Factors Critical to Iontophoretic Assessment of Vascular Reactivity: Implications for Clinical Studies of Endothelial Dysfunction

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Summary: Skin microvascular responses to iontophoresis of acetylcholine, an endothelium-dependent vasodilator, and sodium nitroprusside, an endothelium-independent vasodilator, were measured using a laser Doppler imager whose software controlled iontophoretic current delivery in an integrated fashion. A novel feature involved monitoring voltage across the iontophoresis chambers during current application (total charge: 8 mC). Both drugs elicited vasodilatation but with differing magnitudes and time courses, whereas current delivery with only vehicle (0.5% NaCl) present was ineffective. During drug delivery a three- to fourfold difference in calculated skin resistance was observed between subjects, with higher resistance being associated with lower dilator responses to both drugs. There was a significant (p < 0.0001) linear inverse correlation between perfusion.time and resistance.time integrals for both acetylcholine ($r = -0.86$) and sodium nitroprusside ($r = -0.96$). This was corrected in individual subjects by multiplying individual perfusion values by the resistance.time integral, which reduced response variability. Cyclooxygenase inhibition by aspirin apparently attenuated acetylcholine and sodium nitroprusside vasodilator responses but after correcting for skin resistance there was no longer any difference. Monitoring voltage across the iontophoresis circuit is critical, as effective drug delivery in individual subjects is influenced by the circuit resistance that can be corrected for. These findings have implications for clinical studies that use the iontophoresis technique for assessing vascular function. Key Words: Iontophoresis—Vasodilatation—Endothelium—Resistance.

Increasing evidence relates impaired endothelial vasomotor function to coronary heart disease (1). Endothelial dysfunction is not limited to the coronary circulation but is also detected in the peripheral circulation proportionate to the degree of endothelial dysfunction occurring in the coronary arteries (2). Furthermore, assessment of endothelial vasomotion in these peripheral arteries has been shown to correlate with and relate directly to coronary...
dysfunction (3). Thus there is considerable interest in noninvasive methods for assessing peripheral vascular function. Iontophoresis for transdermal delivery of the vasodilator agents acetylcholine and sodium nitroprusside is increasingly used for this purpose. The technique is based on the fact that a charged molecule migrates across the skin under the influence of an applied electrical field and ionized drug delivery is dependent on the magnitude of the applied current and its duration (current × time = charge, in Coulombs). In the past iontophoresis has been used in conjunction with laser Doppler flowmetry, a noninvasive method for assessing microvascular perfusion at a single point (4). More recently, iontophoresis has been combined with laser Doppler imaging, which reduces measurement variability (5,6) because, unlike Doppler flowmetry, laser Doppler imaging measures perfusion across many points (7) and thus an average measure of perfusion can be computed for any chosen area. Iontophoresis of acetylcholine tests endothelial function since binding to muscarinic receptors with subsequent generation of nitric oxide requires intact endothelial cells and is therefore said to be “endothelium-dependent.” Vasodilatation is ultimately mediated by action of nitric oxide on vascular smooth muscle (via the cyclic guanosine monophosphate pathway) and so iontophoresis of sodium nitroprusside, a nitric oxide donor, is used as an “endothelium-independent” control. This methodology has been widely used to investigate microvascular function in various disease states, most commonly diabetes mellitus, in which endothelial dysfunction has been implied by decreased response to acetylcholine iontophoresis (8–10).

Iontophoresis has a number of advantages. It provides a direct assessment of microvascular function, is simple to use, and, most importantly for clinical application, is noninvasive. However, some important factors that may influence iontophoretic drug responses and interpretation of iontophoresis have not been addressed. One of these is the assumption that drug delivery is solely influenced by the magnitude of current applied and its duration (charge). This ignores the fact that the electrical properties of skin differ between subjects and could impact on effective drug delivery.

By monitoring voltage across iontophoresis chambers, we investigated for the first time whether variation of the electrical properties of skin between normal subjects influences the magnitude of the responses to acetylcholine and sodium nitroprusside. The results of these investigations have critical implications for future clinical studies of endothelial function that use iontophoresis of vasoactive drugs.

