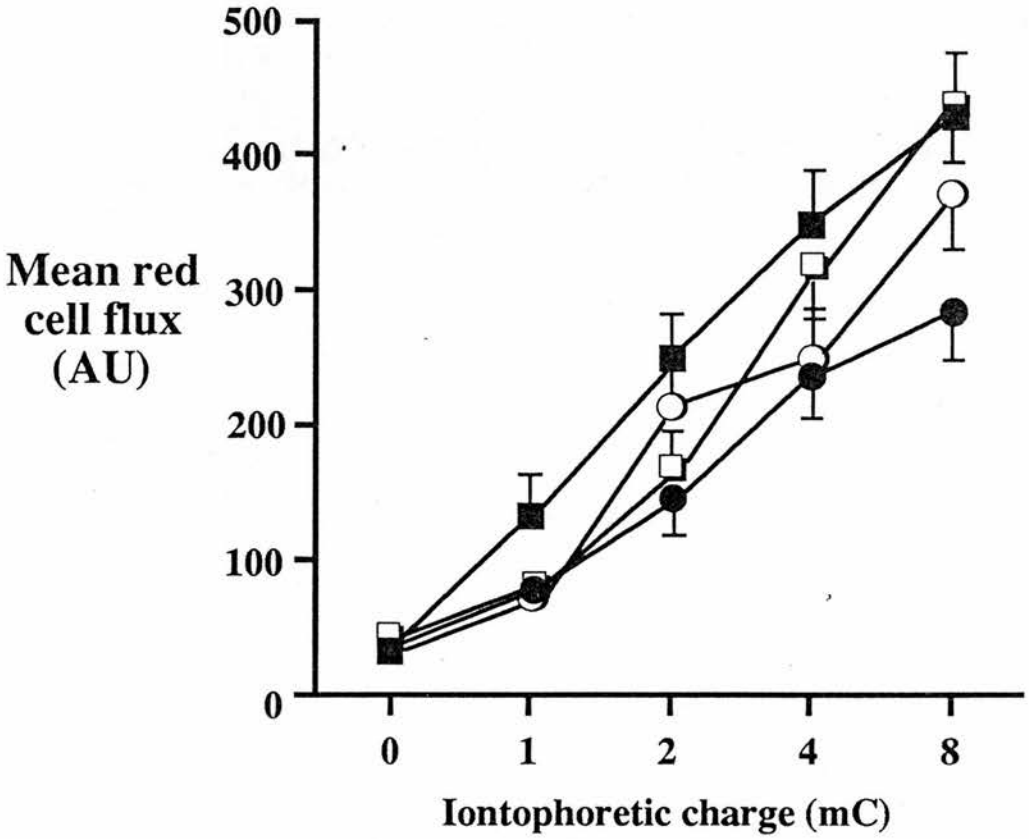


FIGURE 6.3:

Dose response to transdermal iontophoresis of acetylcholine for each of the four corners: HNF: high offspring/low parents (●); HF: high offspring/high parents (❐); LF: low offspring/low parents (○); and LNF: low offspring/high parents (■).

6.3.3 PLASMA AND URINARY NITRATE LEVELS

Nitrate measurements were taken as an index of nitric oxide production across the four corners. There were no significant differences in plasma nitrate levels or in daily rates of nitrate excretion in the urine (Fig. 6.4) between the four corners.

