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Local heating as a predilatation method for measurement of vasoconstrictor responses with laser-Doppler flowmetry

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Running title: Measuring vasoconstriction during local heating

Abstract

Studying microvascular responses to iontophoresis of vasoconstricting drugs contribute to a better understanding of the regulatory mechanisms of cutaneous vessels, but measuring these responses with laser Doppler flowmetry at basal blood flow conditions is technically challenging. This study aimed to investigate whether the measurement of cutaneous vasoconstrictor responses to noradrenaline (NA) and phenylephrine (PE), delivered by iontophoresis, is facilitated by predilatation of the microvascular bed using local heating. We used different drug delivery rates (100 s x 0.12 mA, 200 s x 0.06 mA, 300 s x 0.04 mA) to investigate whether predilatation affects the local drug dynamics by an increased removal of drugs from the skin.

In a predilatated vascular bed, iontophoresis of NA and PE resulted in a 25-33% decrease in perfusion, depending on drug delivery rate ($p < 0.001$). In unheated skin, vasoconstriction was 17% and 14% for NA and PE, respectively ($p < 0.001$).

These results indicate that predilatating the cutaneous vascular bed by local heating facilitates measurement of vasoconstriction with laser-Doppler flowmetry and does not seem to significantly affect the result by an increased removal of drugs from the skin.

Keywords: iontophoresis, laser Doppler, vasoconstriction, local heating, adrenoceptors

Introduction

An impaired regulatory function of microvascular reactivity is associated with many acute and chronic medical conditions, such as diabetes, hypertension, heart failure, and sepsis [1-4]. The regulatory function of the microvasculature often reflects the condition of the entire vascular system, and alterations in microvascular beds are often seen before any systemic effects [5]. To accurately assess the degree of any pathology at an early stage may be beneficial for diagnosis and prognosis, and may also prove useful in guiding treatment [1, 6, 7]. It is therefore important to develop and improve new and existing *in vivo* models for assessment of microvascular function.

Transdermal iontophoresis is the active transport of substances through the skin by application of a small electrical current. When used together with laser Doppler techniques, iontophoresis of vasoactive substances provides a noninvasive examination of microvascular function [8-10]. Most of the investigations using iontophoresis are made with vasodilators, and only a few include the effects of vasoconstrictors. This could be because the cutaneous microcirculation has a relatively low perfusion in its resting state and it is therefore difficult to assess the effects of a vasoconstricting agents [11, 12].

In previous studies, two approaches have been used to facilitate laser-Doppler measurements during iontophoresis of vasoconstrictor drugs, with varying success. The first is to examine a local reactive hyperaemia response after the iontophoretic drug delivery, and the second is to measure the post-iontophoresis response to 5 minutes of 42 °C local heating. Of these

approaches, local heating seemed to be the preferred method, mainly since obtaining reproducible reactive hyperaemia responses may be problematic [11].

There are several potential problems with applying these transient provocations after the iontophoretic delivery of drugs. Due to the inevitable time delay between drug delivery and the vasodilator stimulus, it may be difficult to catch the moment when the drug effect is most pronounced. Also, after 5 minutes of local heating, the vasodilatory response is still unstable due to mediation by local sensory nerves [13], which may add to the variability of the method. On the other hand, local heating is known to cause stable and highly reproducible vasodilatation responses when applied for longer times [13, 14]. We therefore hypothesized that local heating applied for 20 minutes before, and during the iontophoretic delivery of vasoconstrictor drugs may be a better alternative for facilitating blood flow measurements.

A possible problem with delivering drugs to a predilatated vascular bed is that the removal of the drugs by the blood flow may be substantial, which may limit the maximal local drug dose and drug effect. We have previously found that this can be a relevant factor in microvascular studies using iontophoresis of vasodilator drugs [15].

The primary aim of this study was to establish a model for the investigation of vasoconstrictor responses to noradrenalin (NA) and phenylephrine (PE) in the skin using iontophoresis and laser Doppler flowmetry, and using local heating to predilate the microvascular bed and increase the contrast between the baseline flow and the vasoconstrictor response. The secondary aim was to examine whether the vasoconstrictor responses to NA and PE were affected by an increased

removal of drugs from the test site due to the dilated state of the microvascular bed. This was tested by comparing blood flow responses at different iontophoretic drug delivery rates.