**METHODS**

Experiments were performed in 20 healthy subjects ages 22–50 years of both sexes with no history of peripheral vascular abnormalities such as Raynaud’s syndrome, dermatologic diseases, or systemic disease processes such as diabetes mellitus, and all were non-smokers. All subjects fasted overnight and were asked to refrain from drinking any fluids except water before measurements, which were undertaken in a temperature-controlled room (23 ± 1°C) and all subjects were allowed to acclimatize for 30 min before measurement. The study was performed according to the Declaration of Helsinki, the institutional ethics committee approved procedures, and informed consent was obtained.

**Iontophoresis**

Drug delivery was achieved using a battery-powered constant-current iontophoresis controller (MIC-1e; Moor Instruments Ltd., Axminster, U.K). The chambers used for iontophoresis (ION 6; Moor Instruments Ltd.) were constructed of Perspex (internal diameter, 22 mm; area, 3.8 cm²) with an internal platinum wire electrode. Two chambers were attached to the skin of the volar aspect of the forearm by means of doubled-sided adhesive disks, avoiding hair, broken skin, and superficial veins. The chambers were connected to the anode and cathode connections on the iontophoresis controller. A digital multimeter was connected in parallel to monitor voltage across the chambers (Fig. 1A). Because a constant current source was used, resistance values were calculated from the recorded voltages using Ohm’s Law.

Control of current delivery was programmed into the software for the laser Doppler imager such that current was switched on at the beginning of a scan and remained on throughout the scan until the start of the following scan. The current was then either left on for the next scan or was switched off once the total charge had been delivered. Current duration was determined by the time taken to complete each scan (50 s) multiplied by the total number of scans programmed. To limit the iontophoresis dose, resulting from relatively long scan times, low currents were used: the protocol involved incremental current delivery with four scans at 5 μA, four at 10 μA, four at 15 μA, and two at 20 μA, giving a total charge of 8 mC (Fig. 2A). Each frame is associated with the current delivery during that scan, although the resulting vascular response could be delayed owing to the time required for chemical factors to initiate it. A 2.5-ml dose of 1% acetylcholine chloride (Sigma Chemical Co., St. Louis, MO, U.S.A.) was introduced into the anodal chamber and 2.5 ml of 1% sodium nitroprusside (Sigma) was placed in the
cathodal chamber. Thus both agents were delivered simultaneously during each period of current administration. The vehicle for these drugs was 0.5% NaCl. Fluid was prevented from escaping by placing circular 32-mm coverslips over the chambers.

**Perfusion measurements**

Noninvasive measurement of skin perfusion was performed by means of a laser Doppler imager (Moor Instruments Ltd.) equipped with a red laser (wavelength, 633 nm; power, 1 mW; beam diameter, 1 mm). The
technique is based on the Doppler shift imparted by moving blood cells in the underlying tissue to the backscattered light. The laser is scanned in a raster fashion over both chambers and through the coverslips. The backscattered light is collected by photodetectors and converted into a signal proportional to perfusion in arbitrary perfusion (flux) units (PU) that is displayed as a color-coded image on a monitor (Fig. 1B). Perfusion measurements were obtained using the imager manufacturer’s image analysis software by outlining a region of interest (ROI) around the internal circumference of the chamber. Statistical analysis of the ROI was subsequently performed offline to yield the median flux value across approximately 700 measurement points. Twenty repetitive scans were taken, the first being a control (before current administration), followed by the incremental current protocol described above (14 scans), and followed by five further scans with no current administration. The biologic zero was measured by taking a single scan after occluding the arterial blood supply with a sphygmomanometer cuff. This was found to be consistent between and within subjects with a mean (±SEM) of 23.9 ± 0.72 PU.

To determine whether the combination of fluid in the iontophoresis chambers and the coverslip affects the flux signal, scans were obtained comparing basal perfusion in the anodal chamber (containing acetylcholine) and in the cathodal chamber (containing sodium nitroprusside) to that occurring in a surrounding area of skin. The latter showed significantly (p = 0.011, one-way ANOVA; n = 14) higher basal perfusion (71.6 ± 6.4 PU; mean ± SEM) compared with the values at the acetylcholine-containing chamber (54.4 ± 2.7 PU) and the sodium nitroprusside-containing chamber (53.9 ± 3.7 PU), indicating that the chambers produce some attenuation of the laser Doppler imaging signal, but there was no difference between chambers. Although some signal attenuation occurs, perfusion changes in response to intervention are unaffected. After occlusion of the blood supply to the arm, the ratio of the increase in perfusion from biologic zero showed no significant differences between the anodal and cathodal chambers and adjacent skin (1.93 ± 0.09, 2.23 ± 0.15 and 2.18 ± 0.16, respectively; p = 0.26).

