Venous occlusion plethysmography in cardiovascular research: methodology and clinical applications

Ian B. Wilkinson & David J. Webb¹

Clinical Pharmacology Unit, University of Cambridge, Addenbrooke's Hospital, Cambridge CB2 2QQ and ¹Clinical Pharmacology Unit, University of Edinburgh, Western General Hospital, Edinburgh EH4 2XU

Introduction

The use of venous occlusion plethysmography to measure blood flow in humans was first described over 90 years ago by Hewlett & van Zwaluwenburg [1]. Although initially limited by the need for cumbersome equipment, the advent of mercury-in-silastic strain gauges over 50 years ago greatly increased its applicability, and the technique has remained essentially unchanged since except for the introduction of computerization. Venous occlusion plethysmography has been used extensively to study human vascular physiology *in vivo*, and is at its most powerful when combined with intra-arterial drug administration, usually into the forearm vascular bed. This permits detailed study of both vascular pharmacology, usually by infusion of receptor agonists, and physiology, by infusion of receptor antagonists. Indeed, forearm venous occlusion plethysmography with local brachial artery infusion has become one of the 'gold-standards' in the assessment of vascular function in health and disease, and an accurate, reproducible and convenient method with which to assess the effect of new vasoactive drugs and hormones in humans *in vivo* [2]. Besides being minimally invasive, the major advantage of this approach is that drugs can be administered at subsystemic doses minimizing disturbances to systemic physiology. Although venous occlusion plethysmography can be equally well applied to the lower limb, this review will focus on the forearm vascular bed.

Methodology

Measurement of blood flow

The underlying principle of forearm venous occlusion plethysmography is simple: when venous drainage from the arm is briefly interrupted, arterial inflow is unaltered and blood can enter the forearm but cannot escape. This results in a linear increase in forearm volume over time,

which is proportional to arterial blood inflow, until venous pressure rises towards the occluding pressure [3]. Under resting conditions, ~70% of total forearm blood flow (FBF) is through skeletal muscle, with skin blood flow accounting for most of the remainder [4]. However, the hand contains a high proportion of arterio-venous shunts and, because skin blood flow is highly dependent on temperature [5] and has a different basic pharmacology and physiology than muscle blood flow, it is standard practice to exclude the hands from the circulation during measurement of forearm blood flow. Indeed, if the hand is not excluded then blood flow is nonlinear [6].

The basic methodology has changed little since its first description by Hewlett & Zwaluwenburg over 90 years ago [1]. Venous return from the forearm is briefly interrupted by inflating a cuff, placed around the upper arm, to well above venous pressure but below diastolic pressure. Typically, an inflation pressure of around 40 mmHg is used for intervals of 10 s, followed by 5 s of deflation, which does not alter arterial inflow and allows venous emptying [7]. The forearm must be positioned above the level of the heart to ensure adequate venous emptying during the period of deflation, which is usually achieved by resting the elbows on foam pads and supporting the hands with pillows (Figure 1). The hands are excluded from the circulation during measurements by initial rapid inflation of a smaller cuff, placed around the wrist, to well above systolic pressure (220 mmHg for normotensive subjects). The wrist-cuffs must be inflated at least 60 s before starting measurements of flow in order to allow FBF to stabilize [8]. As this manoeuvre renders the hand ischaemic, the measurement period is limited, but periods of up to 13 min have been employed safely [9]. Automated venous occlusion plethysmography equipment has been developed [10] and is of particular use when on-line measurement of flow is required, as discussed later.

Changes in forearm volume are measured by a plethysmograph. Initially, air and then water-filled jackets were used, but these have been largely replaced by mercury-in-rubber (or silastic) strain gauges [11], which may themselves be ultimately replaced by indium-gallium gauges due to concerns over the potential toxicity of mercury. The strain gauges should be placed around the widest part of the forearm, and simply act as resistors

Correspondence: Dr I. B. Wilkinson, Clinical Pharmacology Unit, University of Cambridge, Addenbrooke's Hospital, Cambridge CB2 2QQ. Tel.: 01223 336743; Fax: 08701269863; E-mail: ibw20@cam.ac.uk

Received 15 January 2001, accepted 9 July 2001.

Figure 1 Assessment of forearm blood flow using venous occlusion plethysmography.

Figure 2 Effect of intra-arterial substance P on forearm blood flow. 2 pmol min^{-1} substance P produces a marked increase in blood flow in the infused arm, as illustrated by the increase in the slope of the tracing.

connected as one arm of a Wheatstone bridge [6]. Changes in forearm volume result in a corresponding change in arm circumference and thus strain gauge length, which can be detected as an alteration in electrical resistance of the gauge, and thus potential difference (Figure 2) [11]. If the gauge length is made equal to the resting circumference of the limb, then changes in limb volume are directly proportional to the changes in resistance [12].

Venous occlusion plethysmography provides a measure of blood flow to that part of the forearm enclosed by the two cuffs. This is usually expressed as ml per 100 ml of forearm volume per minute, when electronic calibration is employed [6, 12]. Actual forearm volume can be estimated by calculation, assuming the forearm is a simple truncated cone, or by simple water displacement. When

studies are conducted in a quiet, temperature-controlled room ($22\text{--}26^\circ \text{C}$), with the subject resting in a comfortable supine position, measurement of FBF by strain gauge venous occlusion plethysmography compares favourably with values obtained by other established techniques [13, 14]. More recently, strain gauges employing a non-elastic loop attached to a passive inductive transducer, which acts as an electromagnetic sensor, have been developed and appear to be at least as accurate as mercury gauges [15].

Alternatives to plethysmography

Blood flow can also be measured using ultrasound. Usually this involves combining estimates of mean blood velocity

with the cross sectional area of the vessel. Doppler ultrasound is used to estimate the mean blood velocity, and B-mode ultrasound used to estimate the vessel diameter. Measurement of mean velocity is most accurate when the ultrasound beam-width is greater than the vessel diameter; with modern ultrasound scanners the 3dB beam width is typically 1–2 mm, so that this condition is not met in most vessels of interest, leading to an overestimation of mean velocity. Assessment of vessel diameter relies on the ability to image the anterior and posterior vessel walls and depends on the angle of the probe to the artery. As the area is obtained by squaring the diameter, any error in diameter estimation results in doubling of the error for area; in general it is difficult to measure area in vessels less than about 3–4 mm in diameter, though this is dependent on the location of the vessel and the frequency of the transducer used, with greater accuracy being achieved if high frequency transducers are used for scanning superficial vessels. Typically, in vessels of 5–10 mm in diameter, the flow may be measured at best with an accuracy of ~20–30%. In routine practice, using the above technique, it is easy to generate very large errors of ~50 or even 100% in flow estimation, but in the experimental setting improved accuracy, below 10%, may be possible [16]. Overall, the use of ultrasound does not confer any particular advantage over plethysmography in assessing blood flow, although it does allow conduit vessel diameter to be determined at the same time. Moreover, ultrasonic methods require expensive equipment and highly skilled operators and can be difficult to use with intra-arterial needles.

Minimum vascular resistance

Following limb ischaemia there is a rapid increase in forearm blood flow, which slowly returns to baseline values and is termed *reactive hyperaemia*. The maximum flow and area under the curve for this increase in blood flow are directly related to the duration of ischaemia [17] until ~13 min, after which lengthening the period of ischaemia does not increase the *maximum* recorded FBF further [9]. Indeed, the *maximal* post-ischaemic FBF is not influenced by sympathetic tone [18] or administration of vasodilators [9] and seems to represent the *minimum forearm vascular resistance*, which is a function of the average wall:lumen ratio of the resistance vessels [19]. Therefore, minimum vascular resistance provides indirect information concerning the structure of resistance vessels in the forearm. Moreover, minimum forearm vascular resistance correlates well with more direct measurements of resistance vessel structure, such as the media:lumen ratio [20]. Increased minimum vascular resistance has been demonstrated in individuals with established hypertension [19, 21], young men with 'borderline' hypertension [18],

and those with 'white-coat' hypertension [22]. Interestingly, such structural changes can be reversed with antihypertensive medication [23–25].

Minimum vascular resistance can be assessed reproducibly in the forearm following 13 min of ischaemia, induced by inflating a cuff sited around the upper arm to 300 mmHg for 13 min, and asking subjects to perform 20–30 hand contractions during the last 1 min of ischaemia [9]. As is usual, the hands should be excluded from the circulation before starting the study and measurements of FBF, using venous occlusion plethysmography, should be made at 15 s intervals for 3 min following ischaemia. Maximum FBF usually occurs between 5 and 45 s after cuff deflation and, practically, vascular resistance is calculated as mean arterial pressure ÷ FBF. Although this technique may seem objectionable, it is generally well tolerated.

Forearm venous volume

The compliance of the deep forearm veins can be assessed using venous occlusion plethysmography [26]. Moreover, this can be performed during intra-arterial drug infusions, to provide simultaneous information regarding the effect of drugs on resistance vessels and veins [27–29]. Several techniques exist for determining venous distensibility. However, one of the simple approaches is to increase the upper arm cuff pressure in a stepwise manner to ~30 mmHg, at intervals of ~1 min. Under these conditions, changes in forearm volume reflect changes in venous volume, and thus pressure-volume curves can be constructed to determine venous distensibility (Figure 3).

