Assessment of microvascular endothelial function in human skin

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ABSTRACT

Endothelial dysfunction is an important factor in many cardiovascular diseases, and is commonly associated with impaired endothelium-mediated vasodilatation. Information about the mechanisms behind this dysfunction has come largely from animal studies or, in humans, through invasive techniques that are not specific to one vascular bed. We have developed protocols to assess endothelial function non-invasively in the cutaneous microcirculation by measuring blood flow responses to four receptor-specific vasoactive compounds. Cumulative doses of acetylcholine, methacholine, bradykinin and substance P were administered iontophoretically to the forearm skin of healthy volunteers on two to three occasions. Dose-dependent increases in skin microvascular blood flow in response to these drugs were measured with laser Doppler imaging. Vascular responses to acetylcholine and methacholine were reasonably consistent, with coefficients of variation of approx. 17%. The coefficients of variation for bradykinin and substance P were much poorer, as high as 70% for some doses. This might partly be a consequence of the more unpredictable effects of histamine release in the vasoactive behaviour of these two agonists. Although it might be advantageous to find other agonists with which to test the function of different receptor pathways, we have shown that just acetylcholine and methacholine can currently be used with iontophoresis to allow sensitive and reproducible assessment of endothelial function.

INTRODUCTION

The central role of the endothelium in vascular regulation is highlighted by the many pathological conditions in which its dysfunction is an important feature. Hypertension [1], chronic heart failure [2], diabetes [3], hypercholesterolaemia [4] and generalized atherosclerosis [5], for example, are all associated with impaired endothelium-mediated vasodilatation. Animal studies have provided substantial information about the mechanisms involved in the development of vascular disease [6,7], but it is also important to investigate these mechanisms directly in humans.

Methods using isolated organ preparations have proved valuable in animal models, but the results cannot necessarily be extrapolated directly to humans, and these methods are not appropriate for human subjects. Ideally, endothelial function should instead be assessed in vivo and, as far as possible, non-invasively. Systemic dysfunction is often reflected in the peripheral circulation [8], and a common approach is to administer vasoactive drugs through a cannula in the brachial artery while measuring changes in whole-arm blood flow using strain-gauge plethysmography [9,10]. The drawbacks of this technique are that it is invasive and assesses total limb flow without distinguishing between the muscle and skin circulations, which have very different functions.

The microcirculation is of interest because this is where fine control of blood supply takes place, and tissue ischaemia is dependent on microvascular flow in many circumstances. The skin can provide a good model of this, and in diabetes, for example, measurable changes in the skin have been found to pre-date the symptoms of microvascular disease in other organs by many years [11].

Key words: endothelium, iontophoresis, laser Doppler imaging, receptor, skin.
Abbreviations: ACh, acetylcholine; BK, bradykinin; MCh, methacholine; SP, substance P.
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This vascular bed is therefore very suitable for assessment of the endothelium, as well as being particularly convenient. Our group and others have used a combination of iontophoretic drug delivery and laser Doppler flowmetry to measure blood flow responses in the skin in a variety of vascular disorders [3,11–14]. Iontophoresis allows very small amounts of drug to be administered non-invasively to a localized area, and is thus very safe.

The most common agonists used in such experiments are the endothelium-dependent and -independent vasodilators acetylcholine (ACh) and sodium nitroprusside. We want to extend this methodology to study other compounds so that we can test specific receptor sensitivity. Endothelial damage might have variable effects on different receptors and their signal transduction pathways [15]. In the present study, in addition to ACh, we looked at methacholine (MCh), bradykinin (BK) and substance P (SP).

Our aim was to establish doses of each drug that, administered iontophoretically, produce significant and reproducible changes in skin blood flow. To this end, we conducted each protocol either twice or three times in each individual to estimate the intra- and inter-person variability of the measurements, and therefore to determine whether the measurements are likely to be sensitive assessments of endothelial function.

METHODS

Subjects
We recruited 11 healthy volunteers to take part in this study; four women and seven men aged between 24 and 43 years. Their participation in each of the experiments is detailed in Table 1. Each volunteer gave written, informed consent to participate, and the protocol was conducted in accordance with the Declaration of Helsinki of the World Medical Association and approved by the Tayside Committee on Medical Research Ethics. Individuals with known skin hypersensitivity were excluded. The experiments were conducted in a laboratory at an environmental temperature of 22 ± 1 °C, and the subjects were seated with their arms supported at heart level.

Iontophoretic drug delivery
Iontophoresis allows the non-invasive delivery of drugs to a relatively small area of skin without perturbing the site or inducing systemic effects [16]. The hydrochloride salt of each drug under investigation was dissolved in deionized water to an appropriate concentration: 10 g/l ACh and MCh, 0.6 g/l BK and 1.5 g/l SP (Sigma Chemical Co., Poole, Dorset, U.K.). (In pilot experiments, we determined concentrations that were high enough to produce measurable responses but for which, especially in the case of the latter two drugs, a test would not be excessively expensive. In theory, the concentration of drug might influence the ease of its administration, but not the actual dose delivered.) In solution, all four compounds dissociate into positively charged ions.