Materials and Methods

Subjects

Fifteen healthy volunteers (8 male) with a mean age of 27.3 (5.6) years participated in the study. Exclusion criteria included previous history of, or ongoing, cardiovascular disease, skin disease, taking substances that affect the circulation and impaired general health. All subjects refrained from tobacco and drinks that contained caffeine for 2 hours before participating. They were informed of the methods and procedures before the experiment, and gave their written consent to participate in this study. The study was approved by the Ethics Committee at Linköping University Hospital.

Protocol

All experiments were done at room temperature (22-23°C) and subjects rested for 10 minutes before the data were collected. Subjects were seated comfortably in a half upright position during the measurements with the right forearm resting on an arm support slightly below heart level. The volar side of the forearm was gently cleaned with 70% ethanol before the experiment, and a thermoregulated probe for iontophoresis and laser Doppler flowmetry was attached by double adhesive tape. Big superficial vessels and injured areas of skin were avoided. Test sites for respective drugs were marked with a filter pen and reused throughout the experiment to reduce intrasubject variability. A separate test site was used for each drug. All measurements began with a recording of skin perfusion for 60 seconds to acquire baseline values.

In 12 subjects, a heating period of 20 minutes (44 °C) was applied to increase skin background flow within the test site. Then, either noradrenaline or phenylephrine was delivered by anodal iontophoresis. The heater was left on during iontophoresis. Skin perfusion was monitored by laser

Doppler flowmetry during the entire preheating period and during the iontophoretic delivery of the drugs. Then, the experiment was immediately repeated for the other drug at a different skin site. Three different delivery protocols were performed on 3 separate days with at least 24 hours in between; 0.04 mA x 300 seconds, 0.06 mA x 200 seconds and 0.12 mA x 100 seconds, all with the same total electrical charge of 12 mC. The order in which drugs and current strengths were applied was randomized.

In 4 subjects (3 male), a control experiment was performed without heating of the skin, using a current strength of 0.02 mA applied for 600 seconds, yielding a total electrical charge of 12 mC. One subject participated in both the local heating and control experiments.

Equipment

A PeriFlux system 5000 Laser Doppler Perfusion Monitoring unit (Perimed AB, Järfälla, Sweden) with a thermostatic laser Doppler probe (Probe 481-1, Perimed AB, Järfälla, Sweden) was used to measure changes in skin perfusion and skin temperature. The bandwidth of the system was 15 kHz. The system was calibrated before the start of the study according to the guidelines of the manufacturer. The probe used in the current study has a fibre separation of 0.25 mm and collects perfusion data at a depth of about 0.5-1 mm. Blood flow data were recorded continuously during the experiments at a sample rate of 32 recordings per second. Data from the laser Doppler perfusion monitor were analysed using PeriSoft for Windows, version 2.5.5 (Perimed AB, Järfälla, Sweden).

Drugs were given by disposable drug delivery electrodes (PF 383, Perimed AB, Järfälla, Sweden) using a battery-powered iontophoresis controller to deliver a constant electric current (PeriIont 382, Perimed AB, Järfälla, Sweden).

Noradrenaline and phenylephrine, in concentrations of 10 mg/ml (1%) respectively, were obtained from the JJ Berzelius pharmacy at Linköping University hospital.

Data analysis

When local heating was applied, data were normalized so that the perfusion after 20 minutes of heating period was defined as 100%. The blood flow response to the iontophoresis of the drugs was expressed as percentage decrease from this level. When no heating was applied, data were expressed percentage decrease from baseline, where baseline was defined as the mean perfusion during 1 minute before iontophoresis. A 2-way ANOVA was used to test for differences in the final vasoconstriction responses, with factors current strength and drug. Final response was defined as the mean perfusion value during the last 30 seconds of the iontophoresis period. Slopes of the responses to the drugs during iontophoresis were calculated using linear regression. In the figures, data is presented as mean \pm SEM. Statistical calculations were done using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, “www.graphpad.com”). For all analyses, probabilities of less than 0.05 were accepted as significant.

Results

All the subjects tolerated the tests. No adverse reactions were observed during and after the experiments. An overview of numerical results is given in Table 1.