Microvascular permeability

Following inflation of the upper arm cuff to above venous pressure, there is a rapid rise in forearm volume, which reaches a plateau after 1–2 min, depending on the inflation pressure used [30]. However, during prolonged venous occlusion there is a small but measurable continued increase in forearm volume (Figure 4). This appears to be due to extravasation of fluid from the capillaries, rather than progressive venous distension [31, 32] and, therefore, venous occlusion plethysmography can be used to assess capillary permeability [30]. The pressure in the occluding cuff may be raised in a single step [33], or in progressively by small increments [30], in which case fluid flux is calculated by least-squares fitting of the volume response after the vascular compliance component is completed [34]. Changes in microvascular permeability have been described in diabetic patients [34] and in response to infused peptides [33], as illustrated in Figure 5.

Figure 3 Determination of venous distensibility. Changes in forearm volume (FAV), arterial pressure (AP), and transmurular pressure (TMPV) – occluding cuff pressure, when TMPV was increased in a step-wise manner. From [33] with permission.

Figure 4 Determination of capillary permeability. A simulated response to inflation of the upper arm cuff. There is a rapid increase in forearm volume V_a , followed by a slower continued rise in volume of slope J_v , due to movement of fluid from the capillaries into the forearm. From [30] with permission.

Sympathetic nervous system activity

Application of a small degree of negative pressure (10–15 mmHg) to the lower body produces venous pooling in the legs, and unloading of the baroreceptors [35]. This results in increased sympathetic activity in the nerves supplying the upper limbs and an $\sim 20\%$ reduction in FBF, without any concomitant change in arterial pressure or heart rate [36, 37]. This technique has been used to examine the influence of drugs on peripheral sympathetic

Figure 5 Effect of atrial natriuretic peptide on capillary permeability. Effect of a control infusion, sodium nitroprusside (SNP) and atrial natriuretic peptide (ANP) on forearm volume (FAV) during prolonged cuff inflation to 30 mmHg. From [33] with permission.

function. For example, angiotensin II, in low doses, does not significantly alter FBF, but markedly enhances the sympathetic-mediated vasoconstriction to lower body negative pressure [37].

Local drug administration

Perhaps one of the most important uses of venous occlusion plethysmography has been the study of the local effect of vasoactive mediators or drugs in the forearm vascular bed. Drugs can be given intra-arterially by placement of a fine (27-gauge) needle into the brachial artery under local anaesthesia, which causes minimal discomfort. This is a simple, safe technique and over 90% of subjects can be cannulated at the first attempt. Indeed, a recent survey canvassing experienced users from around the world found that several thousand cannulations have been conducted over the last 15 years without any serious adverse events (P. Vallance; personal communication). Nevertheless, the technique of cannulation is best learnt from an experienced researcher, and general precautions concerning sterility and the preparation of drugs for infusion need to be carefully observed. Infrequent minor problems at the time of cannulation, such as bruising and local discomfort, usually settle rapidly without any problems. However, it is our current practice to always contact the subject the day after cannulation to ensure that they have not experienced any delayed problems. Larger needles can be employed, but there seems no particular advantage of this technique, in most situations, and it is generally less well tolerated and does not allow for frequent repeat studies. Indeed, serial studies can be undertaken safely with the use of a 27-gauge needle [38], and individual investigators (JRC, DJW) have personally

undergone >50 cannulations without harm. The particular advantage of the technique is that drugs can be given at doses 10–100 times below those causing systemic effects because FBF ($<50 \text{ ml min}^{-1}$) is ~ 100 fold lower than cardiac output. Therefore, if long-acting drugs are used for brief infusion periods, or short-acting drugs for longer periods, their effects on resistance vessels can be studied without invoking systemic effects or neuro-hormonal reflexes [39], minimizing the potential for side-effects and toxicity. Moreover, the non-infused arm can be used as a contemporaneous control throughout the experiment [40] to take into account any minor changes in blood flow affecting both arms, such as emotional stress or changes in temperature [41]. It is now rare for unilateral studies to be performed, and this technique should not be encouraged.

Reproducibility

Several groups have assessed the reproducibility of venous occlusion plethysmography. Roberts *et al.* [42] demonstrated good within-subject reproducibility for *unilateral* blood flow measurement, with a coefficient of variation of $\sim 10\%$ for FBF measurement and $\sim 11\%$ for calf blood flow under resting conditions, and 13% for calf blood flow post-exercise. Although others have reported somewhat higher values of $\sim 25\%$ for assessment of *unilateral* FBF over several weeks [43]. Petrie *et al.* [44] investigated the within-subject reproducibility of bilateral forearm venous occlusion plethysmography over three visits 1 week apart, and reported a coefficient of variation of $31\text{--}39\%$ for unilateral flows at rest, but only 19% when the ratio of unilateral flows was used. During infusion of the vasoconstrictors angiotensin II and noradrenaline reproducibility of the response was again improved by analysing the results as percentage change in FBF ratio (14 vs 18% and 16 vs 27% , for interquartile ranges, respectively). However, Walker *et al.* [45] found that the intra-subject variability of the response to dilators (acetylcholine and salbutamol) was much less when quoted as absolute values of FBF than when analysed as percentage change in the FBF ratio. The same also appears to be true for exercise-induced increases in FBF [44].

Data analysis

When only unilateral FBF is measured, results may be expressed in absolute terms, i.e. ml per 100 ml of forearm volume per minute or as a change from baseline. However, when, as is usual practice, blood flow is measured in both arms simultaneously, several approaches to analysing and presenting these data exist. Again, FBF may be expressed in absolute values for each arm, the control arm merely providing evidence concerning any systemic

affects, the occurrence of which would invalidate such an approach. Changes may be reported either in absolute terms or as a percentage of baseline values [46–48]. Alternatively, the ratio of FBF in the infused arm to the control arm (usually around 1) may be quoted, frequently as a percentage change from baseline. This approach was first suggested by Greenfield & Patterson [49], and has been widely used by others since [38, 50, 51]. It has the theoretical advantage of minimizing the influences of small changes in FBF affecting both arms due, for example, to alteration in sympathetic activation or blood pressure [52]; the non-infused arm acting as a contemporaneous control, and appears to give better reproducibility than reporting unilateral flow, at least for vasoconstrictors [44]. However, with vasodilators, especially those that can produce large increases in blood flow, citing changes in absolute blood flow may be better since small changes in the flow in the control arm can have a large influence on the percentage change in the FBF ratio at high flows, increasing inter-study variability [45]. It should be noted that in studies where several different vasoactive agents are infused sequentially it is important to use the flow immediately preceding each infusion as the baseline to assess changes in FBF rather than the original baseline at the start of the study. Flows may also be used to calculate forearm vascular resistance, and results expressed in this format [53, 54]. However, this approach makes several important assumptions, such as assuming laminar flow and that alterations in blood pressure have no effect on the state of smooth muscle contraction, and is, therefore, we believe inappropriate [52]. Indeed, calculating forearm vascular resistance confers no particular advantage over FBF values and may, on occasion, be misleading, especially if baseline blood pressure differs between study groups.

The most frequently employed method for statistical analysis of FBF data is repeated measures analysis of variance, with *posthoc* Student's *t*-tests. Alternatively, summary measures statistics may be used, such as maximal response [55], which may be of more use when the response is biphasic, or area under the curve. However, the exact choice of method may well depend on the question one wishes to address.

Clinical applications

Local drug and hormone infusions

Venous occlusion plethysmography, coupled with brachial artery drug administration, provides an ideal method with which to assess the local effect of drugs and hormones on peripheral resistance vessels without invoking systemic effects (Table 1, Figure 6). Whereas, receptor agonists are usually employed to investigate pharmacological mechanisms, antagonists often provide more useful information

Table 1 Vasoactive peptides and drugs in human forearm resistance vessels.

	Vasoconstrictors	Vasodilators
Mediators	Noradrenaline Angiotensin II Endothelin-1 Neuropeptide Y	Nitric oxide Atrial natriuretic peptide 5-hydroxytryptamine Bradykinin Substance P Adrenomedullin Calcitonin gene-related peptide
Drugs	BQ788 (ET _B receptor antagonist) Phentolamine	Acetylcholine and carbachol Sodium nitroprusside and glyceryl trinitrate Hydrallazine Verapamil BQ123 (ET _A receptor antagonist)
Inhibitors	N ^G -monomethyl-L-arginine (LNMMA) Neutral endopeptidase inhibitors	Prazosin Endothelin converting enzyme inhibitors

Figure 6 Effect of angiotensin II and bradykinin on forearm blood flow. FBF in the infused (■) and noninfused (□) arm, during infusion of the two peptides. From [40] with permission.

regarding physiology of the system under investigation. When given systemically, many vasoactive drugs produce changes in blood pressure and sympathetic tone that will alter FBF [37, 41, 56]. Moreover, the lack of systemic effects allows near full dose response curves to be

constructed for some agonists, such as angiotensin II [40] (Figure 6) and atrial natriuretic peptide [57]. However, for some vasodilators, such as bradykinin (unpublished data) and substance P [58], construction of a full dose-response curve is not possible due to 'spill over' from the arm and consequent systemic effects at high doses. Moreover, large increases in flow will also reduce the effective concentration of a drug in the forearm due to dilution and potentially confound interpretation of the results, although this does not appear to be a problem in practice. However, limited dose-response curves can still be constructed which may be usefully employed to assess the effect of receptor antagonists [59] or interventions such as inhibition of angiotensin converting enzyme [40], or endothelin converting enzyme [38].

