Participation of calcitonin gene related peptide released via axon reflex in the local increase in muscle blood flow following manual acupuncture

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ABSTRACT
Objective The purpose of this study was to determine how calcitonin gene related peptide (CGRP) via axon reflex participates in increasing local muscle blood flow (MBF) following manual acupuncture (MA).

Methods Male Sprague–Dawley rats (N=56, 270–350 g) were used. We examined (1) the effects of MA on MBF in the tibialis anterior (TA) muscle in normal rats; (2) the effects of MA on MBF in the TA injected with saline or hCGRP8-37 (low: 2×10−4 mol/litre; high: 2×10−3 mol/litre), a competitive CGRP receptor antagonist, in rats; and (3) the effects of MA on MBF in the TA in capsaicin-treated rats. The capsaicin-treated rats were injected with capsaicin dissolved in an ethanol solution within 24 h after birth (50 mg/kg subcutaneously). MA was applied to the right TA for 1 min.51Cr-labelled microspheres (15 μm in diameter) were used to measure MBF.

Results MA significantly increased MBF without changing arterial blood pressure in normal rats (p<0.05). MA also significantly increased MBF in saline-injected, low hCGRP8-37-injected and high hCGRP8-37-injected rats (p<0.001, 0.005 and 0.05, respectively). The increases in low and high hCGRP8-37-injected rats were lower than those in saline-injected rats, but the difference was not significant. However, MA did not significantly increase MBF in capsaicin-treated rats (p=0.38).

Conclusions We obtained conflicting results, suggesting that the participation of CGRP released via axon reflex may be limited to a local increase in MBF following MA.

BACKGROUND
Acupuncture has been applied to treat painful musculoskeletal disorders since ancient times. Acupuncture analgesia has been considered to be caused by activating the descending pain inhibitory system, the diffuse noxious inhibitory control (DNIC) and/or segmental spinal analgesia based on the gate control theory.1 Furthermore, the improved muscle circulation provided by acupuncture contributes to pain relief by washing out analgesic and fatigue-related substances.

Acupuncture increases muscle blood flow (MBF) in humans.2–5 Although the mechanism has not been made clear, some hypotheses have been proposed. Kuwasawa et al6 suggested in a novel hypothesis that acetylcholine (ACh) released from sympathetic cholinergic vasodilatory nerves (SCVNs) via axon reflex by manual acupuncture (MA) dilates muscular vessels. Kusumoto et al7 alternatively proposed that ACh released from SCVNs via somatoautonomic reflex by MA dilates muscular vessels. In addition, Noguchi et al8 reported an increase in MBF with elevation of the arterial blood pressure via somatoautonomic reflex during electroacupuncture (EA) in rats. Sakaguchi et al9 and Sato et al10 suggested a possibility that vasodilation via axon reflex is caused by noxious stimuli in skeletal muscle as well as in skin.

The axon reflex is a reflex-like phenomenon, which occurs as follows: (1) thin nociceptive sensory nerve fibres are excited by a noxious stimulus applied to the skin; (2) the impulse is antidromically conducted to the axon’s terminals via the collateral branches of the axon;
(3) calcitonin gene related peptide (CGRP) is released from the branches and dilates skin arterioles: the so-called flare. This flare is often observed in acupuncture treatment. Currently, CGRP released via axon reflex is considered to be the strongest candidate for the vasodilator that increases MBF following MA. In our previous studies using radiolabelled microspheres, we have shown that MA significantly increased local MBF in rats.\textsuperscript{11–13} This increase was observed immediately after denervation, suggesting the possibility of axon reflex participation in the increase.\textsuperscript{11} However, the increase was also observed after chronic denervation, although it seemed to be attenuated by it. Thus, in our previous study, it was unclear whether axon reflex participated in the increase or not.\textsuperscript{11} There are no reports showing any direct evidence for this at present.

Capsaicin is a primary ingredient of hot chilli peppers. It is an inflammatory substance and causes neurogenic inflammation by binding to vanilloid type 1 receptors on Aδ fibres and C fibres.\textsuperscript{14} Subcutaneous administration of capsaicin to neonatal animals prevents the growth of thin sensory nerve fibres (Aδ and C fibres) containing substance P.\textsuperscript{15} Most of these fibres contain CGRP and other substances.\textsuperscript{16} Thus, capsaicin can be used to examine the participation of thin sensory nerve fibres containing substance P and CGRP in physiological and pathological events.

