Effects of nitric oxide synthase inhibition on cutaneous vasodilation in response to acupuncture stimulation in humans

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ABSTRACT

Objectives The aim of the present study was to elucidate the mechanism of cutaneous vasodilation following acupuncture stimulation by investigating the roles of nitric oxide (NO) and axon reflex vasodilation.

Methods The subjects were 17 healthy male volunteers. The role of NO was investigated by administering NG-nitro-L-arginine methyl ester hydrochloride (L-NAME, 20 mM), an NO synthase inhibitor or Ringer’s solution (control site), via intradermal microdialysis (protocol 1; n=7). The role of axon reflex vasodilation by local sensory neurones was investigated by comparing vasodilation at sites treated with ‘eutectic mixture of local anaesthetics’ (EMLA) cream (2.5% lidocaine and 2.5% prilocaine) with untreated sites (control site) (protocol 2; n=10).

After 5 min of baseline recording, acupuncture was applied to PC4 and a control site in proximity to PC4 for 10 min and scanning was performed for 60 min after acupuncture stimulation. Skin blood flow (SkBF) was evaluated by laser Doppler perfusion imaging. Cutaneous vascular conductance (CVC) was calculated from the ratio of SkBF to mean arterial blood pressure.

Results In the first protocol, sites administered L-NAME showed significant reductions in CVC responses following acupuncture stimulation compared to control sites (administered Ringer’s solution) (p<0.05). In the second protocol, changes in CVC responses after acupuncture stimulation did not differ significantly between treated sites with EMLA cream and untreated sites (p>0.05).

Conclusions These data suggest that cutaneous vasodilation in response to acupuncture stimulation may not occur through an axon reflex as previously reported. Rather, NO mechanisms appear to contribute to the vasodilator response.

INTRODUCTION

Acupuncture treatment has been used empirically for pain relief in many disorders and is thought to be effective in improving local blood flow and improvements in local circulation may flush out algic or sensitising substances leading to pain relief. As for the mechanism underlying the improvement in local blood flow caused by acupuncture, Jansen et al. reported that electroacupuncture and injections of the vasodilator calcitonin gene-related peptide (CGRP) caused similar increases in skin blood flow (SkBF) in anaesthetised rats. Loaiza et al. reported that nitric oxide (NO) was released during electroacupuncture of the quadriceps femoris, resulting in increased local blood flow in knee joint capsule arterioles in anaesthetised rats. Furthermore, Tsuchiya et al. reported that acupuncture increased plasma NO concentrations in acupunctured arms compared with non-invasive sham-acupunctured arms in humans. Jou et al. reported that electroacupuncture enhanced NO-cGMP release in human skin. We have also recently demonstrated that NO is involved in the mechanisms of cutaneous vasodilation during warm moxibustion in the forearms of humans. Thus, the local vasodilative reaction at the stimulation site may be induced by vasodilative substances, such as CGRP, released from sensory nerve endings through the axon reflex, but NO production may also be important. The mechanisms of cutaneous vasodilation are complex and many points remain unclear with regard to the action caused by acupuncture stimulation. In addition, vasodilation mechanisms through an axon reflex differ between humans and rats. Previous studies were mostly performed using rats under anaesthesia, with fewer studies on human skin. Therefore, the primary aim of this study was to test the hypothesis that NO...
contributes to cutaneous vasodilation following acupuncture stimulation in human skin. The second objective was to examine the role of axon reflexes in acupuncture stimulation-induced vasodilation.

METHODS

Subjects
Seven men participated in protocol 1. The mean age (± SEM) was 25.8±2.4 years. In total, 10 men participated in protocol 2. The mean age was 23.6±1.8 years. All subjects were healthy, normotensive and non-smokers. Each subject was informed of the purposes and risks of the study before providing written consent. The institutional review board of Kansai University of Health Sciences approved all protocols and informed consent documents in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Measurements
Each subject rested in a supine position during placement of two intradermal microdialysis probes in the dermal space of the ventral side of the left forearm, as described previously. The two microdialysis sites were placed at least 2 cm apart. The membrane window for each probe was 10 mm. For placement of the probes, a 25-gauge needle was used to pierce the dermal space without local anaesthesia, exiting 20–25 mm from the point of entry. The microdialysis probe was threaded through the lumen of the needle and the needle was then removed, leaving the probe in place. The depth of probe placement was not identified, although a depth of 0.3–1.0 mm was reported in a prior study using similar procedures. After membrane placement, the microdialysis probes were taped in place, and Ringer solution was perfused through the fibres at 2 μl/min using an infusion pump (Pump 11; Harvard Apparatus, Natick, Massachusetts, USA) (figure 1A). Measurement of SkBF over the microdialysis membrane was performed using a laser Doppler perfusion imager (LDPI) (Periscan PIM; PERIMED, Järfälla, Sweden). The LDPI used a scanning laser to measure blood flow over a larger area of skin compared with integrating laser Doppler flowmetry. During measurements, the scanner head was mounted 20 cm from the skin surface and a low power (1 mV) laser beam (wavelength, 670 nm) scanned an area of 2.0 × 5.5 cm (20 × 64 measurement sites) at 5-min intervals. Each scan comprised 1280 data points and took approximately 30 s. Mean perfusion values for the area of interest (measurement sites around the point of needle insertion) were measured in volts using the manufacturer’s software, and were used as the output measure for quantification of SkBF. The ambient light level was kept at a minimum in order to avoid any influence on laser light.