The FBF technique avoids potential risks involved with systemic administration of vasoconstrictors and although critical closure of the forearm vascular bed is a theoretical possibility with potent vasoconstrictors [60], it has not been reported, even with large reductions in FBF observed with endothelin-1 [61] and angiotensin II [40], when suitable doses and measurement periods are employed. Moreover, the response of the forearm vascular bed to locally infused drugs correlates well with systemic effects for many agents including angiotensin II [40, 62], endothelin-1 [63, 64] bradykinin [65, 66], and sodium nitroprusside [67, 68]. In contrast, when drugs are given systemically, the observed changes in local blood flow do not always mirror those seen during local intra-arterial infusion. Indeed, despite angiotensin II being a powerful vasoconstrictor when administered directly into the forearm vascular bed, systemic infusion actually increases calf blood flow at high doses even though this is accompanied by a rise in blood pressure and systemic vascular resistance [56]. Therefore, changes in FBF *cannot* be used to reliably

indicate the effects of drugs on resistance vessels when such agents are infused systemically.

Measurement of FBF coupled with intra-arterial infusion of an agonist is often useful for investigating the pharmacological effects of a compound or activation of a receptor subtype and the potential for interactions, physiological or pharmacological. However, studies with receptor antagonists often provide more valuable information concerning the physiology and pathophysiology of pathways, particularly when comparisons are made between patient and control groups. In this respect, the forearm vascular bed provides an ideal model with which to safely evaluate potential antagonists and investigate their effect on resistance vessels. Indeed, endothelin [51], angiotensin II [69], bradykinin [59] and substance P [58] antagonists, and both α - and β -adrenoceptor antagonists [67, 70] have been infused intra-arterially and provide valuable information about the contribution that such systems make to vascular tone at rest (Table 2), or during various manoeuvres such as activation of the sympathetic nervous system. Similarly, infusion of drugs that inhibit the *in vivo* formation of vasoactive mediators such as nitric oxide [71] or endothelin [38] can also provide useful information regarding the physiological importance of such systems. The forearm circulation can also be used to conduct agonist/antagonist interaction studies to assess the efficacy of a particular antagonist, which may be given either by local intra-arterial infusion [38, 69], or systemically [59]. Once again, the local effects of antagonist in the forearm resistance bed correlate well with systemic effects [51, 69, 71–74].

By inserting venous cannulae into both antecubital fossae, it is possible to obtain venous effluent from the infused and non-infused arm. This can provide useful information regarding local drug concentrations and the effect of the infused drug on other vasoactive mediators, the fibrinolytic system (see later), and platelet function. Data from the non-infused arm, serve as a contemporaneous control, and also provides information regarding systemic effects or drug 'spill over'.

Table 2 Systems affecting basal vascular tone in the human forearm.

System
Sympathetic nervous system
Renin-angiotensin system*
L-arginine nitric oxide pathway
Endothelin system

*Only when activated, does the renin-angiotensin system influence basal vascular tone, such as in sodium-depleted or volume-depleted individuals.

Considerations with intra-arterial infusions

The speed with which drugs and peptides act is an important consideration when designing experiments. Some drugs, such as 5-hydroxytryptamine, have a rapid onset of action with a short-lived maximal effect and, therefore, delaying assessment of FBF after starting an intra-arterial infusion may lead to an underestimation the maximum vascular response [75]. In contrast, endothelin has a slow onset of action, maximum vasoconstriction being reached after ~ 45 min [76]. This effect was not investigated in the first intra-arterial studies [77], and a failure to consider the effects of prolonged infusion of endothelin appears to have led to excessive doses being given subsequently with serious adverse consequences [78]. In addition, a slow onset of action makes it difficult to construct dose-response curves during a single study, which, as discussed, is the most powerful way of using the FBF technique. Similarly, tachyphylaxis may occur with repeated exposure to some agents, such as bradykinin [40] and substance P [58]. In such circumstances it becomes necessary to compare responses on different occasions, which, because of the increased variability of measurement, may require a larger number of subjects to be studied, or for comparisons between active and placebo phases to be made. Therefore, a range of preliminary pilot studies may be required to assess the characteristics of the response to intra-arterial infusions before embarking on full dose ranging studies.

When making comparisons between groups, especially control and disease states, differences in basal blood flow or mean arterial pressure are potential sources of difficulty. A *reduced* response to an infused drug might be predicted when resting blood flow is higher, due to the effect of dilution and a reduced local drug concentration, although this should only become an issue at the extremes of flow due to the logarithmic nature of most dose-response curves. However, observational data indicate an increased *absolute* response to dilators and constrictors in subjects with *higher* basal flows [79, 80], but that the *percentage* change from baseline appears to be reasonably independent of baseline flow [81], which may, therefore, be a more suitable method for data analysis. However, which method of data analysis is more appropriate remains unclear. Indeed, different approaches are liable to produce conflicting results. Therefore, perhaps a more rigorous approach, as discussed below, is the use of appropriate 'control' agent(s).

Similarly, arterial pressure influences resting smooth muscle tone and thus the wall-to-lumen ratio, a higher mean arterial pressure, by virtue of an increased wall-to-lumen ratio, should theoretically enhance the response to vasodilators and vasoconstrictors [82]. This may well explain the increased response to a wide-range of agents

including noradrenaline and verapamil in subjects with hypertension [83–85]. Increasing resting tone reduces the *in vitro* response to vasodilators [86] but, clearly, low levels of resting tone may limit the degree to which vasodilatation can be detected and, therefore, explain why pre-constriction can increase the response to some vasodilators [87, 88]. A similar problem exists during co-infusion of two vasoactive drugs, when changes in baseline flow produced by one may alter the response to the other. In such circumstances, use of FBF 'clamp' studies, as discussed in more detail below, may provide a better method with which to remove the potential influence of changes in baseline flow, but use of appropriate control drug(s) is an alternative approach.

We believe that the best way to account for differences in basal flow or arterial pressure between groups is the use of appropriate controls. For example, the inclusion of a 'control' vasoconstrictor, such as noradrenaline, in a protocol designed to investigate differences in the response to drugs, which are vasoconstrictors, between two groups (normotensive and hypertensive subjects, for example) may indicate whether any disparity is specific to that agent or is a general phenomenon, perhaps accounted for by baseline differences [52, 89]. The potential problem resulting from infusion of a drug which alters baseline FBF, such as N^G -monomethyl-L-arginine (LNMA), prior to administration of a second vasoactive agents, such as acetylcholine, can be tackled in a similar manner by using noradrenaline as a control vasoconstrictor [90]. Indeed, occasionally it may be appropriate to use a control constrictor and a control vasodilator drug [90]. However, now that 'on-line' measurement of FBF is possible, an alternative strategy is to adjust blood flow back to baseline after infusion of the first drug with an opposing agent (usually one that acts directly on smooth muscle), and then to administer the second drug. Such a system has already been used to investigate the effects of inhibition of endothelium derived nitric oxide production on the response to various drugs – the so-called '*nitric oxide clamp*' [91, 92].

Assessment of endothelial function

The vascular endothelium releases a number of biologically active mediators, including nitric oxide and endothelin-1, which regulate vessel tone and have been the focus of much research interest in recent years. It is now apparent that vasomotor regulation is only one aspect of endothelial function. Vasoactive mediators released by the vascular endothelium, including nitric oxide and endothelin-1 also have a number of other important actions including modulation of platelet aggregation and adhesion, smooth muscle cell growth, and leucocyte adhesion [43]. In addition, the endothelium

also synthesizes and releases a number of other substances including tissue plasminogen activator (tPA) [93, 94], a key enzyme in the fibrinolytic pathway, release of which can be potentiated by a variety of stimuli [95–97]. This suggests that the vascular endothelium not only plays a crucial role in regulating vessel tone and blood pressure, but also in preventing the development of atheroma, platelet activation and thrombus formation. Therefore, assessment of endothelial function has been the subject of much attention, most frequently as determined by vasomotor responses in the forearm, but techniques for measuring endothelial tPA release from the forearm vascular bed have been developed more recently [97].

