J Physiol Sci (2009) 59:421–427
DOI 10.1007/s12576-009-0052-8

ORIGINAL PAPER

Neural mechanism of localized changes in skeletal muscle blood flow caused by moxibustion-like thermal stimulation of anesthetized rats

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Received: 29 March 2009 / Accepted: 30 June 2009 / Published online: 25 July 2009

Abstract Moxibustion-like thermal stimulation (MTS) was applied to the gastrocnemius muscle to measure local muscle blood flow (MBF) in the stimulated region and the change in the MBF in the region, and its mechanism was examined. In the experiment, we used urethane-anesthetized rats under artificial respiration and observed the change caused by gastrocnemius MTS using a laser Doppler blood-flow meter. MTS applied to the gastrocnemius muscle caused a two-phase response in blood flow that showed a transient decrease followed by an increase without blood pressure change. It is suggested that the increase in response occurs because of an axon reflex that has a reflex arc below the spinal cord, and the decrease in response is caused by direct stimulation of postganglionic muscle sympathetic fibers.

Keywords Axon reflex · Local muscle blood flow · Muscle sympathetic nerve · Laser Doppler blood-flow meter · Rat

Introduction

Since ancient times, moxibustion has been commonly used to ease the pain that occurs with muscle fatigue, etc. It is popular as a home treatment and is easy to use, unlike acupuncture. It is thought that acupuncture and moxibustion contribute to relieving muscle pain by improving the blood circulation in the muscle [1–3].

In experimental research using an anesthetized animal, Noguchi et al. [4] reported an increased response of skeletal muscle blood flow (MBF) that systematically occurs with electro-acupuncture stimulation of the rat hindpaw, and they clarified its neural mechanism. To determine the mechanism of the response in local blood flow occurring with acupuncture and moxibustion stimulation, Jansen et al. measured blood flow at a musculocutaneous flap. They observed that the increased response in skin blood flow at the musculocutaneous flap, equivalent to the situation when administrating the substance P (SP) or calcitonin gene-related peptide (CGRP), occurs because of electro-acupuncture stimulation of the musculocutaneous flap base. They reported that there was an increased response in local blood flow via vasodepressor material at an acupuncture stimulation site [5]. Sato et al. [6] reported that the increase in response in local MBF disappears with CGRP receptor antagonist administration with dorsal root nerve stimulation of anesthetized rats. Moreover, Loaiza et al. [7] reported a blood flow increase in the knee joint capsule arteriole, which involves nitric oxide (NO), caused by electro-acupuncture stimulation of the quadriceps femoris. For moxibustion stimulation, Uchida et al. [8] reported a cerebral cortex blood flow increase with different responses according to stimulation site.

However, the effects of moxibustion stimulation on MBF and its neural mechanism have not been studied yet. This study considered the change in MBF caused by moxibustion-like thermal stimulation (MTS) of a gastrocnemius muscle and its neural mechanism in order to examine the change in skeletal MBF that takes place at a stimulation site when applying MTS to the lower thigh.

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Materials and methods

We conducted the experiment according to the protocol of the Laboratory Animals Committee of the Faculty of Health Science, Tsukuba University of Technology. The subjects were 36 male Wistar rats (age 8–15 weeks, weight 280–320 g), which were anesthetized with urethane (1.2 g/kg, i.p.).

The rats maintained CO_2 concentration in expiration to about 3% under artificial respiration (90 times/min, 10 ml/kg). The infrared lamp affecting the laser Doppler blood-flow meter was switched off, and the body temperature was kept at 36.5–37.5°C using a DC heating pad (ATB-1100, Nihon Kohden, Tokyo, Japan).