The ambient temperature in the laboratory was controlled at 24°C. Heart rate (HR) was obtained from the R–R interval of an electrocardiogram, and arterial pressure was measured manually by auscultation every 5 min. Mean arterial pressure (MAP) was calculated from diastolic blood pressure (DBP) and systolic blood pressure (SBP) as MAP=DBP+(SBP–DBP)/3.

Protocol 1
The goal of this protocol was to identify whether cutaneous vasodilation in response to acupuncture stimulation would be attenuated by NO synthase (NOS) inhibition. After allowing at least 90 min for the hyperaemic response associated with membrane placement to subside, one microdialysis probe was perfused with 20 mM N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME) dissolved in Ringer’s solution at a perfusion rate of 2 μl/min, to inhibit NOS. This concentration of L-NAME has been reported to produce complete NOS inhibition. The second microdialysis probe (control site) was perfused with Ringer’s solution for the duration of the experiment. Considering the heterogeneity of forearm SkBF, the two sites were assigned at random to receive either L-NAME or

Figure 1  A) An infusion pump was used to perfuse solution through the 10-mm semipermeable membrane in the middle of the microdialysis probe. B) Acupuncture stimulation was conducted in the vicinity of the two probes while skin blood flow was measured in the area enclosed by the black ink marks.

Ringer’s solution. Image scanning over both sites was performed at 5 min intervals using LDPI. After baseline recording for 5 min, acupuncture was applied for 10 min. During the acupuncture stimulation period, artefacts introduced by stainless acupuncture needles precluded scanning of SkBF by LDPI. Therefore, scanning was performed for 5 min before and for 60 min after removal of the needle, with a pause during acupuncture stimulation. Simultaneously, HR and MAP were recorded every 5 min.

**Protocol 2**

This protocol was designed to test whether cutaneous vasodilator responses to acupuncture stimulation were blocked when the axon reflex was blocked by application of topical anaesthetic. Then 2 g of ‘eutectic mixture of local anaesthetics’ (EMLA) cream (2.5% lidocaine and 2.5% prilocaine ointment; Astra USA, Westborough, Massachusetts, USA), a topical anaesthetic, was placed as a 1×2 cm band (2 cm² skin surface) on the ventral side of the left forearm. In all cases, the anaesthetised skin was much larger than the size of flare reaction to acupuncture. The cream was then covered by a tegaderm dressing for at least 120 min. After this period of time, the dressing and EMLA cream were removed with a cotton swab. No cream was placed on the control site. The sites were at least 2 cm apart from each other. Blockade of the axon reflex was assessed by a lack of tactile sensation within the blocked area using von Frey hair. In all, 5 min of baseline recording was obtained, followed by acupuncture stimulation in the PC4 and control site for 10 min as described above. SkBF measurements were performed on a larger skin region to cover both areas. Similar to protocol 1, scanning was performed for 5 min before and for 60 min after removal of the needle. HR and MAP were recorded manually as in protocol 1.

**Acupuncture stimulation**

Acupuncture needles (0.20×40 mm; Seirin Kasei Co, Shizuoka, Japan) were inserted into PC4 and a control site, in proximity to PC4, to a depth of approximately 5 mm (figure 1B). The PC4 point was chosen for this study since PC4 is considered to improve circulatory disturbance in Traditional Chinese Medicine. Each insertion site was located adjacent to the centre of the microdialysis probe, since drug diffusion within the dermal interstitial spaces via the microdialysis probe is limited to a small area of either side of the probe. Needle insertion was aided by markings on the polyimide tubing that indicated the centre of the membrane portion of the microdialysis probe. Inserted needles were left for 10 min without additional manipulation. Acupuncture was performed by a certified acupuncturist with >10 years of experience.