Stimulated nitric oxide release

Endothelial function is commonly assessed by determining the vasodilator response, in the forearm, to brachial artery infusion of an agonist, which induces endothelial nitric oxide release via stimulation of nitric oxide synthase (endothelium-dependent agonist). It is usual also to assess the response to a direct nitric oxide donor drug, like sodium nitroprusside (endothelium-independent agonist), in order to exclude any concomitant alteration in vascular smooth muscle sensitivity to nitric oxide (Figure 7). A number of endothelium-dependent agonists have been employed in studies including: acetylcholine [98], bradykinin [65], 5-hydroxytryptamine [99] and substance P [100], although some, such as methacholine, appear to cause vasodilatation that is not nitric oxide mediated [101, 102]. Such agonists not only induce release of nitric oxide but also other vasodilator substances including endothelium derived hyperpolarizing factor (EDHF) and prostaglandins. Moreover, the nitric oxide-dependence varies between agonist [65, 88, 90], on the dose of agonist used [103], and between vascular beds for the same agonist [104]. Of particular importance when acetylcholine is employed are differences in forearm length, since this is rapidly metabolized *in vivo*. Indeed, following brachial artery administration less than 1% of the infused acetylcholine reaches the hand [105], and the vasodilator response to acetylcholine is inversely related to forearm length [80]. However, correction for forearm length is possible and, interestingly, reduces the apparent sex-related difference in the response to acetylcholine between men and women [80].

Using such methodology, endothelial dysfunction has been described in association with a number of cardiovascular risk factors (Table 3) including hypercholesterolaemia [98], diabetes mellitus [106], cigarette smoking [107] and ageing [108], although the results in hypertensive subjects are somewhat conflicting [47, 109–112]. Interestingly, endothelial dysfunction can be reversed by HMG CoA reductase inhibitors in hypercholesterolaemic

Figure 7 Effect of acetylcholine and sodium nitroprusside on forearm blood flow in hypertensive and normotensive individuals. FBF in the infused arm of hypertensive (●) and normotensive (○) individuals in response to an intra-arterial incremental infusion of acetylcholine and sodium nitroprusside. As shown, the response to acetylcholine but not nitroprusside is blunted in the hypertensive subjects. From [109] with permission.

individuals [113], and by angiotensin converting enzyme inhibitors in patients with hypertension [114], diabetes [115] and hypercholesterolaemia [116] (Table 4).

Endothelial dysfunction appears to be a systemic phenomenon, affecting resistance and conduit vessels in the forearm as well as the coronary circulation. Recent data suggest that coronary endothelial dysfunction predicts long-term atherosclerotic disease progression and cardiovascular event rates [117, 118]. Interestingly, there is a correlation between forearm vasomotor responses and those in the coronary arteries [104, 119] and, therefore, the forearm vascular bed can probably be used as a surrogate for assessing endothelial function in the coronary arteries, minimizing the invasive nature of such investigations. However, forearm responses provide information concerning resistance vessels, whilst most coronary studies involve the epicardial arteries, which are conduit vessels. Moreover, in the presence of endothelial dysfunction acetylcholine may cause paradoxical coronary vasoconstriction, whereas reduced vasodilator effects typify the response in the forearm circulation. Nevertheless, endothelial dysfunction appears to occur early in the course of cardiovascular disease, and thus assessment of endothelial function may provide a mechanism for identifying those at increased risk, before they develop manifest vascular disease [120]. Not only does this bring the promise of improved risk stratification, but also the possibility of targeting therapies designed to reduce risk factors or improve endothelial function, at those most in need.

Basal nitric oxide release

Basal release of nitric oxide in the forearm can be assessed by infusing the specific L-arginine analogue nitric oxide synthase inhibitor LNMMA [87] into the brachial artery. In healthy subjects, under resting conditions, LNMMA produces a dose-dependent reduction in FBF of up to 50% [71], which suggest that there is basal release of nitric oxide that opposes vasoconstrictor forces in the forearm. Interestingly, basal synthesis of nitric oxide may be higher in pre-menopausal women than men [121], and reduced in some conditions associated with endothelial dysfunction such as diabetes mellitus [122, 123], but not in hypercholesterolaemia [29, 124]. Again, in hypertension both reduced [89, 125] and normal [126] basal nitric oxide production have been described.

Co-infusion of LNMMA can also be used to assess the contribution of nitric oxide to any vasodilatation induced by a particular agonist, thus providing evidence of 'endothelium-dependence' [101]. However, co-infusion can also be used to address the important issue of up-regulation of alternative vasodilator pathways, such as endothelial-derived prostanoids, in conditions of endothelial dysfunction. For example reduced vasodilator response to carbachol (endothelium-dependent), and reduced vasoconstriction to LNMMA, has been described in microalbuminuric diabetics compared with controls [123]. However, when carbachol was co-infused with LNMMA, dilator responses were reduced in the control

Table 3 Endothelial dysfunction in the human forearm vascular bed and cardiovascular risk factors.

<i>Author</i>	<i>Risk factor (number of subjects)</i>	<i>Drugs used</i>	<i>Main findings</i>
Creager <i>et al.</i> [46]	Hypercholesterolaemia (13)	MCh, SNP	Blunted dilator response to MCh, but not SNP in the hypercholesterolaemic group.
Chowienczyk <i>et al.</i> [98]	Hypercholesterolaemia (12)	ACh, MCh, SNP	Blunted response to ACh in the hypercholesterolaemic, but preserved MCh and SNP vasodilatation.
Heitzer <i>et al.</i> [107]	Smoking (15), hypercholesterolaemia (15), hypercholesterolaemia + smoking (15)	ACh, SNP	Reduced response to ACh in all groups compared with controls, but significantly more impairment in the hypercholesterolaemic smokers. No difference in SNP responses.
Chowienczyk <i>et al.</i> [136]	Hypertriglyceridaemia (17)	ACh, SNP	Responses to both drugs did not differ between the groups.
Panza <i>et al.</i> [109]	Essential hypertension (18)	ACh, SNP	Reduced response to ACh, but not SNP in the hypertensives
Linder <i>et al.</i> [110]	Essential hypertension (14)	ACh, SNP	Reduced response to ACh, but not SNP in the hypertensives
Cockcroft <i>et al.</i> [47]	Essential hypertension (58)	Carbachol, ACh, SNP	Preserved ACh and carbachol responses in the hypertensives.
Taddei <i>et al.</i> [108]	Essential hypertension and age (57)	ACh, SNP	Reduced ACh response in hypertensive group compared with controls, but preserved effect of SNP. Significant negative correlation between ACh-induced vasodilatation and age in both groups.
Makimattila <i>et al.</i> [106]	Type II diabetes (30)	ACh, SNP, LNMMA	Reduced vasodilation to ACh but not SNP in the diabetic group. No difference in the effect of ACh + LNMMA on flows between groups.
Calver <i>et al.</i> [122]	Type I diabetes (10)	ACh, SNP, NA	Similar response to ACh and verapamil between the groups, verapamil, LNMMA but a reduced response to SNP in the diabetics. Reduced effect of LNMMA on basal flow compared with NA in the diabetics.
Elliot <i>et al.</i> [123]	Type I diabetes (28)	Carbachol, SNP,	Vasodilator responses to carbachol and SNP not significantly LNMMA different between the groups, but reduced effect of LNMMA on basal flow in the microalbuminuric diabetics.
Newby <i>et al.</i> [100]	Cigarette smokers (12)	Substance P	Reduced vasodilatation to substance P, and reduced release of tPA antigen, in the smokers compared with controls.

ACh – acetylcholine, MCh – methacholine, SNP – sodium nitroprusside, GTN – glyceryl trinitrate, NA – noradrenaline, LNMMA – N^G -monomethyl-L-arginine.

Table 4 Therapeutic interventions that improve endothelial function in patients with established endothelial dysfunction.

Intervention	Route	Risk factor	Agonists	References
HMG CoA reductase inhibitors	Oral	Familial hypercholesterolaemia	5HT, SNP	[99]
	Oral	Hypercholesterolaemia	ACh, SNP	[113]
LDL Apheresis	–	Familial hypercholesterolaemia	ACh, SNP	[137]
Angiotensin converting enzyme inhibitors	Oral	Hypercholesterolaemia	ACh, SNP	[116]
	Oral	Type I diabetes	ACh, SNP	[115]
Folic acid	i.a.	Familial hypercholesterolaemia	5HT, SNP	[138]
	Oral	Familial hypercholesterolaemia	5HT, SNP	[139]
Vitamin C	i.a.	Hypercholesterolaemia	MCh, SNP, verapamil	[54]
	i.a.	Type I diabetes	MCh, SNP	[140]
	i.a.	Essential hypertension	ACh, SNP	[141]
Vitamin E	Oral	Hypercholesterolaemia + smoking	ACh, SNP	[142]
	Oral	Hypercholesterolaemia	ACh, SNP	[143]
Fish oils	Oral	Type II diabetes	ACh, GTN	[144]

ACh – acetylcholine, SNP – sodium nitroprusside, MCh – methacholine, 5HT – 5-hydroxytryptamine, GTN – glyceryl trinitrate, i.a. – intra-arterial.

group but not in the diabetics, suggesting that there was up-regulation of non-nitric oxide dilator pathways in the diabetic group. Similar results have been reported in individuals with hypertension [125] and hypercholesterolaemia [124].

Since LNMMA has a vasoconstrictor action, changes in baseline FBF may alter the response to subsequent agonists, in a non-specific manner. Likewise, a reduced response to infused LNMMA could also be a non-specific effect, and may also be seen for other ‘control’ vasoconstrictors [126]. Therefore, the use of some form of control in studies using LNMMA is essential. This may involve using either a control dilator or vasoconstrictor, [90] or the ‘nitric oxide clamp’ [92].

