Contributions of nitric oxide and prostaglandins to the local increase in muscle blood flow following manual acupuncture in rats

Hisashi Shinbara,1 Masamichi Okubo,2 Keisaku Kimura,3 Kunio Mizunuma,3 Eiji Sumiya1

ABSTRACT
Objective To investigate the contributions of nitric oxide (NO) and prostaglandins (PGs) to the increase in local muscle blood flow (MBF) observed following manual acupuncture (MA).

Methods Male Sprague-Dawley rats (n=112; 250–310 g) were injected intraperitoneally with a non-selective NO synthase inhibitor (NG-nitro-L-arginine methyl ester hydrochloride: L-NAME; 10, 50 or 500 mg/kg), a non-selective cyclooxygenase inhibitor (indomethacin; 10, 50 or 500 mg/kg), a combination of L-NAME and indomethacin (500 mg/kg each) or saline only under urethane anaesthesia (1.2 g/kg). We used the sparrow pecking technique for 1 min with a stainless steel acupuncture needle (0.20×30 mm) as the acupuncture stimulation method. The stimulus point was on the right tibialis anterior muscle. 51Chromium-labelled microspheres were used for MBF measurement.

Results MA increased MBF in the saline-injected group (p<0.001). This increase was partially inhibited by L-NAME in a dose-dependent manner (p>0.05, p<0.05 and p<0.001 for 10, 50 and 500 mg/kg, respectively). On the other hand, indomethacin did not suppress the increase (p>0.05 each for 10, 50 and 500 mg/kg). No significant difference was observed between the inhibitory effects of combined administration of L-NAME and indomethacin (500 mg/kg each) or saline only under urethane anaesthesia (1.2 g/kg). We used the sparrow pecking technique for 1 min with a stainless steel acupuncture needle (0.20×30 mm) as the acupuncture stimulation method. The stimulus point was on the right tibialis anterior muscle. 51Chromium-labelled microspheres were used for MBF measurement.

Conclusions These results suggest that NO is a major factor in the MA-induced increase in MBF, while PGs do not contribute significantly to this increase. As complete inhibition was not achieved by administration of L-NAME+indomethacin, it appears that non-NO and non-PG vasodilators are additionally involved.

BACKGROUND
Acupuncture induces analgesia and functional improvement in various types of musculoskeletal disorders. Acupuncture analgesia involves activation of endogenous pain relief systems (descending pain inhibition and diffuse noxious inhibitory control) mediated by opioid peptides, as well as segmental spinal analgesia mediated by γ-aminobutyric acid (gate-control theory).1 Furthermore, acupuncture locally relieves pain via opioid receptors2 and adenosine A1 receptors,3 and may also wash out algesic and/or fatigue substances by increasing skeletal muscle blood flow (MBF), leading to improved muscle performance.

The following mechanisms have been proposed to explain the increase in MBF caused by manual acupuncture (MA) and electroacupuncture (EA): (1) acetylcholine (ACh) release from sympathetic cholinergic vasodilator nerves;45 (2) calcitonin gene-related peptide (CGRP) release from sensory nerve fibres via the axon reflex;67 and (3) elevation of arterial blood pressure.8

We previously investigated the effects of MA on MBF in rats using the radiolabelled microsphere technique, which can quantitatively measure MBF.79–11 MA locally and significantly increased MBF without increasing the arterial blood pressure. This increase was proportional to the stimulus intensity of MA, and persisted after acute denervation.9 These findings suggest that the local vasodilatory system is mainly responsible for increased MBF, possibly through the release of CGRP from thin sensory nerve endings via the axon reflex. However, the contribution of CGRP to the increase appears to be limited.7 Additionally, increased MBF is observed after chronic denervation, which induces demyelination.9 Collectively, these results
indicate that multiple local vasodilators are responsible for the increase.