We prepared a measurement site on the volar surface of the forearm by removing surface keratinocytes with adhesive tape and cleaning the area with an alcohol swab. The iontophoresis chamber (Moor Instruments Ltd) consists of a Perspex ring of internal diameter 20 mm with a wire electrode running round its inner surface. This chamber was fixed to the skin with adhesive tape and filled with 2 ml of solution. The positive lead of a current source was connected to the iontophoresis electrode and the negative lead was attached to a conductive hydro-gel pad on the wrist, which serves as a reference.

When an electrical potential difference is established, ions of the drug migrate across the skin, and the dose delivered is therefore a product of the magnitude and duration of current. We used a current of 100 μA which, in our experience, is not high enough to cause non-specific electrical effects with the electrodes we used. Each drug was administered as a successive accumulation of doses, with each dose defined by the duration of current and therefore equivalent to the delivery of a particular electrical charge ($current \times time$). These doses are listed in Table 1, and were chosen on the basis of our preliminary investigations.

Laser Doppler perfusion imaging
We assessed cutaneous microvascular perfusion at the delivery site using laser Doppler imaging (moorLDI; Moor Instruments Ltd). Laser Doppler flowmetry has been used successfully for many years to measure microvascular changes in the skin. Laser Doppler imaging is a recent development of the technique which reduces the variation due to spatial heterogeneity by scanning over a region [17].

A 2 mW helium–neon laser scans the surface of the skin and light, back-scattered from moving erythrocytes,
is shifted in frequency by an amount proportional to their velocity, according to the Doppler principle. These Doppler shifts are collected and processed by the instrument. For each scan, the computer builds up a colour-coded image representing skin perfusion in two dimensions. This relative measure of volume flow is called the laser Doppler flux, and is expressed in arbitrary perfusion units.

The laser head was positioned 50 cm from the measurement site, with a hood mirror deflecting the beam on to the skin surface. The scan region encompassing the iontophoresis chamber was approx. 8 cm × 8 cm. We used a spatial resolution estimated to be approx. 1 mm per pixel and a scan speed of 4 ms per pixel. With these settings, each scan took approx. 25 s.

Before administering the drug, we recorded two baseline images (to ensure stable initial conditions) from which the average was taken. After each drug delivery, the liquid was removed and the chamber dried to avoid spectral reflection from the surface of the liquid. Images were recorded every 30 s for the duration specified in Table 1, thereby allowing time for three scans after each ACh dose, and four scans for each of the other drugs. This time period allowed the blood flow response to plateau before the next dose was delivered, and was determined for each drug from pilot experiments. Subsequent doses were administered in a similar manner.

The recorded images were analysed using dedicated image-processing software. We quantified the perfusion at the site by calculating the median laser Doppler flux in the area enclosed by the chamber. For each dose–response, the mean of the two highest flux values was taken, and this was divided by the baseline measurement to give a ratio representing the change in flow. To assess the reproducibility of these responses, they were repeated 2–7 days later, either once or twice in each subject, as specified in Table 1.

Analysis
For the ACh experiments, we measured blood flow responses to four doses of the drug: 1, 2, 4 and 8 mC. We used five subjects and measured the responses on three separate occasions in each of them (Table 1). To express the variability of these measurements within an individual, we calculated the S. D. and coefficient of variation of the three values at each dose for each subject, and then averaged these across all five subjects. To express the variability between the subjects, we calculated the mean of the three measurements for each subject (again, at each dose) and then derived the S. D. and coefficient of variation of this parameter for the subject group. This analysis was repeated for the blood flow responses to each of the other drugs.

We also tested the difference between the doses of each drug and between the ACh and MCh responses, using SPSS Software (SPSS Inc., Chicago, IL, U.S.A.). The mean of the two or three measurements for each subject was used as the test variable in this analysis. A combination of the Lilliefors and Shapiro–Wilks tests and normal distribution plots showed that this parameter was approximately normally distributed. We therefore used repeated-measures ANOVA to test the effect of different doses.

RESULTS
The blood flow responses to successive doses of each drug are presented in Figures 1–4. These graphs show that each drug produced a dose-dependent increase in flow in most subjects. Overall, this increase was statistically significant ("P" < 0.001 for ACh and MCh; "P" < 0.01 for BK and SP).

This observation is most apparent for ACh and MCh (Figures 1 and 2), for which the spread of data points at each dose is relatively small. Indices of the intra- and
inter-subject variability are presented in Table 2. The coefficients of variation for these two drugs, at the two highest doses, are approx. 17% (range 13–23%), both within and between individuals. We also found that MCh produced a significantly greater response than ACh ($P < 0.05$), by as much as 20% at the higher doses (Table 2).

The increases in flow produced by BK and SP (Figures 3 and 4) were both smaller on average than those for the other two drugs. The spread of data points masks the dose–response somewhat, although at the highest doses there was a 4–5-fold increase, on average. For BK, although the intra-subject coefficients of variation were reasonable, the difference between individuals was very marked, with coefficients of variation from 60% to 70% at worst (Table 2). It can be seen from Figure 3 that some subjects had almost no response at some visits, while others show an 8-fold increase in flow at the highest dose.