Other methods for assessing nitric oxide-dependent pathways

Endothelial function can also be assessed in the forearm by induction of ischaemia, which is the subject of a more detailed review in this series [127]. This method was first suggested by Sinoway in 1989 [128], but introduced as the technique of *flow-mediated dilatation* by Celermajer *et al.* in 1992 [129]. It employs forearm ischaemia as a stimulus to increase flow in the brachial artery, resulting in an increase in shear stress and, ultimately, nitric oxide-mediated dilatation of the brachial artery, which can be assessed using ultrasound. Sublingual GTN is frequently used as a control endothelium-independent dilator, although the precise dose seems to be a matter of some debate [130]. While this technique has the advantage of being non-invasive, it requires considerable expertise and expensive equipment, and doubts still exist over its reproducibility [131] and validity. Moreover, studies demonstrating a close relationship between flow-mediated responses in the forearm and coronary

endothelial function [132] are difficult to interpret since different stimuli (e.g. ischaemia and acetylcholine) are usually employed in the two beds. However, one may consider flow-mediated dilatation and intra-arterial studies as complementary, since they are assessing endothelial function in different parts of the arterial tree: flow-mediated dilatation provides information about arterial conduit vessels, and FBF about endothelial function in the resistance vessels.

Other aspects of vascular endothelial function

The vascular endothelium also plays an important role in the regulation of fibrinolysis. The endothelium synthesizes and releases tPA [93, 94], a key enzyme in the fibrinolytic pathway, which can be potentiated by a variety of stimuli [95, 96]. Using the basic technique of FBF coupled with intra-arterial drug infusion, Newby *et al.* have demonstrated that substance P increases plasma tPA in the infused arm compared with the non-infused arm (by assessing tPA activity in venous effluent) [97], and that this phenomenon is not due to increased blood flow *per se*. Moreover, the effect of substance P can be inhibited by co-administration of LNMMA [133]. Interestingly, reduced stimulated tPA release has been described in cigarette smokers [100], which may provide a mechanism by whereby endothelial dysfunction may increase the risk of atherothrombosis due to reduced fibrinolytic capacity. This has also been confirmed in the coronary circulation recently [145].

Pharmacogenetics

There is increasing interest in the influence of genotype on the effect of drugs *in vivo*. However, drugs can also be used to characterize phenotypic differences between individuals

with various genetic polymorphisms, which may or may not be apparent in the resting state. For example, the degree of vasodilatation in the forearm in response to an intra-arterial infusion of isoprenaline, a β -adrenoceptor agonist, is dependent on polymorphisms of the β_2 -adrenoceptor gene [134]. Interestingly, the same polymorphisms also influence the response to inhaled β_2 -adrenoceptor agonists in patients with asthma [135]. Clearly, therefore, assessing the FBF response to drugs given intra-arterially may provide a useful tool for improved phenotypic characterization of individuals and a better understanding of the potential clinical relevance of genetic polymorphisms.

Conclusions

Venous occlusion plethysmography provides a simple, and robust method for assessing blood flow *in vivo*. It is most frequently applied to the forearm and is a versatile technique that has proved extremely valuable in assessing human vascular physiology and pharmacology, especially when coupled with intra-arterial drug infusion. It has also been employed extensively to assess endothelial function *in vivo*, for which it remains the 'gold-standard'. Unfortunately, the invasive nature of the technique has prevented its use in addressing the important question of whether changes in endothelial function, particularly following drug therapy, are a reliable surrogate of cardiovascular risk [120]. Regrettably, flow-mediated dilatation is unlikely to be useful in this context either, and we must await a simple, reliable, non-invasive test of endothelial function to address this issue. However, plethysmographic assessment of FBF does provide an ideal method for assessing the effects of various drugs and endogenous peptides on the peripheral resistance vessels *in vivo*, without the need to conduct systemic studies. The technique is at its most powerful when used to compare responses within an individual during a single study, for example construction of dose-response curves before and after administration of an antagonist. Use of receptor antagonists has also extended our understanding of the physiological and pathological processes regulating blood flow *in vivo*. Clearly, almost 100 years after its first description, the place of venous occlusion plethysmography in physiology and pharmacology remains firmly established due to the robust validation of the technique and its great utility for addressing the important issues of the time.

We would like to thank Dr John Cockcroft for critical appraisal of the manuscript and helpful suggestions regarding key references, Professor Patrick Vallance for providing the information regarding experience of intra-arterial cannulation and Fiona Strachan for help with Figure 1.

References

- Hewlett AW, van Zwaluwenburg JG. The rate of blood flow in the arm. *Heart* 1909; **1**: 87–97.
- Webb DJ, Hand MF. Assessment of the effects of drugs on the peripheral vasculature. In *Clinical measurement in drug evaluation*, eds Nimmo WS, Tucker GT. London: John Wiley & Sons Ltd, 1995; 136–150.
- Greenfield ADM, Whitney RJ, Mowbray JF. Methods for the investigation of peripheral blood flow. *Br Med Bull* 1963; **19**: 101–109.
- Cooper KE, Edholm OG, Mottram RF. The blood flow in skin and muscle of the human forearm. *J Physiol* 1955; **128**: 258–267.
- Abramson DI, Ferris EB Jr. Responses of blood vessels in the resting hand and forearm to various stimuli. *Am Heart J* 1940; **19**: 541–553.
- Whitney RJ. The measurement of volume changes in human limbs. *J Physiol* 1953; **121**: 1–27.
- Wilkins RW, Bradley SE. Changes in arterial and venous blood pressure and flow distal to a cuff inflated on the human arm. *Am J Physiol* 1946; **147**: 260–269.
- Kerslake DM. The effect of application of an arterial occlusion cuff to the wrist on blood flow in the human forearm. *J Physiol* 1949; **108**: 451–457.
- Pedrinelli R, Taddei S, Spessot M, Salvetti A. Maximal post-ischaemic forearm vasodilation in human hypertension: a re-assessment of the method. *J Hypertens* 1987; **5**: S431–S433.
- Chang PC, Verlinde R, van Brummelen P. A microcomputer-based, R-wave triggered system for hemodynamic measurements in the forearm. *Computer Biol Med* 1998; **18**: 157–163.
- Ensink FBM, Hellige G. History and principle of strain-gauge plethysmography. In *Non-invasive methods on cardiovascular haemodynamics*, ed Jageneau AHM. Amsterdam: Elsevier/North Holland, 1981; 169–183.
- Hokanson DE, Sumner DS, Strandness DE. An electrically calibrated plethysmograph for direct measurement of limb blood flow. *IEEE Trans Biomed Eng* 2000; **22**: 21–25.
- Ensink FBM, Baller D, Wolpers HG, Zipfel J, Hellige G. The reliability of venous capacity and blood flow determination by plethysmograph. In *Non-invasive methods on cardiovascular haemodynamics*, ed Jageneau AHM. Amsterdam: Elsevier/North Holland, 1981; 215–226.
- Dahn I, Hallbrook T. Simultaneous blood flow measurements by water and strain gauge plethysmography. *Scand J Clin Lab Invest* 1970; **25**: 419–428.
- Christ F, Bauer A, Brugger D, Niklas M, Gartside IB, Gamble J. Description and validation of a novel liquid metal-free device for venous congestion plethysmography. *J Appl Physiol* 2000; **89**: 1577–1583.
- Hoskins PR, Fish PJ, McDicken WN, Moran CM. Recent developments in cardiovascular ultrasound II. arterial applications. *Biol Engineering Comp* 1998; **36**: 259–269.
- Patterson GC, Whelan RF. Reactive hyperaemia in the human forearm. *Clin Sci* 1955; **14**: 197–211.
- Takeshita A, Mark AL. Decreased vasodilator capacity of forearm resistance vessels in borderline hypertension. *Hypertension* 1980; **2**: 610–616.
- Conway J. A vascular abnormality in hypertension: a study of blood flow in the forearm. *Circulation* 1963; **27**: 520–529.