Nitric oxide (NO) and prostaglandins (PGs) play a key role in the regulation of muscle circulation. NO is synthesised by mechanical stimulation to the endothelium (eg, shear stress) and by the binding of vasodilators to each receptor on the endothelium (eg, ACh, CGRP and ATP). NO is synthesised from L-arginine, which is catalysed by the enzyme NO synthase (NOS). Three NOS isoforms have been identified: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). NO is continuously produced and released from the vascular endothelium via eNOS, and it regulates vascular tone against the vasoconstriction controlled by the muscle sympathetic nerve. NO acts on the adjacent vascular smooth muscle cells and produces cyclic guanosine monophosphate (cGMP) by activating soluble guanylate cyclase (sGC), which is a NO receptor. The increased cGMP leads to relaxation of vascular smooth muscle by activating protein kinase G.

PGs play a central role in inflammation. Similar to NO, PGs are synthesised by mechanical and/or chemical stimulation. PGs include PG_E2, PG_I2 (also known as prostacyclin), PG_D2 and PG_F2. In addition to prostacyclin, PG_D2 and PG_F2, PGs are formed as follows: (1) phospholipase A_2 (PLA_2) releases arachidonic acid (AA) from the cytoplasmic membrane; (2) PGH synthase, which possesses both cyclo-oxygenase (COX) and peroxidase actions, catalyses the synthesis of PGH_2 from AA via PGG_2; and (3) a tissue-specific isomerase generates the specific PG or thromboxane A_2 (TXA_2) from PGH_2. PGs are vasodilatory effects and produce cyclic adenosine 3’,5’-monophosphate (cAMP) with adenylate cyclase (AC) by binding to EP receptors (E_1, E_2, E_3, IP receptors and DP_1 receptors on vascular smooth muscle cells, respectively. The increased cAMP relaxes vascular smooth muscle by activating protein kinase A.

NO and PGs have different vasodilatory mechanisms and act to maintain adequate blood flow in a complementary manner under normal conditions. On the other hand, they play key synergistic roles during inflammation. NO and PGs are likely to be involved in the MA-induced increase in MBF because MA is a noxious mechanical stimulation accompanied by slight tissue injury, which is likely to trigger the release of substances that stimulate NO and/or PG production. In fact, a previous report has suggested that NO is responsible for the increase in MBF. However, little evidence is available to support this claim.

The aim of this study was to clarify the contributions of NO and PGs to the increase in MBF following MA. To this end, we investigated the single or combined effects of a non-selective NOS inhibitor and a non-selective COX inhibitor on the increase in MBF in rats.

**MATERIALS AND METHODS**

**Experimental animals**

The experimental animals used in this study were male Sprague-Dawley rats (n=112; 250–310 g; Japan SLC Inc, Shizuoka, Japan). Urethane was used as the anaesthetic (1.2 g/kg intraperitoneally). The animals were maintained in an air-conditioned room under a 12:12 light-and-dark cycle and had free access to water and a standard rodent diet. This study was approved by the ethics committee of Meiji University of Integrative Medicine (No. 17-44-2).

**Acupuncture stimulation**

The MA stimulation method used in this study was the sparrow pecking technique at 30 repetitions per minute for 1 min to a depth of 15–18 mm using a stainless steel acupuncture needle (0.20 × 30 mm, Seirin, Japan), as described in our previous studies. The stimulus point was on the right tibialis anterior muscle (TA) at a point 7–8 mm below the knee. Acupuncture needles were angled towards the ankle joint to ensure penetration of the TA.

**Chemicals**

For a single administration, NG-nitro-L-arginine methyl ester hydrochloride (L-NAME; Nacalai Tesque, Kyoto, Japan), a non-selective NOS inhibitor, was dissolved in saline at three different concentrations: 10, 50 and 500 mg/kg. In addition to L-NAME, indomethacin (IND; Nacalai Tesque), a non-selective COX inhibitor, was dissolved in sodium bicarbonate solution at the same concentrations (10, 50 and 500 mg/kg). For combined administration, L-NAME and IND were each used at 500 mg/kg. Both inhibitors were injected intraperitoneally.