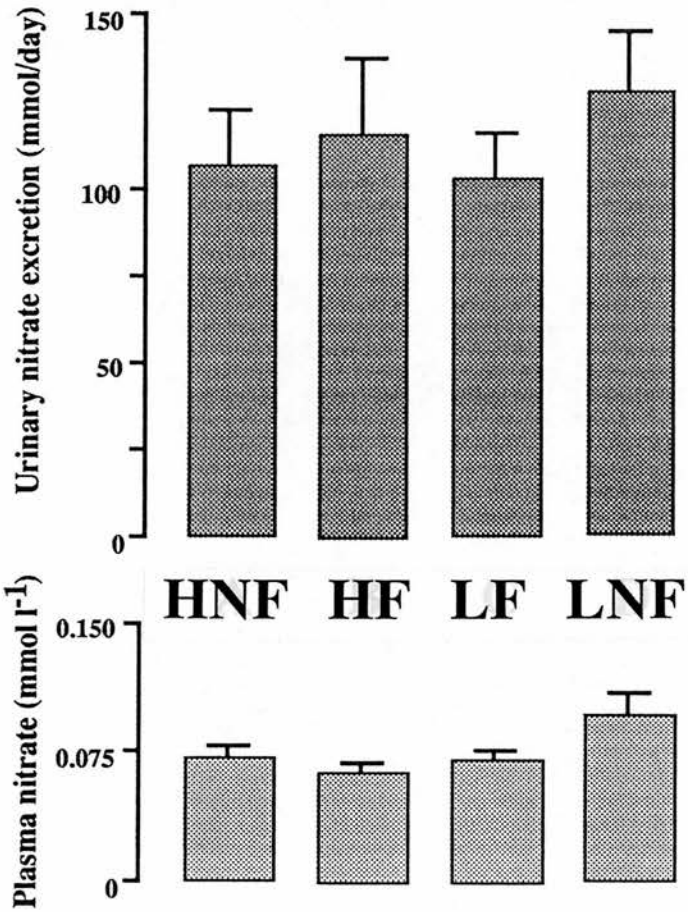


FIGURE 6.4:

Plasma nitrate levels (lower graph) and daily nitrate excretion rate (upper graph) were not significantly different across the four corners (HNF, HF, LF and LNF).

6.3.4 DERMAL VASODILATATION

Increase in flux after both local heating and ischaemia was reduced in the same pattern as capillary number, i.e., lower in HF than in all other offspring (Figure 6.5). The differences between HNF and HF offspring cannot be accounted for by the differences in blood pressure alone, since in multiple regression analyses the response to heating and ischaemia were influenced by corner allocation ($P<0.0001$ for both responses) but not by mean arterial pressure ($P>0.6$ for both).

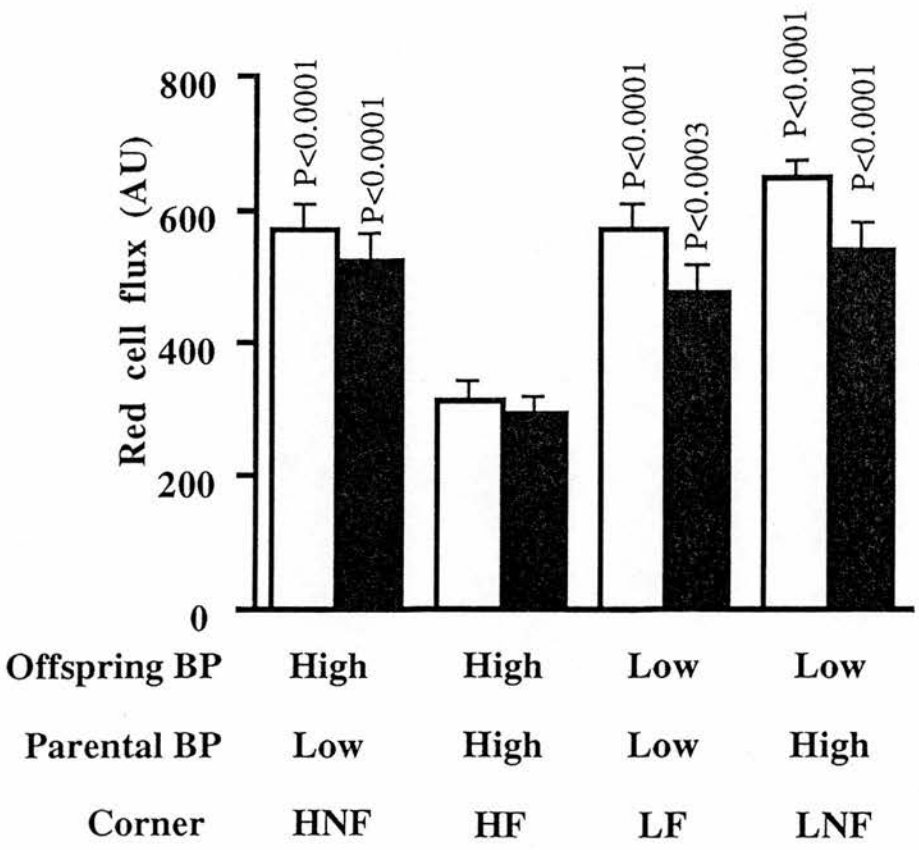


FIGURE 6.5:

Dermal vasodilatation in response to heat and ischaemia in subjects from the four corners. Increasing flux was measured after heating to 42°C (white bars) and after 5 minutes ischaemia (black bars). Error bars are mean ± SEM. Analysis of variance for the four corners was significant to $P<0.0001$ for both variables, and P values shown refer to subsequent comparison of HNF, LF and LNF offspring with HF offspring by Fisher's probability of least squares difference test.

6.3.5 CAPILLAROSCOPY

(i) Capillary blood velocity

There were no differences between corners for capillary blood velocity (Table 6.1). However, 95% confidence intervals of the differences were wide for capillary blood velocity, for which there was a trend towards lower values in HF offspring.

(ii) Red cell column width

Luminal diameter, as judged by red cell column width, was not different between the 4 corners (Table 6.1).

(iii) Capillary numbers

Capillary numbers were not different between corners in the basal state, but during venous occlusion they were markedly lower in offspring with high BP only when parental BP was high ($P<0.003$), i.e., lower in HF compared with all other corners (Figure 6.6). The difference between HNF and HF offspring cannot be accounted for by the differences in blood pressure alone, since in a multiple regression analysis the capillary number after venous occlusion was influenced to a greater degree by corner allocation ($P<0.02$) than by mean arterial pressure ($P<0.04$).

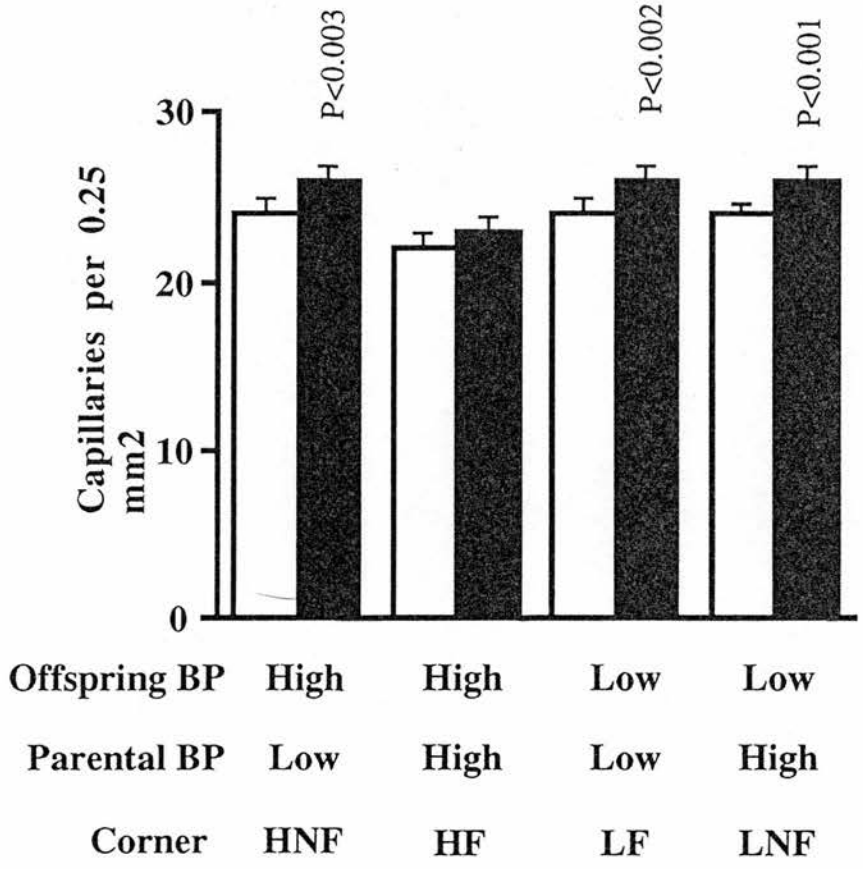


FIGURE 6.6:

Capillary numbers in subjects from the four corners. Capillary numbers were measured in the basal state (white bars) and during venous occlusion (black bars). Error bars are SEM. Analysis of variance for the four corners was significant to P<0.003 for capillary numbers during venous occlusion, and P values shown refer to subsequent comparison of HNF, LF and LNF offspring with HF offspring by Fisher's probability of least squares difference test.