Drugs

In separate experiments, seven subjects were administered 600 mg aspirin orally 30 min before iontophoresis to inhibit prostaglandin synthesis. This has been shown to be an adequate period to produce maximal inhibition of endothelium-derived prostacyclin (11). Aspirin was dissolved in orange juice to disguise its taste, which allowed use of plain orange juice as a placebo. Measurements with placebo or aspirin were performed on one occasion each, separated by a minimum of 14 days.

Statistical analyses

Measurement of responses was performed using raw values, but in some cases an assessment of the overall response to drugs was obtained by taking the area under the perfusion time curve. For resistance data, resistance-time integrals were computed. Comparisons were by ANOVA or Student’s t tests, paired or unpaired as appropriate. All tests were two-tailed and data are expressed as means ± SEM or ± SD. Because of the marked differences in variances between basal perfusion values and the maximal responses to the drugs, but the similar coefficients of variation, log10 transformation of the data was performed to equalize the variances and thereby permit parametric data analysis. The variance ratio, comparing maximal drug responses to the control (before current application) scan, was found to vary between 9–60 for the raw perfusion data, but after log transformation the same variance ratio range was reduced to 0.7–1.2.

Based on the raw perfusion time integrals, the mean (± SD) between-day coefficient of variation for the acetylcholine response, measured in four subjects on 2 separate days, was 6.4 ± 3.3% while the within-day, between-site coefficient of variation, measured in both forearms on the same morning in four subjects, was 8.9 ± 5.3%. The same coefficients of variation for acetylcholine log-transformed perfusion data were 0.81 ± 0.39% and 1.10 ± 0.64%, respectively.

RESULTS

Responses to acetylcholine and sodium nitroprusside

Iontophoresis of acetylcholine and sodium nitroprusside resulted in progressive increase in perfusion, although with differing time courses (Fig. 2A). Acetylcholine showed a more rapid onset than sodium nitroprusside but also a rapid decline once current was terminated. This rapid fall in the acetylcholine response may be due to the presence of acetylcholinesterase in human blood vessels (12). Current administration with only vehicle present failed to elicit vascular responses (Fig. 2B).

The time course of the responses to both drugs for seven subjects is shown in Figure 3A. The drug responses differed significantly (p < 0.001; two-way ANOVA), but it is clear that the current protocol used did not elicit hyperemic responses with only the vehicle
present. With responses expressed relative to cumulative charge, both acetylcholine and sodium nitroprusside reached a plateau by the time about half the charge was delivered (Fig. 3B).

A striking observation is that drug delivery is influenced not only by the applied charge but also by the electrical resistance of the skin, which varied in different subjects (Fig. 4). There is an inverse linear relationship between the perfusion integral and the resistance integral for both acetylcholine (Fig. 5A; \( r = -0.86 \)) and sodium nitroprusside (Fig. 5B; \( r = -0.96 \)) (i.e., higher resistance is associated with smaller vasodilator responses for both drugs and vice versa). These relationships were significant, \( p < 0.0001 \) in both cases. Correcting for this variable, by dividing by the integral of conductance (the reciprocal of resistance) over time, or more simply by multiplying individual perfusion values by the integral of resistance over time, normalizes responses. Correlating these corrected perfusion integrals to their respective resistance integrals now yields nonsignificant \( r^2 \) values of 0.011 for acetylcholine and 0.025 for sodium nitroprusside (Fig. 5C, D), indicating the effectiveness of this correction and lowering apparent intersubject variability. The coefficient of variation for the log perfusion integral of acetylcholine was 3.36\% (\( n = 7 \)) before correction for resistance and 1.86\% thereafter. The respective coefficients for sodium nitroprusside were 2.18\% and 1.56\%, respectively. These corrections reduce variability between subjects by about one half and one third, respectively. The between-day coefficient of variation for the same four subjects described in methods after correction for resistance was 3.04 ± 0.67\% (mean ± SD), which was about half of the uncorrected value.