**Experimental groups**

The experimental conditions and groups are described in table 1. Rats (n=112) were evenly allocated to receive MA (n=56) or no treatment (control (C) group, n=56). Each group was then further divided into eight subgroups (n=7 each), which received injections of saline, L-NAME at 10 mg/kg, L-NAME at 50 mg/kg, L-NAME at 500 mg/kg, IND at 10 mg/kg, IND at 50 mg/kg, IND at 500 mg/kg and L-NAME plus IND (500 mg/kg each), respectively.

**MBF measurement**

**Chromium-labelled microspheres (15 μm in diameter, PerkinElmer Japan) dissolved in saline with 10% Tween 80 were used for the MBF measurement, as described in our previous studies. The measurement was performed using the following procedure: (1) a reference sample of blood (RSB) was drawn from the left femoral artery using a pull pump (0.66 mL/min); (2) 10 s later, the microsphere solution was injected into the aortic arch by a push pump (0.66 mL/min) through a catheter inserted into the**
right common carotid artery and the remaining microspheres in the catheter were continuously flushed with saline for 120 s from the time the injection was started; (3) the rat was killed with an overdose of urethane; (4) the right TA was excised and weighed; (5) the gamma-radiation doses of the RSB and TA were counted using a gamma counter (Auto Well Gamma System ARS-600; Aloka, Japan); and (6) MBF was calculated using the following equation:

\[
MBF = \frac{100 \times Rm \times V}{(Rb \times W)} \text{(mL/min/100g)}
\]

where Rm and Rb are the gamma-radiation doses (cpm) of the muscle and RSB, respectively; V is the pull pump speed for drawing blood; and W is the muscle weight (g). The microspheres were injected 3 min after acupuncture stimulation.

**Arterial blood pressure recording**

The arterial blood pressure was recorded before, during and after MA via a catheter inserted into the right common carotid artery. This catheter was also used to inject the microspheres. Thus, the recording was stopped 1 min before microsphere injection. The mean arterial blood pressure (MAP) was calculated offline every minute.

**Statistical analysis**

GraphPad Prism 5 for Windows (GraphPad Software, USA) was used for statistical analysis. The unpaired t test was used to compare MBF between two groups, and one-way analysis of variance (ANOVA) was used to compare MBF between three groups. Dunnett’s test was conducted as a post hoc test if a significant difference was detected with one-way ANOVA. Two-way repeated measures ANOVA was used for the MAP data followed by the post hoc Bonferroni test. The significance level (p value) was set at <0.05. All data are expressed as mean±SE.

**RESULTS**

**Muscle blood flow (MBF)**

The MBF data derived from the TA in the L-NAME-injected groups versus the saline-injected groups are shown in figure 1. MA significantly increased the MBF in the saline-injected groups (C+Saline vs MA+Saline; p<0.001). L-NAME significantly suppressed the increase in MBF after MA in a dose-dependent manner (MA+Saline vs MA+L-NAME(50) and MA+Saline vs MA+L-NAME(500); p<0.05 and p<0.001, respectively). However, the increase in MBF was not suppressed completely because MA significantly increased the MBF even at 500 mg/kg L-NAME (C+L-NAME(500) vs MA+L-NAME(500); p<0.05).

The MBF data for the saline-injected group and IND-injected group are shown in figure 2. Note that the MBF data in the C+Saline and MA+Saline groups are the same in figures 1 and 2. MA significantly increased the MBF in all the IND-injected groups (C+IND(10) vs MA+IND(10), C+IND(50) vs MA+IND(50), C+IND(500) vs MA+IND(500); p<0.001, p<0.01 and p<0.01, respectively). However, the increase in MBF was not suppressed completely because MA significantly increased the MBF even at 500 mg/kg L-NAME (C+L-NAME(500) vs MA+L-NAME(500); p<0.05).