CGRP released via axon reflex dilates vessels by binding to CGRP\textsubscript{1} receptors.\textsuperscript{17} A fragment of CGRP, hCGRP\textsubscript{8-37}, abolishes the functions of the CGRP receptor by binding to it.\textsuperscript{18} Two pathways for the vasodilation are known, namely, (1) the nitric oxide (NO)-independent and endothelium-independent pathway and (2) the NO-dependent and endothelium-dependent pathway.\textsuperscript{17} In the NO-independent and endothelium-independent pathway, the binding of CGRP to the CGRP\textsubscript{1} receptor on vascular smooth muscle cells activates adenylate cyclase, which increases the intracellular cAMP concentration ([cAMP]). The increase in [cAMP] stimulates protein kinase A, which opens potassium ion (K\textsuperscript{+}) channels and activates Ca\textsuperscript{2+} sequestration mechanisms to relax vascular smooth muscle (ie, causes vasodilation). However, in the NO-dependent and endothelium-dependent pathways, the binding of CGRP to the CGRP\textsubscript{1} receptor on vascular endothelium cells triggers NO production. NO is produced in vascular endothelium cells via endothelium NO synthase (eNOS), with the increase in [cAMP], caused by adenylate cyclase. Then, NO released from vascular endothelium cells diffuses to adjacent vascular smooth muscle cells and leads to relaxation by activating guanylate cyclase.

To make it clear that CGRP released via axon reflex participates in the local increase in MBF following MA, we investigated (1) the effects of MA on MBF in the tibialis anterior muscle (TA) in normal (non-treated) rats; (2) the effects of MA on MBF in the TA of rats that had been injected with saline or hCGRP\textsubscript{8-37}; and (3) the effects of MA on MBF in the TA of capsaicin-treated rats, using \textsuperscript{51}Cr-labelled microspheres for measurement of MBF. The microsphere technique has the following advantages: (1) it can quantitatively measure MBF, (2) it does not damage the target muscles and (3) it is not influenced by movement. These advantages are helpful while performing acupuncture studies, causing little damage to and movement of the target muscles. Therefore, we used this technique in the present study as well as in our previous studies.\textsuperscript{11–13}

**MATERIALS AND METHODS**

**Experimental animals**

The animals used in this experiment were male Sprague–Dawley rats (total N=56, Japan SLC, Inc, Shizuoka, Japan). A total of 12 rats were purchased as fetuses 3 days before birth and were used for the capsaicin experiment, and 44 8-week-old rats were purchased and used for the other experiments. All the rats were fed in an air-conditioned room under a 12 h:12 h light–dark cycle until they were 9–10 weeks old (weight, 270–350 g). They had free access to water and a standard rodent diet. Urethane was used as the anaesthesia (1.2 g/kg intraperitoneally). This study was approved by the ethical committee of Meiji University of Integrative Medicine (no. 17-44-3).

**Acupuncture stimulation**

As in our previous study,\textsuperscript{11–13} the acupuncture stimulation method used was the sparrow pecking technique at 30 repetitions per minute to a depth of 15–18 mm using a stainless-steel acupuncture needle (0.20×30 mm, Seirin Inc., Japan). The stimulus point was on the right TA at a point 7–8 mm below the knee. An acupuncture needle was inserted toward the ankle joint and penetrated the TA.

**Chemicals and treatment**

The CGRP receptor antagonist used was hCGRP\textsubscript{8-37} (LKT Laboratories Inc., USA). A solution of 0.2 ml at low (2×10\textsuperscript{−3} mol/litre) or high (2×10\textsuperscript{−2} mol/litre) concentration was injected intramuscularly into the TA 10–15 min before commencing acupuncture stimulation.\textsuperscript{10}

Capsaicin (Nacalai Tesque, Japan) was used to destroy thin sensory nerve fibres containing substance P and CGRP. Capsaicin dissolved in ethanol solution (10% ethanol, 10% Tween 80, 80% saline, w/w) was injected into the subcutaneous tissue of newborn rats within 24 h after birth (50 mg/kg subcutaneously).\textsuperscript{15} The rats were put on a cold plate to reduce pain during the injection. The validity of the capsaicin treatment was checked by observing an escape behaviour following contact with a capsaicin-soaked cotton.
bud to one eyeball of each 9–10-week-old rat before taking MBF measurements.