When skin sites were heated to 44 °C (N = 12), the blood flow response was biphasic with an initial peak at 5 minutes followed by a stable plateau after 20 minutes of heating (Figure 1). At the first minute of iontophoresis, a slight initial vasodilatation was observed with both drugs in most of the subjects. After this, there were significant decreases in perfusion (all $p < 0.001$) with final vasoconstrictions between 25.1 (21.1)% and 32.5 (27.2)%, depending on the drug and current strength (Figure 2). The slope of the decrease was steeper for higher current strengths but there were no significant differences between final perfusion levels (2-way ANOVA, $p = 0.98$). The decrease in perfusion was similar for NA and PE (2-way ANOVA, $p = 0.39$).

At unheated skin sites (N = 4), a significant reduction in perfusion was observed (both $p < 0.001$) resulting in a final vasoconstriction of 16.8 (20.5)% and 14.1 (8.8)% (Figure 3).

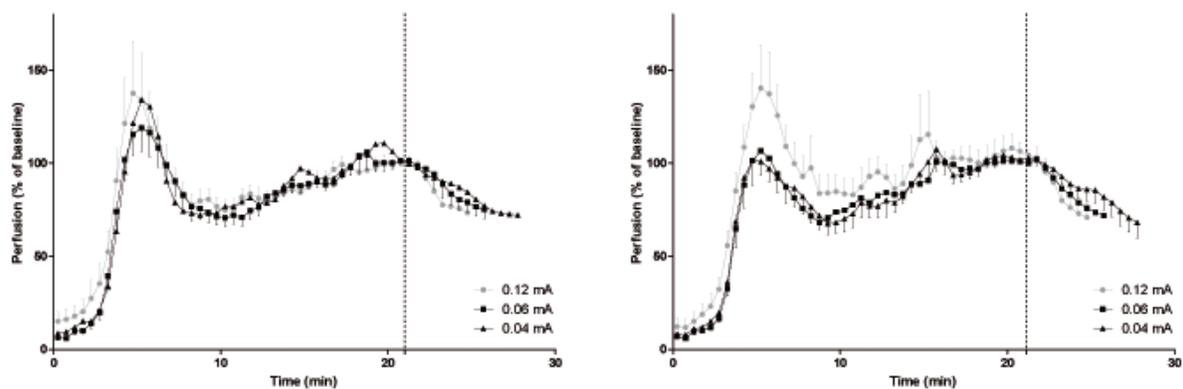


Figure 1. Blood flow responses during local heating and iontophoresis of noradrenaline (A) and phenylephrine (B) with different current strengths (N = 12). The response to local heating was biphasic with an initial peak after 5 minutes, followed by a gradual increase towards maximum blood flow. Iontophoresis of noradrenaline and phenylephrine elicited a significant reduction in blood flow compared to the maximum blood flow obtained after 20 minutes of heating ($p < 0.001$). Data are presented as mean \pm S.E.M. The vasoconstriction responses (right sides of the vertical, dashed lines) are shown in more detail in figure 2.