The arterial pressure was measured and continuously recorded by a polygraph (RM-6000, Nihon Kohden, Tokyo, Japan) via a pressure transducer (Model TP-400T, Nihon Kohden, Tokyo, Japan) using a catheter inserted in the common carotid artery. A jugular catheter was used for fluid replacement. Body movement, blood pressure, and respiratory status were monitored, and urethane (about 0.1 g/kg) was additionally administered according to the change in anesthetic depth. When the systolic arterial pressure went below 90 mmHg, 4% Ficoll 70 solution (GE Healthcare Bio-Sciences KK, Japan) or 5% lactated Ringer's solution with glucose (Otsuka Pharmaceutical, Tokyo, Japan) was injected intravenously as needed to maintain the blood pressure. Furthermore, in order to prevent artifacts in the blood flow measurement because of body movement, 0.5 mg/kg muscle relaxant (Mioblock Intravenous 4 mg, Schering-Plough, Osaka, Japan) was administered before starting the stimulation.

MBF measurement

The rats were fixed in a prone position. Their gastrocnemius muscle was exposed by a small skin incision in the central gastrocnemius muscle region at one side of the leg, and the muscle surface was covered with paraffin oil to prevent drying. The local surface MBF of the gastrocnemius was measured with a needle probe (tip diameter 0.8 mm) of a laser Doppler blood-flow meter (ALF-2100, Advance Co., Tokyo, Japan) that was fixed using a balancing holder so that it would not be pressed. The local surface MBF just underneath the probe, at a volume of approximately 1 mm^3, was continuously measured by laser Doppler flowmetry.

The output of the laser Doppler blood-flow meter was expressed in mV and recorded continuously together with blood pressure (Fig. 1A).

MTS method

For MTS, we modified a clinical electronic moxibustion treatment appliance (Sofcom: MXA-8000, Zeniryoki, Fukuoka, Japan) and stimulus probe, and used them to make a pattern similar to thermal stimulation using usual moxa.

We performed MTS by placing the heating part (5 mm in diameter) of the stimulus probe on the skin surface, with a heating temperature of 41–52°C, three times (heating 7.5 s, rest 12.5 s) in 1 min. The mean temperature of the peak values ($n = 5$) of the stimulus probe measured by a thermistor thermometer was 42.7 ± 1.2°C the first time, 49.2 ± 1.8°C the second time, and 51.0 ± 0.8°C the third time. We stimulated the surface of the central gastrocnemius muscle region skin on a single limb. MTS was performed at a site 5 mm from the laser Doppler blood-flow meter probe and at a site 10 mm away in the direction of the Achilles tendon or the popliteal space for comparison (Fig. 1B).

We performed MTS three times around the gastrocnemius muscle, which recorded MBF, at intervals of more than 30 min. However, the MBF reactivities of decrease

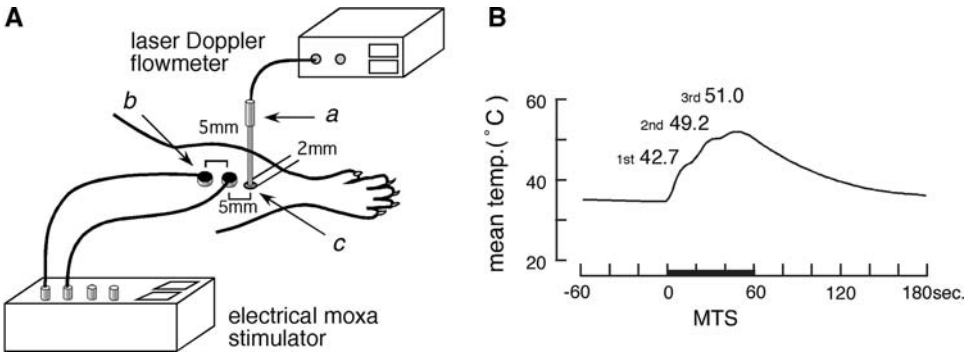

Fig. 1 Experimental methodology. **A** Schema of MBF measurement. *a* Laser Doppler flowmeter (LDF) probe; *b* moxibustion-like thermal stimulation (MTS) (for 5 and 10 mm from the LDF probe in the direction of the popliteal space); *c* exposed gastrocnemius (the surface is filled with liquid paraffin). **B** Temperature curve of MTS. Temporal stimulation electrode surface temperature measured by the thermistor thermometer with stimulation electrodes

and increase both showed a tendency to decrease when we performed MTS on one rat repeatedly.