6.3.6 FOREARM BLOOD FLOW AND VASCULAR RESISTANCE

There were no differences between corners in forearm vascular resistance either in the basal state or after ischaemia (Table 6.1).

6.3.7 BIRTH WEIGHT

The trend was towards lower birth weight in subjects with high blood pressure if they had a familial predisposition (HF offspring). However, this difference was not statistically significant (Table 6.1). Further, it can be seen from these results that LNF offspring had comparably low birth weights.

6.4 DISCUSSION

The 'four-corners' epidemiological design is a novel attempt to overcome the difficulties of identifying 'pre-hypertensive' humans in order to explore the primary mechanisms of their high blood pressure without observing secondary effects of high blood pressure. The model has been discussed in greater detail elsewhere (Watt et al, 1992). From the present blood pressure levels, the predictions from the original allocations of offspring to different corners in 1985 are proving to be remarkably robust. In 1987, when 170 subjects from the four corners last had blood pressure recorded, mean arterial pressure was 89 mm Hg in HF; 89 mm Hg in HNF; 81 mm Hg in LF; and 82 mm Hg in LNF offspring (Watt et al, 1992). By comparison with measurements 7-9 years later (Table 6.1), the contrast in blood pressure between the corners is increasing with time. Blood pressure has increased most in individuals with high blood pressure whose parental blood pressure was also high [HF]. These observations confirm that blood pressure 'tracks' with age (Lever & Harrap, 1992) and suggest that the combination of parental and offspring blood pressures, rather than

either alone, provides a more accurate prediction of the subsequent development of essential hypertension.

However, the fact that blood pressure is now higher in HF than in HNF offspring, means that the use of HNF subjects as a 'control' group with matched blood pressure is no longer straightforward. To exclude the possibility that a difference between HNF and HF offspring is due to the difference in blood pressure alone, where differences between these corners were observed, multiple regression analyses were performed. These confirmed that the differences were attributable to corner allocation rather than blood pressure differences alone. The magnitude of the difference between HF offspring and the other groups is greater than has been observed between essential hypertensive patients and controls. Furthermore, for all variables measured, there is no difference between the other 'high' group [HNF] and the 'low' groups [LF and LNF].

The functional abnormality, in dermal vessels, of reduced maximum vasodilatation, i.e., increased resistance, is likely to be, at least in part, related to structural rarefaction observed (see later), rather than to impaired function alone. No significant differences in endothelial function between the corners, as assessed by transdermal drug iontophoresis, were observed. The subjects with high blood pressure with a familial predisposition (HF) did not have an impaired iontophoretic response to acetylcholine. From previous results for iontophoresis (Chapter 4), the mechanism for dermal cholinergic vasodilatation appears to be mediated by a prostanoid vasodilator - probably prostacyclin (PGI_2), as the response is markedly attenuated by aspirin, but not by L-NMMA. In the present study, only basal responses to acetylcholine and sodium nitroprusside were measured, since manipulation by pharmacological tools would have affected other microvascular measurements. Therefore, the nature of the present study may have masked any significant differences. Furthermore, there were

limits to the nature and number of investigations which could be undertaken in subjects from this unique population in a single 3-hour study.

The results for iontophoresis of sodium nitroprusside are even more difficult to explain. In the previous chapter, I demonstrated that hypercholesterolaemic patients with impaired nitric oxide generation in forearm skeletal resistance vessels had reduced vascular smooth muscle sensitivity in dermal vessels. No such reduction in sensitivity was observed in subjects predisposed to high blood pressure. On the contrary, offspring a familial predisposition to *low* blood pressure [LF], had the lowest response to sodium nitroprusside. Further, the 4 corners appear to have separate response curves, with the closest match observed between HF and LF offspring - the two opposite extremes in this model of contrasting predisposition! As none of the subjects studied had a clinical diagnosis of essential hypertension, it may be that iontophoresis can only detect functional abnormalities in established disease.