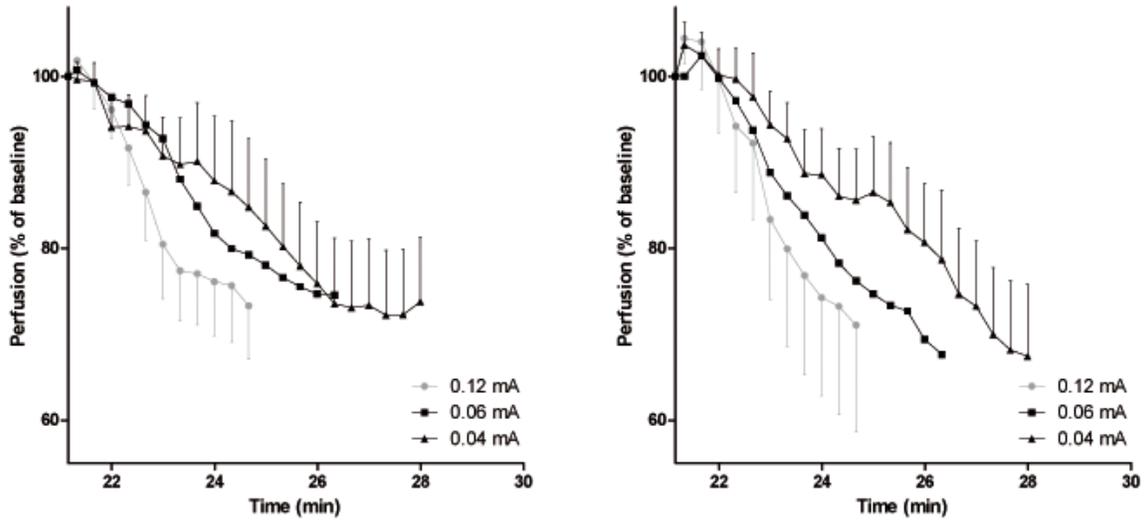


Figure 2. Vasoconstriction responses during iontophoresis of noradrenaline (A) and phenylephrine (B) after predilatating the vascular bed using 20 minutes of local heating. The decrease in blood flow varied between 25.1 (21.2)% and 32.5 (27.2)%, depending on the drug and current strength (Table 1). The slope of the decrease was steeper for higher current strengths but there were no significant differences between final perfusion levels (2-way ANOVA, $p = 0.98$). The decrease in perfusion was similar for NA and PE (2-way ANOVA, $p = 0.39$). Data are presented as mean \pm S.E.M.

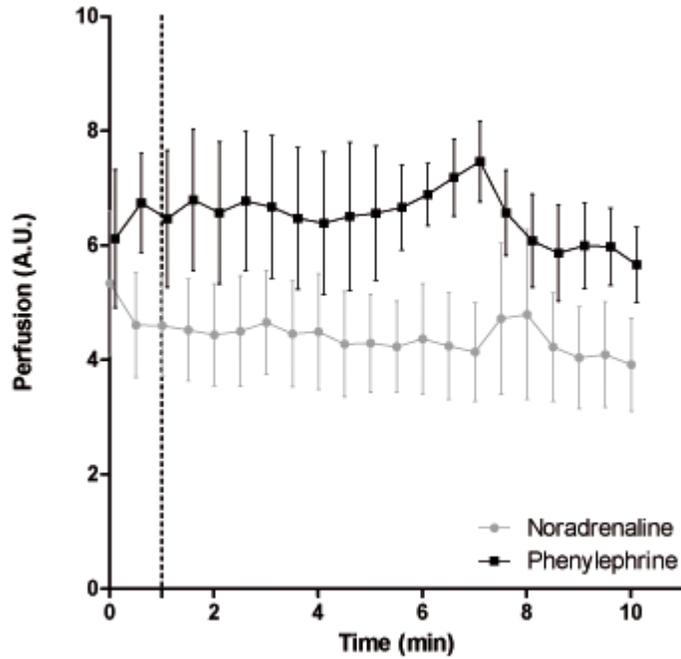


Figure 3. Blood flow response to noradrenaline and phenylephrine delivered by iontophoresis in unheated skin, as measured by laser Doppler flowmetry ($N = 4$). Slopes were similar for noradrenaline and phenylephrine and were significantly different from zero (both $p < 0.001$). The final decrease in perfusion at the end of the iontophoresis period was 16.8 (20.5)% and 14.1 (8.8)% for noradrenaline and phenylephrine, respectively. Data are presented as mean \pm S.E.M.

Discussion

The main findings in the present study are that noradrenaline and phenylephrine delivered by iontophoresis cause a reduction of 25-33% in skin perfusion as measured by laser Doppler flowmetry if the vessels in the test area are predilatated by local heating at 44°C for 20 minutes before, and during drug delivery. This reduction is independent of the rate of drug delivery.

Iontophoresis of vasoactive substances enables a direct examination of microvascular function when used together with laser Doppler techniques [8-10]. Investigations including the effects of vasoconstrictors on the local skin blood flow could contribute to a further understanding of these functions. Such investigations are, however, complicated by the relatively low sensitivity of the laser Doppler technique to detect decreases in perfusion values within the perfusion range of unprovoked skin [11, 12]. This low sensitivity is caused by at least two effects. In its resting state, the cutaneous microcirculation has a relatively low perfusion, with perfusion values that are typically close to the biological zero (the contribution of the flow signal caused by the natural movement of molecules red blood cells in the tissue) [16]. Second, when there is vasoconstriction in the capillary bed, the laser light, instead of being absorbed by RBC's in the capillaries, penetrates to deeper areas such as the subdermal plexus, where the changes in flow may be smaller [17].