- 20 Rosei EA, Rizzoni D, Castellano M, *et al.* Media: lumen ratio in human small resistance arteries is related to forearm minimal vascular resistance. *J Hypertens* 1995; **13**: 341–347.
- 21 Folkow B. Physiological aspects of primary hypertension. *Physiol Rev* 1982; **62**: 347–504.
- 22 Julius S, Mejia A, Jones K, *et al.* 'White coat' versus 'sustained' borderline hypertension in Tecumseh, Michigan. *Hypertension* 1990; **16**: 617–623.
- 23 Sivertsson R, Hansson L. Effects of blood pressure reduction on the structural vascular abnormality in skin and muscle vascular beds in human essential hypertension. *Clin Sci Mol Med* 1976; **51**: 77–79.
- 24 Agabiti-Rosei E, Muiesan ML, Rizzoni D, Romanelli G, Beschi M, Castellano M. Regression of cardiovascular structural changes after long-term antihypertensive treatment with the calcium antagonist nitrendipine. *J Cardiovasc Pharmacol* 1991; **18**: S5–S9.
- 25 Marceau M, Kouame N, Lacourciere Y, Cleroux J. Vascular structure in the forearm and calf after 6 months of angiotensin converting enzyme inhibition in elderly hypertensive subjects with left ventricular hypertrophy. *J Hypertens* 1998; **16**: 673–679.
- 26 Brown E, Greenfield ADM, Goei JS, Plassaras G. Filling and emptying of the low-pressure blood vessels of the human forearm. *J Appl Physiol* 1966; **21**: 573–582.
- 27 Mason DT, Braunwald E. Effect of nitroglycerine and amyl nitrate on arteriolar and venous tone in the human forearm. *Circulation* 1965; **32**: 755–766.
- 28 Cheesman AR, Benjamin N. Lack of tolerance in forearm blood vessels in man to glyceryl trinitrate. *Br J Clin Pharmacol* 1994; **37**: 441–444.
- 29 John S, Schlaich M, Langenfeld M, *et al.* Increased bioavailability of nitric oxide after lipid-lowering therapy in hypercholesterolemic patients: a randomized, placebo-controlled, double-blind study. *Circulation* 1998; **98**: 211–216.
- 30 Gamble J, Gartside IB, Christ F. A reassessment of mercury in silastic strain gauge plethysmography for microvascular permeability assessment in man. *J Physiol (Lond)* 1993; **464**: 407–422.
- 31 Brown E, Wise CS, Wheeler EO. Effect of local cooling on the filtration and absorption of fluid in the human forearm. *J Clin Invest* 1947; **26**: 1031.
- 32 Hyman C, Wong WH. Capillary filtration coefficient in the extremities of man in high environmental temperatures. *Circ Res* 1968; **22**: 251–261.
- 33 Ando S, Imaizumi T, Harada S, Hirooka Y, Takeshita A. Atrial natriuretic peptide increases human capillary filtration and venous distensibility. *J Hypertens* 1992; **10**: 451–457.
- 34 Jaap AJ, Shore AC, Gartside IB, Gamble J, Tooke JE. Increased microvascular fluid permeability in young type 1 (insulin-dependent) diabetic patients. *Diabetologia* 1993; **36**: 648–652.
- 35 Abboud FM, Eckberg DL, Johannsen UJ, Mark AL. Carotid and cardiopulmonary baroreceptor control of splanchnic and forearm vascular resistance during venous pooling in man. *J Physiol (Lond)* 1979; **286**: 173–184.
- 36 Brown E, Goei JS, Greenfield ADM, Plassaras G. Circulatory responses to stimulated gravitational shifts of blood in man induced by exposure of the body below the iliac crests to subatmospheric pressure. *J Physiol (Lond)* 1966; **183**: 607–627.
- 37 Seidelin PH, Collier JG, Struthers AD, Webb DJ. Angiotensin II augments sympathetically mediated arteriolar constriction in man. *Clin Sci* 1991; **81**: 261–266.
- 38 Haynes WG, Webb DJ. Contribution of endogenous generation of endothelin-1 to basal vascular tone. *Lancet* 1994; **344**: 852–854.
- 39 Allen WJ, Barcroft H, Edholm OG. On the actions of adrenaline on the blood vessels in human skeletal muscle. *J Physiol (Lond)* 1946; **105**: 255–267.
- 40 Benjamin N, Cockcroft JR, Collier JG, *et al.* Local inhibition of converting enzyme and vascular responses to angiotensin and bradykinin in the human forearm. *J Physiol (Lond)* 1989; **412**: 543–555.
- 41 Blair DA, Glover WE, Greenfield ADM, Roddie IC. Excitation of cholinergic vasodilator nerves to human skeletal muscle during severe emotional stress. *J Physiol (Lond)* 1959; **148**: 633–647.
- 42 Roberts DH, Tsao Y, Breckenridge AM. The reproducibility of limb blood flow measurements in human volunteers at rest and after exercise by using mercury-in-Silastic strain gauge plethysmography under standardized conditions. *Clin Sci* 1986; **70**: 635–638.
- 43 Cooke JP, Dzau VJ. Nitric oxide synthase: role in the genesis of vascular disease. *Annu Rev Med* 1997; **48**: 489–509.
- 44 Petrie JR, Ueda S, Morris AD, Murray LS, Elliott HL, Connell JMC. How reproducible is bilateral forearm plethysmography? *Br J Clin Pharmacol* 1998; **45**: 131–139.
- 45 Walker H, Jackson G, Ritter JM, Chowienzyk PJ. Reproducibility of bilateral forearm plethysmography to endothelium-dependent vasodilator. *Br J Pharmacol* 1999; **126**: 73P.
- 46 Creager MA, Cooke JP, Mendelsohn ME, *et al.* Impaired vasodilation of forearm resistance vessels in hypercholesterolemic humans. *J Clin Invest* 1990; **86**: 228–234.
- 47 Cockcroft JR, Chowienzyk PJ, Benjamin N, Ritter JM. Preserved endothelium-dependent vasodilatation in patients with essential hypertension. *N Engl J Med* 1994; **330**: 1036–1040.
- 48 Newby DE, Flint L, Fox KAA, Boon NA, Webb DJ. Reduced responsiveness to endothelin-1 in peripheral resistance vessels of patients with syndrome X. *J Am Coll Cardiol* 1998; **31**: 1585–1590.
- 49 Greenfield ADM, Patterson GC. Reactions of the blood vessels of the human forearm to increases in transmural pressure. *J Physiol (Lond)* 1954; **125**: 508–524.
- 50 Cockcroft JR, Sciberras DG, Goldberg MR, Ritter JM. Comparison of angiotensin-converting enzyme inhibition with angiotensin II receptor antagonism in the human forearm. *J Cardiovasc Pharmacol* 1993; **22**: 579–584.
- 51 Haynes WG, Strachan FE, Webb DJ. Endothelin ET (A) and ET (B) receptors cause vasoconstriction of human resistance and capacitance vessels in vivo. *Circulation* 1995; **92**: 357–363.
- 52 Benjamin N, Calver A, Collier JG, Robinson B, Vallance P, Webb D. Measuring forearm blood flow and interpreting the responses to drugs and mediators. *Hypertension* 1995; **25**: 918–923.
- 53 Kingwell BA, Tran B, Cameron JD, Jennings GL, Dart AM. Enhanced vasodilation to acetylcholine in athletes is associated with lower plasma cholesterol. *Am J Physiol* 1996; **270**: H2008–H2013.