HF subjects tended to have the lowest levels of plasma nitrate (no differences were seen for urinary nitrate levels), which were assayed as an index of nitric oxide production. Although not statistically significant ($P>0.05$), this trend was more convincing than the acetylcholine test (above) for prostanoid-mediated vasodilation. The result for nitrate levels may have been more clear, had subjects been controlled for diet which strongly influences nitrate and nitrite levels in urine and plasma. Furthermore, the assay is not specifically an index of oxidation of endothelium-derived nitric oxide to nitrate, as nitric oxide may originate in a variety of tissues (Chapter 1). Nevertheless, it is tempting to speculate that abnormalities of nitric oxide generation may be present in subjects with high blood pressure who have a familial predisposition.

In the present study, this unique population was used to help to distinguish abnormalities of microvascular function and structure which are likely to be primary in the development of high blood pressure from those which are secondary to high blood pressure. A pattern consistent with a primary abnormality for reduced capillary number (rarefaction) was observed. In addition, there was a substantial impairment in dermal vasodilatation to heating and to ischaemia (i.e., abnormal in offspring with high blood pressure only when parental pressure was high [HF>LF], and abnormal in families with higher blood pressure only when offspring blood pressure was high [HF>LNF]). There was also a trend towards lower capillary blood velocity in HF offspring. However, there were no differences in capillary luminal diameter.

The expected haemodynamic impact of microvascular rarefaction is controversial, and will differ greatly between vascular beds according to the extent of inter-arteriolar and arteriolar-venular anastomoses. However, calculations by computer modelling of *in vivo* beds predict an exponential relationship between vessel number and vascular resistance (Hudetz et al, 1993). For example, loss of 42% of third-order arterioles in hamster cheek pouch would increase vascular resistance by 21% (Greene et al, 1989). In the present study, I have observed a 14% difference in capillary number during venous occlusion in subjects with a familial predisposition to high blood pressure (Fig. 6.6) which compares with 17% rarefaction in established hypertension (Williams et al, 1990). This was associated with a marked increase in minimum dermal vascular resistance (Table 6.1), and may have been due to an absence of vessels rather than to increased wall:lumen ratio, the hallmark of structural change in larger vessels. In forearm resistance vessels, vascular resistance and minimum vascular resistance are increased in patients with essential hypertension (Folkow et al, 1958; Pedrinelli et al, 1990) and in their normotensive relatives (Takeshita et al, 1982). However, I found no difference between groups in total peripheral or minimum forearm vascular resistance, which is determined mainly by resistance in skeletal muscle. This raises the possibility

that microvascular abnormalities associated with a familial predisposition to hypertension are limited to specific vascular beds, for example affecting dermal but not forearm skeletal vessels.

Arterioles with luminal diameter 20-400 μm have previously been demonstrated to have reduced luminal diameter in established hypertension due to vascular hyperplasia and/or remodelling (Folkow, 1990; Heagerty et al, 1993; Shore & Tooke, 1994). Increased minimum vascular resistance is considered the hallmark of this structural change *in vivo*. The present data suggest that such luminal narrowing as occurs in capillaries in essential hypertension (Lack et al, 1949; Landau & Davis, 1957; Harper et al, 1978) is not present in subjects predisposed to high blood pressure, and is therefore unlikely to be of pathogenic importance. However, in the present study the structure of larger arterioles was not examined directly. The absence of any difference in minimum forearm vascular resistance suggests that luminal narrowing does not occur in skeletal muscle in subjects predisposed to high blood pressure. However, there is a substantial contribution to minimum dermal vascular resistance from larger arterioles (Shore & Tooke, 1994), and I cannot exclude the possibility that luminal narrowing in these vessels contributes to the marked differences in dermal vascular resistance observed.