Somatosensory nerve block and spinal cord pithing

In order to block the input from the somatosensory nerve that innervates the gastrocnemius muscle, which is an MTS site, to the central nervous system, after cutting the skin of the stimulation side of the thigh outside of the rat, the sciatic nerve was severed at the center of the thigh backside by advancing between the femur and biceps femoris.

Furthermore, to examine involvement of the central nervous system, we took the 10th thoracic spine laminectomy, and pithed the 10th thoracic spinal cord and following.

Vasomotor nervous system and vasodepressor material block

In order to block the input of the somatosensory nerve that innervates the gastrocnemius muscle, which is an MTS site, 0.5 ml of a local anesthetic drug (2% lidocaine solution; AstraZeneka, Osaka, Japan) was subcutaneously administered around the MTS site.

We intravenously administered an alfa adrenergic receptor blocker (Phentolamine 10 mg/kg; Novartis, Tokyo, Japan) or beta adrenergic receptor blocker (Propranolol 3 mg/kg; AstraZeneka, Osaka, Japan) to examine involvement by the adrenergic sympathetic nerve and muscarinic cholinergic receptor antagonist (Atropine 2.5 mg/kg; Tanabe Mitsubishi Seiyaku, Osaka, Japan) for involvement by the cholinergic nerve and pharmacologically blocked the blood vessels of the dominant vegetative nervous system.

Before MTS, we also intravenously administered h-CGRP (8-37) (10-4 M/0. 2 ml; Sigma, St Louis, MO), which is a CGRP receptor antagonist, and L-NAME (Nω-L-arginine methyl ester 30 mg/kg; Sigma), which is a nitric oxide synthase inhibitor.

Data processing

For blood flow and blood pressure, we measured the change in the values under and after stimulation against the values before stimulation every 20 or 60 s, and calculated the mean values. The response is expressed in percentage to the value before stimulation and indicated by the mean ± standard error of the mean.

As a statistical test, analysis of variance (ANOVA) and Dunnett's multiple comparison test were performed for temporal change. We judged a significance level of 5% or less as significant.

Results

Local MBF response to MTS

The local MBF at the site 5 mm from the central part of the gastrocnemius muscle was a transient decrease response that starts in the MTS and an increase in response that subsequently appears and exceeds the value before stimulation.

The mean peak value of this decrease in response was $-23.3 \pm 2.4\%$ of the significant response. Moreover, the continuous local MBF response turned into an increase after a decrease. It had peaks 2–7 min after the end of MTS. The average of the maximum peak value was $25.5 \pm 8.6\%$ (Fig. 2a).

All the examples (15/15, $n = 9$: number of responses/number of stimulations, number of rats) show the response of transient MBF decrease that starts with MTS. The succeeding increase in response started after the end of MTS and changed into an increase that exceeded the value before stimulation. This increase in response appeared in 73.3% of the examples (11/15). Moreover, this response maintained a significant increase for an average of 14 min, and 33.3% of the examples (5/15) had not restored to the value before stimulation after 60 min. In 26.6% of the examples (4/15) that did not show an increase, the response was restored to the value before stimulation from an increase in response.

In order to examine the details of this response, the local MBF response at the regions 5 and 10 mm from the MTS site was measured every 20 s. As a result, a transient decrease in response (7/7, $n = 4$) appeared after 20 s at the local gastrocnemius MBF 5 mm from an MTS site, and after 120 s it changed to a significant increase in response (5/7) (Fig. 2b, 5 mm). Moreover, there was no MBF response at the MTS site (towards the popliteal space or Achilles' tendon), which was 10 mm from the blood flow measurement site (Fig. 2b, 10 mm).

The change in the local MBF caused by these MTSs at gastrocnemius muscle regions did not accompany the change in mean blood pressure.

Influence of somatosensory nerve block and thoracolumbar spine destruction on an MBF response

After blocking the somatic nerve around the MTS site with a local anesthetic drug analgesic, a local MBF response caused by MTS was not observed (6/6, $n = 3$) (Fig. 3a).