- 54 Ting HH, Timimi FK, Haley EA, Roddy M-A, Ganz P, Creager MA. Vitamin C improves endothelium-dependent vasodilation in forearm resistance vessels of humans with hypercholesterolemia. *Circulation* 1997; **95**: 2617–2622.
- 55 Matthews J, Altman D, Campbell M, Royston P. Analysis of serial measurements in medical research. *Br Med J* 1990; **300**: 230–235.
- 56 Motwani JG, Struthers AD. Dose–response study of the redistribution of intravascular volume by angiotensin II in man. *Clin Sci* 1992; **82**: 397–405.
- 57 Webb DJ, Benjamin N, Allen MJ, Brown J, O'Flynn M, Cockcroft JR. Vascular responses to local atrial natriuretic peptide infusion in man. *Br J Clin Pharmacol* 1988; **26**: 245–251.
- 58 Newby DE, Sciberras DG, Ferro CJ, *et al.* Substance P-induced vasodilatation is mediated by the neurokinin type receptor but does not contribute to basal vascular tone in man. *Br J Clin Pharmacol* 1999; **48**: 336–344.
- 59 Cockcroft JR, Chowienzyk PJ, Brett SE, Bender N, Ritter JM. Inhibition of bradykinin-induced vasodilation in human forearm vasculature by icatibant, a potent beta2-receptor antagonist. *Br J Clin Pharmacol* 1994; **38**: 317–322.
- 60 Ashton H. Peripheral circulation in man: critical closure in human limbs. *Br Med Bull* 1963; **19**: 149–154.
- 61 Strachan FE, Newby DE, Sciberras DG, McCrea JB, Goldberg MR, Webb DJ. Reproducibility of forearm vasoconstriction to intra-arterial endothelin-1. *Br J Clin Pharmacol* 1998; **45**: 194P.
- 62 Collier JG, Robinson BF, Vane JR. Reduction of pressor effects of angiotensin I in man by synthetic nonapeptide (B.P.P._{9a} or SQ 20, 881) which inhibits converting enzyme. *Lancet* 1973; **i**: 72–74.
- 63 Clarke JG, Benjamin N, Larkin SW, Webb DJ, Davies GJ, Maseri A. Endothelin is a potent long-lasting vasoconstrictor in men. *Am J Physiol* 1989; **257**: H2033–H2035.
- 64 Vierhapper H, Wagner O, Nowotny P, Waldhausl W. Effect of endothelin-1 in man. *Circulation* 1990; **81**: 1415–1418.
- 65 Cockcroft JR, Chowienzyk PJ, Brett SE, Ritter JM. Effect of N (G)-monomethyl-L-arginine on kinin-induced vasodilation in the human forearm. *Br J Clin Pharmacol* 1994; **38**: 307–310.
- 66 Bonner G, Preis S, Schunk U, Toussaint C, Kaufmann W. Hemodynamic effects of bradykinin on systemic and pulmonary circulation in healthy and hypertensive humans. *J Cardiovasc Pharmacol* 1990; **15**: S46–S56.
- 67 Collier JG, Lorge RE, Robinson BF. Comparison of effects of tolmesoxide (RX1107), diazoxide, hydralazine, prazosin, glyceryl trinitrate and sodium nitroprusside on forearm arteries and dorsal hand veins of man. *Br J Pharmacol* 1978; **5**: 35–44.
- 68 Palmer RF, Lasseeter KC. Drug therapy: sodium nitroprusside. *N Engl J Med* 1975; **292**: 294–297.
- 69 Baan JJ, Chang PC, Vermeij P, Pfaffendorf M, Van Zwieten PA. Effects of losartan on vasoconstrictor responses to angiotensin I in the forearm vascular bed of healthy volunteers. *Cardiovasc Res* 1996; **32**: 973–979.
- 70 Chang PC, Van Brummelen P, Vermeij P. Acute vasodilator action of pindolol in humans. *Hypertension* 1985; **7**: 146–150.
- 71 Vallance P, Collier J, Moncada S. Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. *Lancet* 1989; **ii**: 997–1000.
- 72 Burnier M, Waeber B, Brunner HR. Clinical pharmacology of the angiotensin II receptor antagonist losartan potassium in healthy subjects. *J Hypertens* 1995; **13**: S23–S28.
- 73 Haynes WG, Noon JP, Walker BR, Webb DJ. Inhibition of nitric oxide synthesis increases blood pressure in healthy humans. *J Hypertens* 1993; **11**: 1375–1380.
- 74 Strachan FE, Spratt JC, Wilkinson IB, Johnston NR, Gray GA, Webb DJ. Systemic blockade of the endothelin-B receptor increases peripheral vascular resistance in healthy men. *Hypertension* 1999; **33**: 581–585.
- 75 Roddie IC, Shepherd JT, Whelan RF. The action of 5-hydroxytryptamine on the blood vessels of the human hand and forearm. *Br J Clin Pharmacol* 1955; **10**: 445–450.
- 76 Haynes WG, Clarke JG, Cockcroft JR, Webb DJ. Pharmacology of endothelin-1 in vivo in humans. *J Cardiovasc Pharmacol* 1991; **17**: S284–S286.
- 77 Hughes AD, Thom SAM, Woodall N, *et al.* Human vascular responses to endothelin-1: Observations in vivo and in vitro. *J Cardiovasc Pharmacol* 1989; **13**: S225–S228.
- 78 Dahlof B, Gustafsson D, Hedner T, Jern S, Hansson L. Regional haemodynamic effects of endothelin-1 in rat and man: unexpected adverse reactions. *J Hypertens* 1990; **8**: 811–817.
- 79 Robinson BF. Assessment of responses to drugs in forearm resistance vessels and hand veins of man: techniques and problems. In *Dose-response relationships of drugs*, eds. Kuhlmann J, Wingender W. Munich: Zuckschwerdt-Verlag, 1990: 40–43.
- 80 Chowienzyk PJ, Cockcroft JR, Ritter JM. Blood flow responses to intra-arterial acetylcholine in man: effects of basal flow and conduit vessel length. *Clin Sci* 1994; **87**: 45–51.
- 81 Pedrinelli R, Taddei S, Salvetti A. Calcium entry blockade and alpha-adrenergic vascular reactivity in human beings: differences between nicardipine and verapamil. *Clin Pharmacol Ther* 1989; **45**: 285–290.
- 82 Folkow B. Cardiovascular structural adaptation: its role in the initiation and maintenance of hypertension. *Clin Sci Mol Med* 1978; **55**: 3S–22S.
- 83 Doyle AE, Fraser JRE, Marshall JR. Reactivity of forearm vessels to vasoconstrictor substances in hypertensive and normotensive subjects. *Clin Sci* 1959; **18**: 441–454.
- 84 Egan B, Schork N, Panis R, Hinderliter A. Vascular structure enhances regional resistance responses in mild essential hypertension. *J Hypertens* 1988; **6**: 41–48.
- 85 Hulthen UL, Bolli P, Buhler FR. Vasodilatory effect of nicardipine and verapamil in the forearm of hypertensive as compared with normotensive man. *Br J Clin Pharmacol* 1985; **20** (Suppl 1): 62S–66S.
- 86 Furchgott RF. The pharmacology of vascular smooth muscle. *Pharmacol Rev* 1955; **7**: 183–256.
- 87 Rees DD, Palmer RMJ, Moncada S. Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc Natl Acad Sci U S A* 1989; **86**: 3375–3378.
- 88 Newby DE, Boon NA, Webb DJ. Comparison of forearm vasodilatation to substance P and acetylcholine: contribution of nitric oxide. *Clin Sci* 1997; **92**: 133–138.
- 89 Calver A, Collier J, Moncada S, Vallance P. Effect of local intra-arterial N (G)-monomethyl-L-arginine in patients with hypertension: the nitric oxide dilator mechanism appears abnormal. *J Hypertens* 1992; **10**: 1025–1031.

- 90 O'Kane KPJ, Webb DJ, Collier JG, Vallance PJT. Local L-N (G)-monomethyl-arginine attenuates the vasodilator action of bradykinin in the human forearm. *Br J Clin Pharmacol* 1994; **38**: 311–315.
- 91 Stroes ES, Luscher TF, de Groot FG, Koomans HA, Rabelink TJ. Cyclosporin A increases nitric oxide activity in vivo. *Hypertension* 1997; **29**: 570–575.
- 92 Verhaar MC, Strachan FE, Newby DE, *et al.* Endothelin-A receptor antagonist-mediated vasodilatation is attenuated by inhibition of nitric oxide synthesis and by endothelin-B receptor blockade. *Circulation* 1998; **97**: 752–756.
- 93 Hekman CM, Loskutoff DJ. Fibrinolytic pathways and the endothelium. *Semin Thromb Hemost* 1987; **13**: 514–527.
- 94 Smith D, Gilbert M, Owen WG. Tissue plasminogen activator release in vivo in response to vasoactive agents. *Blood* 1985; **66**: 835–839.
- 95 Jern C, Selin L, Jern S. In vivo release of tissue-type plasminogen activator across the human forearm during mental stress. *Thromb Haemost* 1994; **72**: 285–291.
- 96 Wall U, Jern C, Jern S. High capacity for tissue-type plasminogen activator release from vascular endothelium in vivo. *J Hypertens* 1997; **15**: 1641–1647.
- 97 Newby DE, Wright RA, Ludlam CA, Fox KAA, Boon NA, Webb DJ. An in vivo model for the assessment of acute fibrinolytic capacity of the endothelium. *Thromb Haemost* 1997; **78**: 1242–1248.
- 98 Chowieńczyk PJ, Watts GF, Cockcroft JR, Ritter JM. Impaired endothelium-dependent vasodilation of forearm resistance vessels in hypercholesterolaemia. *Lancet* 1992; **340**: 1430–1432.
- 99 Stroes ESG, Koomans HA, De Bruin TWA, Rabelink TJ. Vascular function in the forearm of hypercholesterolaemic patients off and on lipid-lowering medication. *Lancet* 1995; **346**: 467–471.
- 100 Newby DE, Wright RA, Labinjoh C, *et al.* Endothelial dysfunction, impaired endogenous fibrinolysis, and cigarette smoking: a mechanism for arterial thrombosis and myocardial infarction. *Circulation* 1999; **99**: 1411–1415.
- 101 Chowieńczyk PJ, Cockcroft JR, Ritter JM. Differential inhibition by N (G)-monomethyl-L-arginine of vasodilator effects of acetylcholine and methacholine in human forearm vasculature. *Br J Pharmacol* 1993; **110**: 736–738.
- 102 Rongen GA, Smits P, Thien T. N (G)-monomethyl-L-ARG reduces the forearm vasodilator response to acetylcholine but not to methacholine in humans. *J Cardiovasc Pharmacol* 1993; **22**: 884–888.
- 103 Chowieńczyk PJ, Cockcroft JR, Ritter JM. Inhibition of acetylcholinesterase selectively potentiates N (G)-monomethyl-L-arginine-resistant actions of acetylcholine in human forearm vasculature. *Clin Sci* 1995; **88**: 111–117.
- 104 Tagawa T, Mohri M, Tagawa H, *et al.* Role of nitric oxide in substance P-induced vasodilation differs between the coronary and forearm circulation in humans. *J Cardiovasc Pharmacol* 1997; **29**: 546–553.
- 105 Duff F, Greenfield ADM, Shepherd JT, Thompson ID. A quantitative study of the response to acetylcholine and histamine of the blood vessels of the human hand and forearm. *J Physiol* 1953; **120**: 160–170.
- 106 Makimattila S, Liu ML, Vakkilainen J, *et al.* Impaired endothelium-dependent vasodilation in type 2 diabetes. Relation to LDL size, oxidized LDL, and antioxidants. *Diabetes Care* 1999; **22**: 973–981.
- 107 Heitzer T, Yla-Herttuala S, Luoma J, *et al.* Cigarette smoking potentiates endothelial dysfunction of forearm resistance vessels in patients with hypercholesterolemia: role of oxidized LDL. *Circulation* 1996; **93**: 1346–1353.
- 108 Taddei S, Virdis A, Mattei P, *et al.* Aging and endothelial function in normotensive subjects and patients with essential hypertension. *Circulation* 1995; **91**: 1981–1987.
- 109 Panza JA, Quyyumi AA, Brush JEJ, Epstein SE. Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. *N Engl J Med* 1990; **323**: 22–27.
- 110 Linder L, Kiowski W, Buhler FR, Luscher TF. Indirect evidence for release of endothelium-derived relaxing factor in human forearm circulation in vivo. Blunted response in essential hypertension. *Circulation* 1990; **81**: 1762–1767.
- 111 Panza JA, Casino PR, Kilcoyne CM, Quyyumi AA. Impaired endothelium-dependent vasodilation in patients with essential hypertension: evidence that the abnormality is not at the muscarinic receptor level. *J Am Coll Cardiol* 1994; **23**: 1610–1616.
- 112 Bruning TA, Chang PC, Hendriks MGC, Vermeij P, Pfaffendorf M, Van Zwieten PA. In vivo characterization of muscarinic receptor subtypes that mediate vasodilatation in patients with essential hypertension. *Hypertension* 1995; **26**: 70–77.
- 113 O'Driscoll G, Green D, Taylor RR. Simvastatin, an HMG-coenzyme A reductase inhibitor, improves endothelial function within 1 month. *Circulation* 1997; **95**: 1126–1131.
- 114 Hirooka Y, Imaizumi T, Masaki H, *et al.* Captopril improves impaired endothelium-dependent vasodilation in hypertensive patients. *Hypertension* 1992; **20**: 175–180.
- 115 O'Driscoll G, Green D, Rankin J, Stanton K, Taylor R. Improvement in endothelial function by angiotensin converting enzyme inhibition in insulin-dependent diabetes mellitus. *J Clin Invest* 1997; **100**: 678–684.
- 116 Lee AFC, Dick JBC, Bonnar CE, Struthers AD. Lisinopril improves arterial function in hyperlipidaemia. *Clin Sci* 1999; **96**: 441–448.
- 117 Schachinger V, Britten MB, Zeiher AM. Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. *Circulation* 2000; **101**: 1899–1906.
- 118 Suwaidi JA, Hamasaki S, Higano ST, Nishimura RA, Holmes DR Jr, Lerman A. Long-term follow-up of patients with mild coronary artery disease and endothelial dysfunction. *Circulation* 2000; **101**: 948–954.
- 119 Hirooka Y, Egashira K, Imaizumi T, *et al.* Effect of L-arginine on acetylcholine-induced endothelium-dependent vasodilation differs between the coronary and forearm vasculatures in humans. *J Am Coll Cardiol* 1994; **24**: 948–955.
- 120 Luscher TF. The endothelium and cardiovascular disease: a complex relation. *N Engl J Med* 1994; **330**: 1081–1083.
- 121 Kneale BJ, Chowieńczyk PJ, Cockcroft JR, Coltart DJ, Ritter JM. Vasoconstrictor sensitivity to noradrenaline and N (G)-monomethyl-L-arginine in men and women. *Clin Sci* 1997; **93**: 513–518.