**MBF measurement**

The $^{51}$Cr-labelled microspheres (15 μm in diameter, PerkinElmer Japan Co Ltd, Japan) dissolved in saline with 10% Tween 80 were used for measurement of MBF in the same manner as in our previous study.\(^{11–13}\)

The measurement was performed using the following procedure: (1) a reference sample of blood (RSB) was withdrawn from the left femoral artery by 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) γ radiation doses of RSB and TA were counted by a γ counter (Auto Well Gamma System ARS-600, ALOKA Co Ltd, Japan); and (6) MBF was calculated using the following equation:

$$\text{MBF} = \frac{100 \times Rm \times V}{(Rb \times W)}$$ (ml/min/100g)

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

**Arterial blood pressure**

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

**Experimental groups**

See table 1 for details on the experimental conditions and groups. ‘Acp’ and ‘Cnt’ show acupuncture and non-acupuncture rat groups, respectively. ‘Cap’ and ‘Nrm’ show capsaicin-treated and normal (non-treated) rat groups, respectively.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Number of rats</th>
<th>Treatment</th>
<th>MA technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nrm_Cnt</td>
<td>6</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Nrm_Acp</td>
<td>6</td>
<td>–</td>
<td>Sparrow pecking</td>
</tr>
<tr>
<td>Saline_Cnt</td>
<td>8</td>
<td>Saline (intramuscularly)</td>
<td>–</td>
</tr>
<tr>
<td>Saline_Acp</td>
<td>8</td>
<td>Saline (intramuscularly)</td>
<td>Sparrow pecking</td>
</tr>
<tr>
<td>low_hCGRP8.37_Acp</td>
<td>8</td>
<td>hCGRP8.37 (2.0×10^-3 mol/litre intramuscularly)</td>
<td>Sparrow pecking</td>
</tr>
<tr>
<td>high_hCGRP8.37_Acp</td>
<td>8</td>
<td>hCGRP8.37 (2.0×10^-3 mol/litre intramuscularly)</td>
<td>Sparrow pecking</td>
</tr>
<tr>
<td>Cap_Cnt</td>
<td>6</td>
<td>Capsaicin (50 mg/kg subcutaneously)</td>
<td>–</td>
</tr>
<tr>
<td>Cap_Acp</td>
<td>6</td>
<td>Capsaicin (50 mg/kg subcutaneously)</td>
<td>Sparrow pecking</td>
</tr>
</tbody>
</table>

Sparrow pecking was performed at 30 repetitions/min. Acp, acupuncture groups; Cap, capsaicin-treated group; CGRP, calcitonin gene related peptide; Cnt, non-acupuncture groups; MA, manual acupuncture; Nrm, normal (non-treated) group.

**RESULTS**

**Muscle blood flow**

As shown in figure 1, there was a significant difference in MBF between Nrm_Cnt and Nrm_Acp groups ($p<0.05$ in the unpaired t test). MA significantly increased MBF in the normal (non-treated) rats.

As shown in figure 2, there were significant differences in MBF between Saline_Cnt and Saline_Acp, between Saline_Cnt and low_hCGRP8.37_Acp, and between Saline_Cnt and high_hCGRP8.37_Acp groups ($p<0.001$, 0.05 and 0.05 on Dunnett’s test, respectively). MA significantly increased MBF in these acupuncture groups. Although MBFs of the low_hCGRP8.37_Acp and high_hCGRP8.37_Acp groups were less than that of the Saline_Acp group, there were no significant differences in MBF between these acupuncture groups ($p>0.05$ in the one-way ANOVA). Thus, hCGRP8.37 did not significantly inhibit the increased effect of MA on MBF.

As shown in figure 3, there was no significant difference in MBF between the Cap_Cnt and Cap_Acp groups ($p=0.38$ on unpaired t test). Thus, capsaicin treatment attenuated the increased effect of MA on MBF. However, MBF of Cap_Acp group seemed to be higher than that of Cap_Cnt group.