**Statistical analysis**

Cutaneous vascular conductance (CVC) was calculated from the ratio of SkBF (mean perfusion values) to MAP. For both protocols, a two-way analysis of variance (ANOVA) was used to detect differences in CVC responses between the drug treatment and control sites. Average HR and MAP values were also analysed using a one-way ANOVA. For ANOVA, Tukey’s post hoc analysis was used. Baseline CVC and peak CVC values during the 60 min after acupuncture were compared between two sites using a paired Student t test. The data are presented as mean±SEM. Statistical significance was accepted at p<0.05.

**RESULTS**

**Protocol 1**

Figure 2 shows laser Doppler scanner images from a representative subject 5 min after acupuncture stimulation, illustrating the differences in cutaneous vasodilation between L-NAME and control sites. The change in CVC after acupuncture stimulation was significantly smaller at the site perfused with 20 mM L-NAME compared to the control site (p<0.05, figure 3). A smaller rise in CVC after acupuncture was observed in the L-NAME-treated site (figure 3). At baseline, CVC values were similar between L-NAME-treated (0.012±0.001 V/mm Hg) and control sites (0.012±0.001 V/mm Hg; p>0.05), whereas there was a significant difference in peak CVC values after acupuncture between L-NAME (0.015±0.001 V/mm Hg) and control sites (0.018±0.002 V/mm Hg; p<0.05). Average HR and MAP remained unchanged throughout the protocol (p>0.05).

**Figure 2** Laser-Doppler scanner image (top: flux image; bottom: photo image) taken from a representative subject 5 min after removal of the acupuncture needle at L-NAME and control sites. The left probe was perfused with L-NAME and the right probe with Ringer solution. An increase in skin blood flow was observed at the control site (green, yellow and red), whereas decreased skin blood flow was seen at the L-NAME site (shades of green and yellow).
Prior to baseline recording, EMLA cream was applied to the skin to locally block cutaneous nerves. Each subject reported an absence of tactile sensation after a 120 min application of EMLA cream. In addition, they did not report sensations of pain at the EMLA-treated site when acupuncture needles were inserted into the skin. Figure 4 shows laser Doppler scanner images from a representative subject 5 min after acupuncture stimulation at EMLA and control sites. There was no difference in CVC between the EMLA and control sites after acupuncture stimulation (p>0.05; figure 5).

There was no significant difference in CVC values between EMLA (0.008±0.001 V/mm Hg) and control sites (0.008±0.001 V/mm Hg) at baseline (p>0.05). There was also no significant difference in peak CVC values between EMLA (0.019±0.002 V/mm Hg) and control sites (0.019±0.002 V/mm Hg) after acupuncture (p>0.05). As in protocol 1, HR and MAP remained unchanged throughout the protocol (p>0.05).

DISCUSSION

Acupuncture is used clinically to improve circulation in patients with peripheral circulatory disturbance. However, the exact mechanism by which local blood flow increases in response to acupuncture is still unknown. The primary finding of the present study was that the vasodilation provoked by acupuncture stimulation in human skin was in part dependent on NO production. The lack of effect of the EMLA treatment on the SkBF response further suggested that axon reflexes are not responsible for cutaneous vasodilation under these conditions.

The increase in local circulation as a result of elevated NO levels could contribute to the therapeutic mechanisms involved in acupuncture treatment. Attention has been directed in recent years to the involvement of NO released from vascular endothelial cells in the cutaneous vasodilation response to acupuncture stimulation. NO is an important element in neurogenic vasodilation in human skin, and has been reported to be involved in vasodilation occurring in response to acupuncture in rats and humans. Data from the present study showing a pivotal role of NOS are consistent with previous findings.

Our findings also raise the question of how acupuncture enhances NO generation. NOS activity has been found to be increased at meridians and acupuncture points, suggesting that NOS regulates NO
levels in acupunctured subjects. A possible explanation may be an involvement of the sympathetic nervous system. Acupuncture has been suggested to change sympathetic activity, and changes in catecholamine status can affect NOS activity in the endothelium. Thus, acupuncture may trigger a sequential reaction, beginning with a change in sympathetic activity and leading to NOS activation and NO production. Another explanation could be that NO production may be caused by mechanical stimulation of the vascular endothelium during acupuncture. Initial vasoconstriction of the arterioles due to sympathetic activity may increase shear stress imposed on the vascular wall, thereby stimulating NO production by endothelial cells. Secondly, NO production may be promoted by shear stress of the vascular endothelium associated with vasodilation due to local inflammatory response caused by needle insertion.