Local heating has been claimed to be the preferred method to increase blood flow during laser Doppler measurements that involve vasoconstricting agents [11]. The cutaneous vasodilatory response to a local heating stimulus has been thoroughly investigated in previous studies. The response typically consists of an initial peak vasodilatation followed by a sustained plateau of

increased blood flow. The initial peak is mediated by activation of antidromic neurotransmitter release from sensory afferents mediated by temperature-sensitive vanilloid type 1 receptors, while the sustained plateau is mediated by production of nitric oxide from the endothelium [13].

In the present study all subjects showed a strong response to the local heating treatment (44 °C for 20 minutes). The rapid initial increase in perfusion was maximal around 5 minutes of heating, and was followed by a more gradual increase of perfusion that eventually levelled to a value similar to the perfusion value observed at the time of the initial peak. At this point, noradrenaline and phenylephrine were delivered by iontophoresis, resulting in a 25-33% decrease in perfusion.

The reduction in perfusion found in this study is somewhat less than that found by Wilson et al. [18], who observed vasoconstriction responses to noradrenaline of up to 50% during local heating to 42 °C. The small difference in temperature is unlikely to causing the difference in vasoconstriction, as in the study by Wilson et al., temperatures of 37 °C and 40 °C resulted in similar vasoconstriction levels. In their study, microdialysis was used to deliver noradrenaline into the skin, which may have resulted in higher doses than the doses obtained using iontophoresis in the current study. We did not observe plateaus in the responses at the end of the iontophoretic delivery period, so it is likely that higher local doses of noradrenaline would have resulted in a further decrease in perfusion. We could have tried to increase the total electrical charge to increase the number of responders with stable plateaus, but this would certainly have introduced the risk of obtaining nonspecific effects of the iontophoretic current itself that would have complicated the analysis of the response. The protocol used here has been optimised in previous investigations to avoid any nonspecific effects caused by the current [19].

It is known from vascular in vitro studies that any preconditioning method may interfere with the mechanisms that are studied [20]. A possible problem with using local heating for the measurement of vasoconstrictor responses in the skin is that it has been found to reduce the responses to exogenous noradrenaline, and decreased the ability of the vessels in the skin to vasoconstrict. [18, 21]. The effect has been claimed to be specific to non-glabrous skin, such as the skin on the forearms. A recent study by Wingo et al. has suggested that the reduction in vasoconstrictor responses is not caused by temperature per se [22] but is possibly modulated by the increased production of nitric-oxide during local heating [23-25]. Also, the reduction in vasoconstrictor response to noradrenalin in the skin during local heating seems to be modest. Even though the noradrenalin dose-response is right-shifted compared with the dose-response at normal skin temperature, high doses of noradrenalin result in a reduction in blood flow that is similar to the reduction observed in unheated skin [18].

In the present study, a minor reduction in perfusion was observed when noradrenaline or phenylephrine was delivered to the skin at basal conditions, without any predilatation of the vascular bed. The reduction in perfusion, 16.8% and 14.1% for noradrenalin and phenylephrine, respectively, was statistically significant, indicating that measuring vasoconstrictor responses in unheated skin using laser Doppler is feasible. The fact that the vasoconstriction was not as pronounced as the response after predilatation, is likely because of the lower sensitivity of the laser Doppler technique at lower blood flow levels due to shifts in measurement volume, as described above [17]. Another plausible explanation is that the blood vessels have less ability to constrict from their resting state, as compared to when they are almost fully dilated. Local heating can increase forearm skin blood flow as much as 5 - 15 times as measured by different methods [26-28]. Therefore, regardless of a reduced technical sensitivity to changes in perfusion at low

blood flow, predilatation of the vascular bed simply allows for a larger physiological contrast in blood flow before and after delivery of vasoconstrictors.