The MBF data for the saline-injected group and IND-injected group are shown in figure 2. Note that the MBF data in the C+Saline and MA+Saline groups are the same in figures 1 and 2. MA significantly increased the MBF in all the IND-injected groups (C+IND(10) vs MA+IND(10), C+IND(50) vs MA+IND(50), C+IND(500) vs MA+IND(500); p<0.001, p<0.01 and p<0.01, respectively). In addition, there were no significant differences between any of the IND-injected plus acupuncture groups (MA+Saline, MA+IND(10), MA+IND(50) and MA+IND(500); p=0.80). Thus, IND did not influence the MA-induced increase in MBF.

The MBF data for the L-NAME+IND-injected groups are shown in figure 3. For the sake of comparison, data from the C+L-NAME(500) and

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Table 1: Experimental groups and conditions

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Number of rats</th>
<th>Drug and dose</th>
<th>Acupuncture stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>C+Saline</td>
<td>7</td>
<td>Saline</td>
<td>–</td>
</tr>
<tr>
<td>MA+Saline</td>
<td>7</td>
<td>Saline</td>
<td>SP</td>
</tr>
<tr>
<td>C+L-NAME(10)</td>
<td>7</td>
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</tr>
<tr>
<td>MA+L-NAME(10)</td>
<td>7</td>
<td>L-NAME 10 mg/kg IP</td>
<td>SP</td>
</tr>
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<td>7</td>
<td>L-NAME 50 mg/kg IP</td>
<td>–</td>
</tr>
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</tr>
<tr>
<td>MA+L-NAME(500)</td>
<td>7</td>
<td>L-NAME 500 mg/kg IP</td>
<td>SP</td>
</tr>
<tr>
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<td>7</td>
<td>IND 10 mg/kg IP</td>
<td>–</td>
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<td>L-NAME and IND 500 mg/kg IP each</td>
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<td>L-NAME and IND 500 mg/kg IP each</td>
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</table>

C, control (no acupuncture); IND, indomethacin, a cyclo-oxygenase inhibitor; IP, intraperitoneal injection; L-NAME, NG-nitro-L-arginine methyl ester hydrochloride, a nitric oxide synthase inhibitor; MA, manual acupuncture; SP, sparrow pecking, which was performed at 30 repetitions/min.

MA+L-NAME(500) are also reproduced in figure 3. The combined administration of L-NAME and IND eliminated the differences that were observed when a single dose of L-NAME was administered (C+L-NAME(500)+IND(500); p=0.09). However, the suppression caused by the combined administration was not significantly greater than that of a single administration. This is evidenced by the fact that there was no significant difference between these acupuncture groups (MA+L-NAME(500) vs MA+L-NAME(500)+IND(500); p=0.25).

Mean arterial blood pressure (MAP)

The MAP data before drug administration and before, during and after MA in the experimental groups are presented in table 2. The MAP was not significantly different before, during or after MA between the acupuncture and no acupuncture groups (all p>0.05). Although significant differences in the MAP were observed as an interaction effect of group and time between the C+Saline and MA+Saline groups and between the C+L-NAME(500) and MA+L-NAME(500) groups (p<0.05 and p<0.01, respectively), no significant differences were observed in the MAP at each time (p>0.05 each). Thus, MA did not increase the MAP in any of the experimental groups.

DISCUSSION

MA significantly increased the local blood flow of the muscles subjected to acupuncture stimulation, in keeping with our previous studies.7 9–11 The NOS inhibitor L-NAME significantly attenuated the MA-induced increase in MBF in a dose-dependent manner in the acupuncture groups, suggesting that NO is mainly responsible for this increase. These data support earlier reports from Tsuchiya et al16 and Kimura et al17 which found that NO makes a major contribution to increased MBF and skin blood flow, respectively, following MA in human subjects.