**Influence of prostaglandins on cutaneous vascular responses**

The vascular responses to both acetylcholine and sodium nitroprusside appeared to be significantly depressed after administration of aspirin (\( p < 0.0001 \); two-way ANOVA; Fig. 6 A, B). However, it was found

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**FIG. 3.** A. Time course of response to iontophoretic administration of acetylcholine (filled squares) and sodium nitroprusside (open squares) dissolved in 0.5% NaCl as well as the response to this vehicle at the anode (filled circles) and cathode (open circles). Dose-dependent increase in perfusion is significant (\( p < 0.0001 \), one-way ANOVA) for drugs but not for vehicle. Mean ± SEM; \( n = 7 \) subjects. B. Same perfusion data as in A, but plotted against cumulative charge.

**FIG. 4.** Relationship between perfusion.time integrals and the resistance.time integrals for the response to acetylcholine (ACh) in a series of subjects. The inverse nature of the relationship is clear.
that resistance during acetylcholine and sodium nitroprusside administration was significantly greater ($p < 0.03$; Student paired $t$ test, $n = 7$; means ± SD) after aspirin ($6.25 ± 2.08$ M$\Omega \min$) than with placebo administration ($4.09 ± 1.04$ M$\Omega \min$). After correcting for resistance, there was no longer any significant difference between the responses for acetylcholine or sodium nitroprusside ($p = 0.64$ and $p = 0.75$, respectively; two-way ANOVA; Fig. 6 C, D). Measurement of drug responses in four subjects on two separate days but without aspirin administration showed no significant differences in resistance between days ($4.32 ± 0.94$ and $4.74 ± 0.6$ M$\Omega \min$).

**DISCUSSION**

There is mounting interest in the measurement of vascular function in cardiovascular research, because endothelial dysfunction may be an early feature of the atherogenic process and is amenable to therapeutic intervention. It can be argued that endothelial function integrates the stress of other risk factors. Laser Doppler imaging with iontophoresis is the most recent technique used for assessing endothelial function and is particularly attractive because of its noninvasive nature. Although iontophoresis only assesses the cutaneous microcircula-
tion, this is effectively a robust surrogate marker of vascular function in other vascular beds. Reduced responsiveness to iontophoretic administration of acetylcholine has been observed in diabetes (8–10) and hypercholesterolemia (13), and in both these conditions there is a parallel reduction of the acetylcholine response in the forearm circulation (predominantly a skeletal muscle vascular bed) assessed by venous occlusion plethysmography (14,15). Moreover, attenuated response in the skin of heart transplant patients to acetylcholine iontophoresis (16) is paralleled by reduced responsiveness of coronary blood vessels to acetylcholine in this group (17). In addition, acetylcholine-induced vasodilatation is reduced in both forearm musculature (18,19) and skin (19) in patients with essential hypertension. Thus many conditions affecting the cardiovascular system appear to result in global endothelial dysfunction and assessment of the cutaneous microcirculation yields valuable insights into peripheral vascular function.

The current protocol used in the current study resulted in an overall lower charge (8 mC) than that used in previous studies (5,6) and this, combined with a large surface area for iontophoresis and the use of a weak saline vehicle, prevented the development of hyperemic artefacts at the cathode. This obviated the need to use a topical anesthetic such as lidocaine-prilocaine cream (5,6), which produces cutaneous vasoconstriction (20) and which presents a problem because any vasodilator responses would then be superimposed on a basal vasoconstrictor tone. In preliminary experiments we have observed that iontophoresis of acetylcholine and sodium nitroprusside to skin treated with lidocaine-prilocaine cream elicited substantially reduced vasodilator responses (by 30%–50%, unpublished observations) using the protocol used in the present investigation.