The situation was similar for SP (Figure 4), best illustrated by a subject with no response at one visit and a 10-fold increase at a dose of 6 mC on another occasion.

DISCUSSION

Our group and others have used iontophoretic administration of ACh to measure microvascular endothelial function in a variety of conditions, including diabetes [3], peripheral arterial disease [14] and Raynaud’s phenomenon [13]. These studies have shown that this type of assessment can be used to distinguish differences between patient groups and controls, and is sensitive to treatment-related changes in endothelial function. In the present study, we have verified that iontophoresis of ACh into the skin produces reproducible, dose-dependent increases in blood flow, and for the first time have shown the same for MCh.

The variability of the responses to ACh was relatively low, both within (17%) and between (15%) individuals, confirming the suitability of this protocol for experimental assessment of endothelial function in vivo. Our results also compare favourably with those from other studies. Morris and Shore [18], for example, measured a within-subject variability of 23% in the response to ACh, administered by iontophoresis in doses of 7 or 14 mC.

Laser Doppler imaging reduces variability due to the spatial heterogeneity of skin blood flow, which is a problem with conventional, single-point flowmetry. In addition, we used a specially made iontophoresis chamber with a larger diameter. This reduces the current density at the site of administration, and should therefore minimize the non-specific galvanic effects on blood flow associated with current density. Indeed, our own observations confirm that these are negligible at the 100 µA current that we use (D. J. Newton, F. Khan and J. J. F. Belch, unpublished work).

We did not measure biological zero (the laser Doppler signal when arterial inflow is arrested) because our experience, and that of another group [18], is that it makes only a small contribution to the total signal, and does not change significantly under different conditions.

We obtained similar results using MCh, with coefficients of variation at the 8 mC dose of 14% (within-subject) and 13% (between-subject). We also found that MCh produces higher blood flow responses than ACh, most significantly at the higher doses. MCh binds to the same endothelial receptor as ACh, but is less influenced by the enzyme acetylcholinesterase, which inactivates the
agonist at the receptor site. MCh therefore has a greater propensity to accumulate at the receptor, producing a sustained response, and a larger one after administration of successive doses, as we found here.

While MCh gives us no further information about the function of the M₁ muscarinic receptor upon which both it and ACh act, this drug might provide a more stable test of the endothelium, and our experiments have demonstrated a slightly lower variability for MCh. More importantly, perhaps, a comparison of the responses to MCh and ACh, and their subsequent rates of decline as the drugs are washed out, might tell us about the activity of acetylcholinesterase.

All four drugs studied here produce their effects via G-protein receptors on the vascular endothelium, and a sequence of events that includes activation of phospholipase A or C and the production of nitric oxide [19], prostacyclin [12] and/or hyperpolarizing factor [20]. BK and SP bind respectively to B₂ and NK₁ receptors, and it would be interesting to study how these particular pathways are affected by different pathological conditions. In particular, these two agents are important mediators of the inflammatory process, which is thought to play a key role in the development of atherosclerotic disease [21].

To our knowledge, this is the first time that the iontophoretic delivery of BK and SP to the skin has been investigated. However, our experiments show that these drugs do not produce satisfactory results. In both cases, the blood flow responses elicited, although significant and dose-dependent in most cases, were highly variable, both within and between individuals (with coefficients of variation as high as 60–70%). Neither would constitute a very reliable test of vascular function in the context of a clinical study.

BK and SP have been used successfully when administered intra-arterially [15,20], so it is possible that the problem here lies either in some variability in the physiological response of the skin or in inconsistent delivery of the drug. Most volunteers were within a 10-year age range, with only two over 35, so age is unlikely to be a significant factor in the inter-subject variability. Three of the four women were taking oral contraceptives, which will have limited the effect of intra-subject variation due to the menstrual cycle.

An interesting observation in our experiments was that, in almost every case, BK and SP produced marked oedema of the skin and a stinging sensation. These signs indicate the involvement of histamine in the skin reaction, and other groups have documented that histamine release contributes to the vasodilatation caused by intradermal injection of both BK [22] and SP [23].
We have removed the effect of needle trauma by administering the drugs by iontophoresis, but we still see evidence of histamine release, which may be a cause of the variability of our blood flow responses. However, more importantly, this also suggests that these tests are not specific to the $B_2$ and $NK_1$ receptors, but might also ultimately involve the $H_1$ histamine receptor. In addition, oedema may have the effect of limiting the laser Doppler signal, so adding an extra source of variability, although this is unlikely because the greatest oedema tended to correlate with the highest blood flow.

In summary, iontophoresis of either ACh or MCh produces consistent and reproducible vasodilatation of the skin microcirculation. These responses provide sensitive, non-invasive in vivo assessments of endothelial function. The vasodilator responses elicited by BK and SP, on the other hand, are too variable for any practical assessment and may not be receptor-specific. It is therefore important to identify other endothelium-dependent vasoactive agonists that can be administered in this way and that might complement ACh and MCh in our studies of the mechanisms of endothelial function and dysfunction. Although we used a relatively small subject group, the measures of intra- and inter-subject variability will enable us to estimate adequate sample sizes for future, larger studies using these tests.

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