- 122 Calver A, Collier J, Vallance P. Inhibition and stimulation of nitric oxide synthesis in the human forearm arterial bed of patients with insulin-dependent diabetes. *J Clin Invest* 1992; **90**: 2548–2554.
- 123 Elliott TG, Cockcroft JR, Groop P-H, Viberti GC, Ritter JM. Inhibition of nitric oxide synthesis in forearm vasculature of insulin-dependent diabetic patients: blunted vasoconstriction in patients with microalbuminuria. *Clin Sci* 1993; **85**: 687–693.
- 124 Casino PR, Kilcoyne CM, Quyyumi AA, Hoeg JM, Panza JA. The role of nitric oxide in endothelium-dependent vasodilation of hypercholesterolemic patients. *Circulation* 1993; **88**: 2541–2547.
- 125 Panza JA, Casino PR, Kilcoyne CM, Quyyumi AA. Role of endothelium-derived nitric oxide in the abnormal endothelium-dependent vascular relaxation of patients with essential hypertension. *Circulation* 1993; **87**: 1468–1474.
- 126 Kneale BJ, Chowienczyk PJ, Brett SE, Cockcroft JR, Ritter JM. Forearm vasoconstriction in response to noradrenaline and N (G)-monomethyl-L-arginine in essential hypertension. *Clin Sci* 1999; **97**: 277–282.
- 127 Celermajer DS. Flow-mediated dilatation. *Br J Clin Pharmacol* 2000; **50**: 397–404.
- 128 Sinoway LI, Hendrickson C, Davidson WRJ, Prophet S, Zelis R. Characteristics of flow-mediated brachial artery vasodilation in human subjects. *Circ Res* 1989; **64**: 32–42.
- 129 Celermajer DS, Sorensen KE, Gooch VM, *et al.* Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. *Lancet* 1992; **340**: 1111–1115.
- 130 Ghiadoni L, Donald AE, Cropley M, *et al.* Mental stress induces transient endothelial dysfunction in humans. *Circulation* 2000; **102**: 2473–2478.
- 131 Hardie KL, Kinlay S, Hardy DB, *et al.* Reproducibility of brachial ultrasonography and flow-mediated dilatation (FMD) for assessing endothelial function. *Aust N Z J Med* 1997; **27**: 649–652.
- 132 Anderson TJ, Uehata A, Gerhard MD, *et al.* Close relationship of endothelial function in the human coronary and peripheral circulations. *J Am Coll Cardiol* 1995; **26**: 1235–1241.
- 133 Newby DE, Wright RA, Dawson P, *et al.* The L-arginine/nitric oxide pathway contributes to the acute release of tissue plasminogen activator in vivo in man. *Cardiovasc Res* 1998; **38**: 485–492.
- 134 Cockcroft JR, Gazis AG, Cross DJ, *et al.* Beta₂-adrenoceptor polymorphism determines vascular reactivity in humans. *Hypertension* 2000; **36**: 371–375.
- 135 Lipworth BJ, Hall IP, Aziz I, Tan KS, Wheatley A. Beta₂-adrenoceptor polymorphism and bronchoprotective sensitivity with regular short- and long-acting beta₂-agonist therapy. *Clin Sci* 1999; **96**: 253–259.
- 136 Chowienczyk PJ, Watts GF, Wierzbicki AS, Cockcroft JR, Brett SER. Preserved endothelial function in patients with severe hypertriglyceridemia and low functional lipoprotein lipase activity. *J Am Coll Cardiol* 1997; **29**: 964–968.
- 137 Tamai O, Matsuoka H, Itabe H, Wada Y, Kohno K, Imaizumi T. Single LDL apheresis improves endothelium-dependent vasodilatation in hypercholesterolemic humans. *Circulation* 1997; **95**: 76–82.
- 138 Verhaar MC, Wever RMF, Kastelein JJP, *et al.* 5-methyltetrahydrofolate, the active form of folic acid, restores endothelial function in familial hypercholesterolemia. *Circulation* 1998; **97**: 237–241.
- 139 Verhaar MC, Wever RMF, Kastelein JJP, *et al.* Effects of oral folic acid supplementation on endothelial function in familial hypercholesterolemia: a randomized placebo-controlled trial. *Circulation* 1999; **100**: 335–338.
- 140 Timimi FK, Ting HH, Haley EA, Roddy M-A, Ganz P, Creager MA. Vitamin C improves endothelium-dependent vasodilation in patients with insulin-dependent diabetes mellitus. *J Am Coll Cardiol* 1998; **31**: 552–557.
- 141 Taddei S, Virdis A, Ghiadoni L, Magagna A, Salvetti A. Vitamin C improves endothelium-dependent vasodilation by restoring nitric oxide activity in essential hypertension. *Circulation* 1998; **97**: 2222–2229.
- 142 Heitzer T, Herttua SY, Wild E, Luoma J, Drexler H. Effect of vitamin E on endothelial vasodilator function in patients with hypercholesterolemia, chronic smoking or both. *J Am Coll Cardiol* 1999; **33**: 499–505.
- 143 Green D, O'Driscoll G, Rankin JM, Maiorana AJ, Taylor RR. Beneficial effect of vitamin E administration on nitric oxide function in subjects with hypercholesterolaemia. *Clin Sci* 1998; **95**: 361–367.
- 144 McVeigh GE, Brennan GM, Johnston GD, *et al.* Dietary fish oil augments nitric oxide production or release in patients with Type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia* 1993; **36**: 33–38.
- 145 Newby DE, McLeod AL, Uren NG, *et al.* Impaired coronary tissue plasminogen activator release is associated with coronary atherosclerosis and cigarette smoking: direct link between endothelial dysfunction and atherosclerosis. *Circulation* 2001; **103**: 1936–1941.