The mechanism of capillary rarefaction may be either structural, associated with capillary attrition or with impaired angiogenesis, or functional, associated with impaired recruitment of non-perfused capillaries during stimulation (Prewitt & Wang, 1991). In subjects with a familial predisposition to high blood pressure, rarefaction is structural, since basal capillary numbers were not significantly different but recruitment of capillaries during venous occlusion was markedly impaired in HF offspring. Thus, structural capillary rarefaction is associated with almost maximal recruitment of capillaries in the basal state so that the incremental functional change with venous

occlusion is smaller. The observation that a smaller proportion of capillaries are non-perfused in the basal state in subjects with a familial predisposition to high blood pressure raises the possibility that their microvessels are exposed to higher systemic perfusion pressures for longer periods than normal. In due course this may promote the microvascular pathology which develops with essential hypertension.

If the capillary rarefaction observed is structural then it may be explained by impaired angiogenesis. This is one hypothesis used to explain the relationships between adult hypertension and growth in intra-uterine life, childhood, and puberty (Lever & Harrap, 1992). Of particular interest is the observation that hypertensive patients, and the offspring with high blood pressure irrespective of parental blood pressure (Table 6.1 and Watt et al, 1992), tend to have a higher body mass index despite the epidemiological data that they start life smaller than their normotensive peers (Barker et al, 1990). It may be that, because of faster rates of 'catch-up' growth in later life, hypertensive subjects out-grow their vascular tree, thereby having to recruit more capillaries to continuous perfusion resulting in accelerated structural microvascular changes and hypertension. Although HF subjects had the lowest birth weights, this result was not statistically significant. Further, LNF subjects had comparably low birth weights, and it may be that this is dictated more by parental than by offspring blood pressure. However, in epidemiological terms, the numbers of offspring studied were extremely small, and it is likely that there was insufficient power to draw useful conclusions. Birth weight is only one variable in Barker's equation. A pilot study was performed to obtain placental weights from maternity records for the 105 offspring who took part in this study. However, due to poor archiving, it was impossible to obtain placental weights.

In summary, this study shows that capillary rarefaction and impaired microvascular vasodilatation are associated with familial predisposition to essential hypertension in

young adult males, and are not features of increased blood pressure *per se*. In contrast, capillary luminal diameter and blood flow are not deranged at this stage of the natural history of the disease, and these are more likely to be consequences rather than causes of hypertension. Thus, the mechanisms dictating microvascular growth and anatomy deserve more detailed attention as potential candidate mechanisms in the pathogenesis of hypertension.

Chapter 7

GENERAL CONCLUSIONS

DYSLIPIDAEMIA, which predisposes individuals to atherosclerosis, and hypertension, which accelerates atherosclerotic vascular disease, are major causes of morbidity and mortality in the industrialised world. Being able to recognise which hypertensive and/or hypercholesterolaemic individuals are at high risk of developing clinically significant target organ damage would be useful to their clinical management, and to determining their prognosis. The few studies which have used arterial infusion techniques to assess dysfunction of the vascular endothelium in essential hypertension and hypercholesterolaemia, have not produced data on a large enough scale to allow conclusive epidemiological interpretation. For example, some patients with essential hypertension do not show an impairment in endothelial function as judged by the acetylcholine test (Cockcroft et al, 1994). Hypertension has a multi-factorial aetiology (Chapter 6), and it may be that certain sub-populations of patients are more at risk than others of developing target organ damage.

With this in mind, a major aim of this thesis was to validate the non-invasive technique of iontophoresis for assessing endothelial function, so that larger groups of patients could more easily be studied. Although this aim may now appear to have been ambitious, some important information on the pharmacological mechanisms of microvascular function has, nevertheless, emerged.

An intact endothelium is necessary for the maintenance of vascular tone and the regulation of blood pressure (Chapter 2). Inhibition of basal generation of nitric oxide by L-NMMA caused an increase in blood pressure and peripheral vascular resistance. The blood vessels contributing to these effects are likely to be microvessels as these make the greatest contribution to peripheral resistance, and the purpose of Chapters 3-5 was to investigate the existence of functional microvascular defects and to establish their pharmacological mechanisms.