**Arterial blood pressure**

As shown in table 2, there were no significant differences in temporal change (before, during and after MA) of MAP.

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in the Saline_CNT, Saline_Acp, low_hCGRP8-37_Acp, high_hCGRP8-37_Acp, Nrm_CNT, Nrm_Acp, Cap_CNT and Cap_Acp groups (p=0.75, 0.41, 0.60, 0.12, 0.06, 0.25, 0.70 and 0.90, respectively). Thus, MA did not significantly influence MAP.

**DISCUSSION**

Local or systemic administration of CGRP improves tissue survival in surgical skin flaps in animals and humans.19–21 This improvement is because of the increase of blood flow and/or the inhibition of surgically induced neutrophil recruitment. Jansen et al22 reported that EA or MA significantly increased the survival of ischaemic musculocutaneous flaps. They assumed that this effect is due to the suppression of sympathetic vasoconstriction by muscle afferent nerve fibres and/or due to the vasodilation by substances (substance P and CGRP) released from sensory nerve fibres activated by EA or MA.

In previous studies, we have shown that MA significantly increased local MBF in rats.11–13 This increase was dependent on stimulus intensity (repetitions of sparrow pecking) without changing arterial blood pressure.11–12 In addition, the increase was observed immediately after denervation by cutting off the sciatic nerve. This result suggested that the local vasodilatory system containing axon reflexes participated in the increase. MA is a noxious mechanical stimulation and often induces a flare on the skin. It was shown that vasodilation via axon reflex could be caused by excitatory stimuli to thin sensory nerve fibres in skeletal muscle.9, 10 Kinoshita23 reported that contraction recovery after high-frequency electrical stimulation was accelerated by MA in guinea pigs and that this recovery was abolished by subcutaneous administration of capsaicin but was not influenced by destroying central and peripheral nerves; thus, it was considered that improvement of muscle circulation via axon reflex was responsible for the recovery. However, this recovery was inhibited by atropine, a muscarinic acetylcholine receptor antagonist. In response, Kuwasawa et al,6 who belonged to the same research groups as Kinoshita, proposed a hypothesis that ACh released from sympathetic cholinergic vasodilatory nerves (SCVNs) via axon reflex by MA dilates muscular vessels. However, in our previous study using rats, the increase in MBF after MA was not affected by atropine at all.13 Many studies have reported that topical application of ACh dilates the arterioles of rat skeletal muscles,24–26 and that vasodilation induced by physical or mental stress is attenuated by atropine in human skeletal muscles,27 thereby suggesting the existence of muscarinic receptors on muscular vessels. However, there is no histological evidence of SCVNs in mice,28 rats,28 or humans.27 The reason for the difference between ours and Kinoshita’s results may be the difference in animal species used in the experiments. Kusumoto et al7 proposed a hypothesis that vasodilation occurs via
somatoautonomic reflex by MA. However, there are few reports suggesting the involvement of autonomic nerves in muscle vasodilation by MA. MA and EA both seem to transiently increase muscle sympathetic nerve activity.8 29–31 Sympathetic efferent fibres modified neurogenic inflammation mediated by dorsal root reflexes via peripheral α1 adrenoceptors.32 However, the participation of the autonomic or central nervous systems in the increase of MBF by MA is not yet clearly understood. Therefore, on the basis of the above-mentioned facts, the axon reflex is considered the strongest candidate for the vasodilatory mechanism underlying localised increase of MBF by MA.1 3 11

We obtained the following conflicting results in this study: (1) hCGRP8–37 did not significantly inhibit the increased effect of MA on MBF and (2) capsaicin treatment attenuated the increased effect of MA on MBF. These results did not clearly prove that CGRP via axon reflex is the main factor in vasodilation induced by MA, as had been predicted. There is a possibility that other vasodilators released from thin sensory nerve fibres participated in the increase, but we consider the probability of this to be low. However, low and high hCGRP8–37 both seemed to suppress the increase in MBF after MA, and capsaicin treatment seemed not to completely eradicate the increase, although these findings were not significant. In addition, in our previous study, the significant increase in MBF by MA was observed after acute and chronic denervation in rats.11 These findings suggest that local vasodilators other than the axon reflex are involved in the increase. Therefore, we concluded that CGRP released via axon reflex may partially participate in the increase in MBF, along with other local vasodilators.