CGRP, which is released from thin sensory nerve endings via the axon reflex, is the strongest candidate vasodilator for MA-induced NO synthesis. However, denervation caused by cutting the sciatic nerve,9 destruction of the thin sensory nerves caused by capsaicin7 and topical application of hCGRP8-27 (a CGRP receptor antagonist)7 did not completely suppress the MA-induced increase in MBF, suggesting that other vasodilators contribute to this effect. A recent report showed that application of MA using the rotating needle technique locally raised the extracellular concentration of ATP, ADP, AMP or adenosine in mice3 and humans.18 ATP and ADP activate NO production and adenosine activates NO by binding to P2 receptors and A2 receptors, respectively, on the endothelium and vascular smooth muscle.19 ATP is thought to leak from the skeletal muscle cells or blood vessels injured by MA, and both ADP and adenosine are degradation products of ATP. Thus, we predict that the MA-induced release and leakage of CGRP, ATP, ADP and adenosine increase MBF via NO. In addition, MA might mechanically promote NO synthesis in the same way as shear stress. Furthermore, ACh induces

Figure 1  Effects of a single administration of the non-selective nitric oxide synthase inhibitor NG-nitro-L-arginine methyl ester hydrochloride (L-NAME) at three different concentrations (10 to 500 mg/kg) compared to saline on the increase in muscle blood flow of 56 Wistar rats (n=7 per group) receiving manual acupuncture (MA) or no acupuncture (control, C). Data are expressed as mean±SE. NS: no significant difference.

Figure 2  Effects of a single administration of the cyclooxygenase inhibitor indomethacin (IND) at three different concentrations (10 to 500 mg/kg) compared to saline on the increase in muscle blood flow of 56 Wistar rats (n=7 per group) receiving manual acupuncture (MA) or no acupuncture (control, C). Data are expressed as mean±SE. NS: no significant difference.
NO production by binding to muscarinic receptors on the endothelium. However, sympathetic cholinergic vasodilator nerves have not been histologically demonstrated in the skeletal muscle of rats and humans. The fact that MA-induced increases in MBF are not antagonised by atropine (a muscarinic ACh receptor antagonist) suggests that ACh does not contribute significantly to the increase.11

In addition to the endothelium, other candidate sources of NO include erythrocytes, neurons and skeletal muscle.20 21 Under hypoxic conditions such as tetanic muscle contraction, erythrocytes releases NO, which is directly liberated from S-nitrosohaemoglobin or is reduced from nitrate by deoxyhaemoglobin.22 However, MA is generally not accompanied by sustained muscle contraction. Although nitrergic nerves are known to contribute to penile erection by releasing NO, it is unclear whether nitrergic nerves dominate for NO production under the present experimental conditions. eNOS and nNOS are constantly expressed in skeletal muscle cells, and NO contributes to a variety of activities such as glucose metabolism.13 23 On the other hand, iNOS is expressed during inflammation.13 Thus, MA might hypothetically induce NO via iNOS because MA produces a minor degree of muscle tissue injury that would be expected to induce inflammation.24 However, the mechanistic details were not examined in this study.

As mentioned above, a NOS inhibitor significantly (but not completely) suppressed the MA-induced increase in MBF in this study, suggesting that a non-NO vasodilator contributes to this action. Because acupuncture stimulation might induce the

![Figure 3](image)

**Figure 3** Effect of the combined administration of the non-selective nitric oxide synthase inhibitor NG-nitro-L-arginine methyl ester hydrochloride (L-NAME) and the cyclo-oxygenase inhibitor indomethacin (IND) compared to L-NAME alone (all at 500 mg/kg) on the increase in muscle blood flow of 28 Wistar rats (n=7 per group) receiving manual acupuncture (MA) or no acupuncture (control, C). Data are expressed as mean±SE. NS: no significant difference.

**Table 2** Mean arterial blood pressure before, during and after acupuncture

<table>
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<th>Experimental group</th>
<th>Before Drug</th>
<th>Before MA 0–1 min</th>
<th>During MA 0–1 min</th>
<th>After MA 1–2 min</th>
<th>After MA 2–3 min</th>
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<td>89±2</td>
<td>90±3</td>
<td>91±3</td>
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Data expressed as mean±SE.