Past work aimed at establishing the role of prostaglandins in mediating the response to acetylcholine has produced contradictory results. It was previously observed that the vasodilator response to iontophoresis of acetylcholine was reduced by administration of oral (21) or intravenous aspirin (22). However, Morris and Shore (5) found no difference in the acetylcholine response after

![Graph A](image1.png)

**FIG. 6.** Vascular responses to administration of acetylcholine (ACh; A) and sodium nitroprusside (SNP; B) in 0.5% NaCl vehicle before (open symbols) and after (closed symbols) administration of aspirin. For both ACh and SNP the responses before and after administration of aspirin differ significantly (p < 0.0001, two-way ANOVA). Vascular response to administration of ACh (C) and SNP (D) before (open symbols) and after (closed symbols) administration of aspirin after correcting flux values for resistance in each subject. (Mean ± SEM; n = 7 subjects).
oral aspirin. Our finding of an apparent difference in the vascular responses to both acetylcholine and sodium nitroprusside after oral aspirin suggests a possible explanation for discrepancies between these earlier studies. After correction for resistance, there was no longer any difference in the acetylcholine and sodium nitroprusside responses and this may indicate that variations in resistance across the iontophoresis circuit during drug administration could have been a confounding factor in previous studies. Aspirin itself could have changed skin resistance, and the time control experiment supports this, as no change in resistance was found when measured on separate days but without aspirin administration. The change in resistance could not be ascribed to variations in room temperature, because all measurements were undertaken in a temperature-controlled room and subjects were allowed to acclimatize. It is therefore possible that in previous studies, variations in skin resistance might have influenced effective drug delivery, leading to differing results. This emphasizes the importance of correcting for resistance to avoid potentially spurious results, particularly if measurements are taken at different sites or on different days.

The variation in calculated resistance between subjects suggests that there may be variable numbers of resistance pathways in the skin of different subjects. As the equipment and the composition of the solutions used was the same between subjects, the site of the resistance must be related to the skin. It might be the case that lower resistance is associated with a greater availability of low-resistance pathways, such as sweat ducts or hair follicles, which are close to blood vessels. This is quite likely given that sweat glands and their associated ducts, as well as hair follicles, are known to be richly vascularized (23). Higher resistance may be associated with high-resistance pathways for ion flow, mostly likely through the stratum corneum, and these are more remote from blood vessels. Thus effective drug delivery could differ, even though the charge remains constant. In effect, although the same total amount of drug may be delivered, the vascular response could differ depending on the relative number of low-resistance pathways available.

The inverse relationship between drug response and resistance, with calculated resistance integrals showing on average more than twofold variation between subjects, indicates that skin resistance may contribute to intersubject variability of drug responses. This has important implications for interpreting responses to drug administration in clinical studies. This factor has not been taken into account in any previous study using iontophoresis and variations in observed responses between subject groups could have been influenced by a systematic variation in resistance rather than a true difference in vascular reactivity. This said, it remains likely that diabetic patients do have vascular dysfunction, as most studies using venous occlusion plethysmography and intraarterial drug administration show a reduced response to acetylcholine compared with control subjects (14,24,25). However, observed differences to iontophoresis of drugs at different sites could be explained by variations in skin resistance. Reduced vascular responses have been observed in the skin over the dorsum of the foot compared with the forearm for both normal (10,26) and diabetic subjects (27). Similarly, marked differences in response to iontophoretic administration of acetylcholine occur at various sites on the hand and forearm in normal subjects (28), assessed by using laser Doppler flowmetry. Even when laser Doppler imaging is used, between-site variations in the magnitude of responses occur, although variability can be reduced by ensuring that consecutive measurements are taken from the same site (6). This reinforces the need to monitor voltage during iontophoresis so that resistance can be estimated. Only by doing this is it possible to accurately assess the extent to which effective drug delivery is affected by skin resistance and to correct for this variable.

In conclusion, this study has shown for the first time that resistance is an important but previously unrecognized variable that influences iontophoresis. The inverse relationship between skin resistance and blood flow responses to both acetylcholine and sodium nitroprusside indicates that resistance influences effective drug delivery. We anticipate that correction for resistance, coupled with use of appropriate vehicles, chambers, and iontophoresis protocols, will lead to significant improvement of the iontophoresis technique and permit its additional development, thereby further increasing its robustness as a noninvasive tool for assessing endothelial function in clinical studies.

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