It would appear that nitric oxide plays a more important role in the regulation of arteriovenous anastomotic flow in the skin microcirculation than in nutritive flow (Chapter 3). Therefore, the role of nitric oxide may be related to the function of the tissue being investigated, as well as to the size of vessel (see the Discussion section (Section 3.4) in Chapter 3).

In Chapter 4, using transdermal iontophoresis of acetylcholine in the presence of aspirin, the investigation of endothelial function in forearm dermal microvessels showed that the principal mechanism of vasodilatation in nutritive tissue (volar surface of forearm) was prostanoid-mediated. With this established in healthy individuals, the next logical step was to examine whether any alterations occurred in patients with known endothelial dysfunction as assessed by the 'gold standard' arterial infusion technique, and this was the subject of Chapter 5. An impaired response to acetylcholine in dermal vessels of hypercholesterolaemic patients was only observed in the presence of aspirin, suggesting enhanced generation of vasodilator prostanoids, or reduced generation of other mediators of vasodilatation in these subjects. Thus, under resting conditions of blood flow, use of iontophoresis for direct detection of endothelial dysfunction (as impaired nitric oxide generation) would not seem appropriate. However, hypercholesterolaemic patients also showed an impaired sodium nitroprusside response in dermal vessels (with or without aspirin), suggesting reduced sensitivity of vascular smooth muscle to nitric oxide. This might explain the importance of vasodilator prostanoids in effecting vasodilatation in these vessels. Iontophoresis may be more useful, therefore, in assessing vascular smooth muscle sensitivity in the dermis.

Previous theory for the structural basis of increased peripheral resistance characterising essential hypertension, has concentrated on increased arterial wall:lumen ratio

(Folkow, 1958). However, the results from Chapter 6 suggest microvascular rarefaction as an alternative explanation for increased resistance. The structural defect of rarefaction (Chapter 6) appears to be causal as it is associated with familial predisposition to essential hypertension in young adult males. In contrast, capillary luminal diameter and blood flow do not appear abnormal at this stage in the pathogenesis of hypertension, and these are likely to be consequences rather than causes of the disease. Functional abnormalities were not demonstrated in the young people studied in Chapter 6. These young people did not have the clinical diagnosis of essential hypertension, but in due course structural rarefaction may promote the microvascular pathology which develops with essential hypertension. Therefore, functional abnormalities, particularly endothelial dysfunction as discussed above, may be only part of the aetiological profile, and shoud not be seen in isolation in a heterogeneous disorder (Lüscher, 1992). The mechanisms dictating microvascular growth and anatomy deserve more detailed attention in future studies as possible candidate mechanisms in the pathogenesis of hypertension.

In atherosclerosis, endothelial dysfunction is secondary to the damaging effects of hypercholesterolaemia, particularly to oxidised LDLs (Chapter 5), a process which is accelerated by hypertension. In turn, if the endothelium cannot produce the correct balance of agents required to neutralise oxLDLs and oxygen free radicals, and to prevent thrombus formation (Chapter 5), this may lead to the structural damage of atheromatous plaque formation, ultimately contributing to the risk of coronary heart disease and stroke. Therefore, functional and structural defects are not separate entities providing risk, but interact in different ways to produce the occlusive vascular disease of hypertension (hypertrophy) and hypercholesterolaemia (atheroma). The mechanisms by which hypertension induces lesions of atherosclerosis are poorly understood (Chapter 1), and investigation of angiogenic factors involved in genesis of atherosclerosis also deserves further attention.

Chapter 8

PUBLICATIONS ARISING FROM THIS THESIS

Papers

Noon JP; Haynes WG; Webb DJ; Shore AC. Local inhibition of nitric oxide generation in man reduces blood flow in the finger pulp but not in hand dorsum skin. *J Physiol* 1996; **490**: 501-08.

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Abstracts

Noon JP; Walker BR; Shore AC; Holton DW; Edwards HV; Watt GMC; Webb DJ. Abnormalities of microvascular structure and related function in subjects with familial predisposition to hypertension. *J Hypertens* 1995; **13**: 1485.

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Chapter 9

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