C, control (no acupuncture); IND, indomethacin, a cyclo-oxygenase inhibitor; L-NAME, NG-nitro-L-arginine methyl ester hydrochloride, a nitric oxide synthase inhibitor; MA, manual acupuncture.

production of various inflammatory substances by slightly injuring the tissues, we postulated that vasodilatory PGs would contribute to the increase. However, a COX inhibitor did not suppress the increase. Furthermore, the suppressive effect of the combined administration of a NOS inhibitor and a COX inhibitor was not greater than that of a single administration of a NOS inhibitor, and the effect was rather weak. A possible explanation for this finding is that the complementary action of NO and PGs was lost through application of these inhibitors. Thus, the contribution of PGs to the MA-induced increase in MBF appears to be slight or negligible. However, the increase continues for at least 1 h after MA.9 Because the present study estimated the MBF at 3 min after MA (early phase), it is possible that PGs are involved in the increase after the early phase. On the other hand, the following systems are independent of NO or PG: (1) several vasodilators such as CGRP, ATR, ADP and adenosine, which directly bind to their respective receptors on vascular smooth muscle cells; and (2) endothelium-derived hyperpolarising factors (EDHF), which are proposed to be potassium ions (K+), cytochrome P450 metabolites, hydrogen peroxide (H2O2) or gap junctions between endothelial cells and smooth muscle cells (myoendothelial gap junction).23 We believe that EDHF partially contributes to the MA-induced increase in MBF, but this was not assessed by this study. Further investigation is therefore needed to elucidate these details.

Muscle sympathetic nerves, which generally are adrenergic sympathetic vasoconstrictor nerves, tonically control the blood vessels in skeletal muscle. It has been reported that MA may or may not transiently increase muscle sympathetic nerve activity (MSNA) at rest, both in normal subjects26 and patients with heart failure.27 Additionally, MA has been reported to reverse the increase in MSNA induced by mental stress patients with heart failure28 but not in normal subjects.27 Despite such reports, there have been no animal studies that have investigated the effects of MA on MSNA. In this study, the extent of the influence of the muscle sympathetic nerve on the MA-induced increase in MBF was unclear. However, we would anticipate it to be low, since the animals tested in this study were at rest and under anaesthesia and no significant change in MAP was observed.

CONCLUSIONS
We investigated the effect of a non-selective NOS inhibitor, a non-selective COX inhibitor and a combination of these agents on the MA-induced increase in MBF (measured using radiolabelled microspheres) in rats. A single administration of the non-selective NOS inhibitor significantly (but not completely) attenuated the MA-induced increase in MBF in a dose-dependent manner. On the other hand, the non-selective COX inhibitor had no effect. In addition, no synergistic effects were observed. Our findings suggest that NO mainly contributes to the increase in MBF following MA, and that the role of PGs is slight or negligible.

Summary points

- We aimed to examine the relative contributions of nitric oxide (NO) and prostaglandins (PGs) to the increase in local muscle blood flow (MBF) observed following manual acupuncture (MA) in rats using non-selective inhibitors of NO synthase (L-NAME) and cyclo-oxygenase (indomethacin).
- The MA-induced increase in MBF was partially inhibited by L-NAME in a dose-dependent manner, while indomethacin had no significant effect whether given alone or in combination with L-NAME.
- NO appears to be the major factor involved in the MA-induced increase in MBF, while PGs do not appear to play a significant role in this phenomenon.

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Contributors HS performed all the experiments and wrote the submitted article. MO, KK and KM participated in the experiments on muscle blood flow measurement in rats injected with a nitric oxide synthase inhibitor, a cyclo-oxygenase inhibitor and saline, respectively. ES supervised the experiments.

Competing interests None.

Provenance and peer review Not commissioned; externally peer reviewed.

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