The other candidates for local vasodilators participating in the increase in MBF following MA include bradykinin,33 prostaglandin,34 35 adenosine,36 37 adenosine 5′-triphosphate (ATP),37 38 and NO.39 Bradykinin and prostaglandin are inflammatory substances and are locally produced in damaged tissue via the kallikrein–kinin system and the arachidonic acid cascade, respectively. These have a vasodilatory action, and also execute various bioactivities by binding to their receptors and lead to inflammation. It is a possibility that these substances are produced by small amounts of tissue damage that accompany acupuncture stimulation. Although we consider that these all are possible candidates, there are few reports studying their participation in the increase in MBF following MA.

### Table 2  Mean arterial blood pressure before, during and after manual acupuncture in rats

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Mean arterial blood pressure (mm Hg)</th>
<th>After MA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before MA –1–0 min</td>
<td>During MA 0–1 min</td>
</tr>
<tr>
<td>Nrm_Cnt</td>
<td>91±2</td>
<td>93±3</td>
</tr>
<tr>
<td>Nrm_Acp</td>
<td>83±3</td>
<td>78±3</td>
</tr>
<tr>
<td>Saline_Cnt</td>
<td>79±4</td>
<td>76±4</td>
</tr>
<tr>
<td>Saline_Acp</td>
<td>85±6</td>
<td>80±3</td>
</tr>
<tr>
<td>low_hCGRP8–37_Acp</td>
<td>71±5</td>
<td>71±3</td>
</tr>
<tr>
<td>high_hCGRP8–37_Acp</td>
<td>83±3</td>
<td>86±3</td>
</tr>
<tr>
<td>Cap_Cnt</td>
<td>88±4</td>
<td>87±3</td>
</tr>
<tr>
<td>Cap_Acp</td>
<td>84±4</td>
<td>83±3</td>
</tr>
</tbody>
</table>

There were no significant differences in mean arterial blood pressure taken before, during and after manual acupuncture (MA) in the repeated measures one-way analysis of variance. See table 1 for details on the experimental conditions and groups. Data are expressed as mean±SE. Acp, acupuncture groups; Cap, capsaicin-treated group; CGRP, calcitonin gene related peptide; Cnt, non-acupuncture groups; MA, manual acupuncture; Nrm, normal (non-treated) group.
Adenosine is a component of adenosine phosphate compounds, for example, ATP, ADP and AMP, and has a potent vasodilatory effect by binding to adenosine receptors.\textsuperscript{36} ATP has a vasodilatory effect by binding to purine receptors (P2Y receptor).\textsuperscript{38} Under hypoxic conditions, adenosine and these adenosine phosphate compounds are released from skeletal muscle cells or red blood cells to the extracellular space and dilate the surrounding vessels.\textsuperscript{37} Recently, in a microdialysis study, Goldman \textit{et al.}\textsuperscript{40} reported that MA significantly elevated extracellular concentration of adenosine and adenosine phosphate compounds in the acupunctured muscle and showed that adenosine plays a role in the local antinociceptive effects of acupuncture by binding to A1 receptors. Activation of the descending pain inhibitory system and/or DNIC is needed to excite thin sensory nerve fibres.\textsuperscript{1} MA can activate the central and the local analgesia system and increase MBF by exciting thin sensory nerve fibres, leading to pain relief. We also believe that adenosine is a strong candidate for vasodilatation by MA.

Nitric oxide, the endothelium-derived relaxing factor, is a potent vasodilator. NO production is induced by binding various substances, for example, adenosine, ATP and CGRP to their receptors on vascular endothelium cells.\textsuperscript{37} However, NO stimulates CGRP release from sensory fibres, and thus NO and CGRP seem to influence each other in vasodilation.\textsuperscript{41,42} Therefore, we consider that NO probably participates in vasodilation by MA. In fact, there is a report suggesting that NO might be responsible for the increase in blood flow in subcutaneous tissue after MA.\textsuperscript{43}

**CONCLUSIONS**

We investigated the participation of CGRP released via axon reflex in the local increase in MBF following MA and obtained conflicting results. These results suggest that CGRP released via axon reflex may partially participate in the increase, but that other local vasodilators may also be involved in it.


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