Under normal conditions, skin blood flow fluctuates due to factors such as vasomotion, temperature variations, and other changes in basal vascular conditions [29]. Technical factors such as motion artefacts, drift and electronic noise [30, 31] may add to the variations in measured perfusion. In this study, in unheated skin, the mean standard deviation of the perfusion signal was 0.9 A.U during baseline. When vasoconstrictor drugs were delivered to unheated skin, the absolute decrease in perfusion was 1.0 A.U. When the vasoconstriction response are expressed relative to the fluctuations at baseline, as a signal-to-noise ratio (SNR), this translates to a SNR of 1.1.

The vasoconstriction response after predilatation, on the other hand, corresponded to a mean absolute change in perfusion of 12.8 A.U, while the mean standard deviation in the perfusion measurement was 2.4 A.U. during the last minute of heating, resulting in a SNR of 5.4. Thus, the vasoconstrictor responses after predilatation of the vascular bed are much more pronounced compared with the physiological and technical variations in measured perfusion. A low SNR, as it was found in unheated skin, may complicate the interpretation of responses. Specifically, when responses are to be compared between groups of subjects or between types of drugs, a larger number of subjects may have to be included to reach sufficient statistical power for finding significant effects.

An alternative to predilatation of the vascular bed may be found in the use of other techniques for perfusion measurement. We have previously used polarization spectral imaging [32] to assess the vasoconstrictive response in skin by both NA and PE [33]. This technique was been found to

have a better sensitivity than laser Doppler flowmetry in the skin at low perfusion. In those experiments we were able to obtain dose response curves for both NA and PE at an iontophoretic charge of less than 12 mC and without predilatating the cutaneous vascular bed.

Our group has previously applied a time-response model for the response to iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP) that takes into account: the constant influx of drug ions by the electric current; the removal of drugs from the skin by passive diffusion and blood flow, and the relation between the local dose of drug and the blood flow response based on common pharmacodynamic principles [15]. The results of that study indicated a significant effect of washout of drugs from the local vascular bed, particularly for acetylcholine. In the current study, we had expected that due to the high baseline blood flow, the removal of drugs from the local vascular bed would be substantial and that this might result in reduced vasoconstrictor responses, depending on the current strength used to deliver the drugs. With higher current strength, the influx of drugs is higher. Hence, in the presence of an effect of a local removal of drugs, higher current strengths are expected to result in higher local doses and stronger responses. However, given that the final perfusion levels were similar regardless of the current strength, this effect does not seem to be significant. A possible explanation lies in the mechanism by which noradrenaline and phenylephrine cause vasoconstriction in human skin. Both drugs are alpha-adrenoceptor agonists, acting on α_1 -receptors (phenylephrine) or both α_1 - and α_2 -receptors (noradrenaline). These receptors are located on the vascular smooth muscle. Drug molecules entering the intracellular space in the skin as a result of the electric field may therefore act on these receptors before they enter the vessels and before any removal by blood flow can occur.

In summary, this study shows that noradrenaline and phenylephrine, delivered by iontophoresis, elicit a dose-dependent vasoconstriction in skin. In a predilatated vascular bed, the vasoconstriction is more pronounced compared with the physiological and technical fluctuations in the perfusion signal than in unheated skin. This enables more sensitive measurements of vasoconstriction responses to drugs delivered by iontophoresis. Removal of drugs by high local blood flow during local heating does not seem to significantly affect the local drug dynamics, since final vasoconstrictor responses were independent of drug delivery rate.

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Table

Noradrenaline			
		Slope (A.U./min)	Maximum vasoconstriction (% decrease from baseline)
Local heating	0.04 mA	-4.9 (1.0)	29.0 (23.8)
	0.06 mA	-6.9 (1.5)	26.4 (16.4)
	0.12 mA	-12.9 (2.6)	26.4 (19.3)
No heating	0.02 mA	-0.05 (-0.01)	16.8 (20.5)

Phenylephrine			
		Slope (A.U./min)	Maximum vasoconstriction (% decrease from baseline)
Local heating	0.04 mA	-5.2 (0.9)	25.1 (21.2)
	0.06 mA	-8.4 (1.1)	28.5 (28.3)
	0.12 mA	-9.4 (3.9)	32.5 (27.2)
No heating	0.02 mA	-0.07 (0.01)	14.1 (8.8)

Table 1. Overview of the mean (S.D.) vasoconstriction responses to noradrenaline and phenylephrine, delivered by iontophoresis into unheated and locally heated skin, using different current strengths.