Furthermore, a recent study has demonstrated the role of adenosine in the localised analgesic effects of acupuncture stimulation. In addition to analgesic effects, adenosine is a strong vasodilator. Increased shear stress due to adenosine-induced increases in blood flow may stimulate vascular endothelial cells to produce NO. With respect to duration of NO production in response to acupuncture, Özüm et al reported that NO levels in brain and dermal tissues returned to baseline during cervical spinal cord electrical stimulation. Although tissue NO levels were not directly measured in the present study, they may return to baseline during acupuncture stimulation.

Importantly, our observation that NOS inhibition did not completely abolish the cutaneous vasodilator response suggests that NO-independent mechanisms may also contribute to SkBF responses to acupuncture stimulation. Some studies have proposed that substance P and CGRP released from sensory nerve terminals may contribute to increases in local blood flow caused by acupuncture stimulation. For instance, Jansen et al found that electroacupuncture to a dermal flap in anaesthetised rats caused a cutaneous vasodilatory response similar to that which occurred in response to administration of substance P or CGRP. Sato et al reported an increase in muscle blood flow following electrical stimulation of muscle afferents in anaesthetised rats, and showed that inhibition of CGRP receptors abolished this response. These animal studies suggest that local blood flow increases in response to acupuncture are mediated by axon reflex mechanisms, and that CGRP is mainly implicated in this process. Based on these observations, blocking cutaneous nerves involved in axon reflexes with EMLA would have been expected to attenuate the SkBF response to acupuncture, but our findings did not show this. The present findings could be attributed to acupuncture-induced activation of intramuscular ergoreceptors, which transmit excitation via A delta fibres. A delta fibres induce vasodilation without causing neurogenic inflammation and are less sensitive to EMLA cream. Based on these findings, one possibility is that the cutaneous vasodilation observed at EMLA-treated sites is partially induced by A delta fibres, which are less sensitive to EMLA. Another potential explanation is that afferent C fibres in human skin are functionally classified into mechanoresponsive and mechanointensive C units, and a persistent flare response has been shown to be caused by excitation of the mechanointensive, but not the mechanoresponsive, C units. The mechanoresponsive C unit is excited by von Frey hair, whereas the mechanointensive C unit is not. Based on these findings, acupuncture stimulation, which is mechanical, would be expected to excite the mechanoresponsive, but not the mechanointensive, C units, consistent with a minimal involvement of axon reflex vasodilation during acupuncture stimulation. EMLA cream has been reported to inhibit axon reflex vasodilation in human skin. The EMLA cream treatment did decrease tactile sensation at the treated site, arguing for its efficacy. Mean insertion depth with acceptable pain after 120 min of EMLA application has been reported as 4.5 mm within subcutaneous tissue. We therefore cannot exclude the possibility that EMLA cream failed to completely block cutaneous nerves present in deeper subcutaneous tissue mediating axon reflexes. Also, there are likely to be small differences in the localisation of the cutaneous nerves depending on the surface application site of the EMLA cream as well as differences in the depth of the anaesthetic effect. However, these differences were accounted for in the present study by comparing the cutaneous surface on the medial side of the forearm.

The combined use of LDPI and drug administration via intradermal microdialysis could be used in further studies investigating the neural mechanisms of local cutaneous vasodilation during acupuncture treatment. Finally, while the present study investigated healthy individuals, blood flow responses to acupuncture differ between healthy individuals and those with fibromyalgia. Potential reasons for this include pain-induced increases in sensitivity and sympathetic nerve activity. Even in healthy individuals, NO production capacity is known to decrease with age, indicating the need for a comparison of blood flow responses in patients with other diseases and investigation of the related mechanisms of action. Also, disruption of the NO pathway plays a major role in the pathogenesis of peripheral artery disease (PAD). In particular, endothelial NOS (e-NOS) from endothelial cells, has been shown to be an important modulator of NO. Thus, e-NOS-mediated NO production due to acupuncture can be expected to improve circulation for patients with PAD.

The present study demonstrated that inhibition of NOS with L-NAME attenuated cutaneous vasodilation induced by acupuncture stimulation, whereas blocking...
axon reflexes with application of EMLA cream did not have any effect. These findings suggest that NO contributes to cutaneous vasodilation induced by acupuncture stimulation, whereas axon reflexes are less important.

**Summary points**

- Skin flushing around acupuncture needles is often thought to be due to axon reflex
- We explored this effect in human volunteers
- Nitric oxide (NO) is involved in flushing, but the axon reflex may not be

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**Competing interests** None.

**Patient consent** Obtained.

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