After severing the sciatic nerve, which innervates the gastrocnemius, the blood flow response in the gastrocnemius muscle caused by MTS was only the significant transient MBF decrease in all the examples (6/6, $n = 3$).

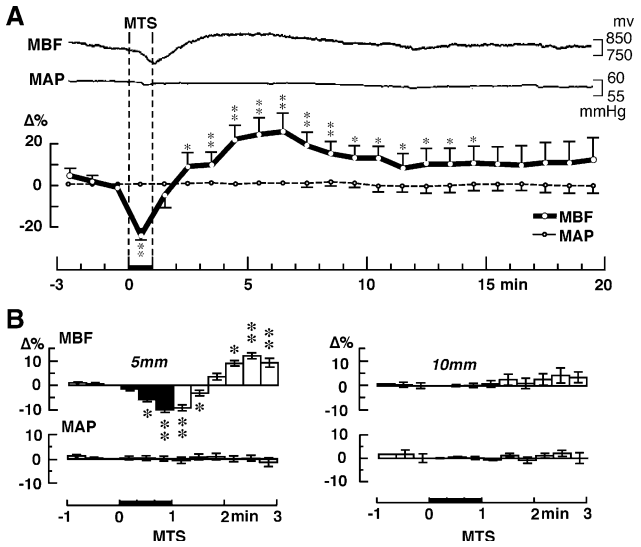

Fig. 2 Time-course changes in local MBF accompanying MTS. **a** A typical example and averaged percentage changes (mean ± SEM, 15 trials in 9 rats, recorded every minute) in gastrocnemius muscle blood flow (*MBF*) and mean arterial blood pressure (*MAP*). The *horizontal bar* and *dotted line* indicate the MTS period of 60 s. Responses of MBF shows two-phase changes such as the continual increase in response after the transient blood flow decrease in response. **b** Averaged percentage changes in MBF (mean ± SEM, seven trials in four rats, recorded every 20 s). The *right* is MBF recorded at 10 mm and the *left* at 5 mm from the stimulation site. The *black column* is the response in the MTS period. Each *column* and *vertical bar* represents a mean ± SEM; $*P < 0.05$ and $**P < 0.01$, significantly different from the control values using ANOVA and Dunnett's multiple comparison test

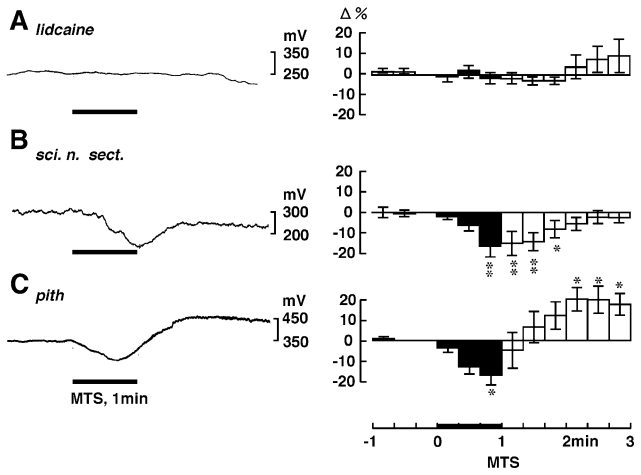

Fig. 3 Influence of somatosensory nerve block and thoracolumbar spine destruction on MBF response. A typical example of MBF recorded every 20 s (*left column*) and mean ± SEM (*right column*). The *horizontal bar* and *black column* show the MTS period. *From top to bottom*: **a** lidocaine (0.5 ml, s.c.) (three rats: six trials); **b** sectioning of sciatic nerve (three rats: six trials); **c** pithing of the tenth thoracic spine and later (three rats: seven trials). Statistical data details are the same as in Fig. 2

However, a subsequent increase in response in MBF (0/6) disappeared (Fig. 3b).

The response to MTS after pithing the spine below the 10th thoracic spine level was the transient MBF decrease in response and the significant increase in response that subsequently appeared (7/7, $n = 3$) (Fig. 3c).

Influence of vasomotor nerve block on an MBF response

To block the cholinergic sympathetic vasodilator nerve that innervates blood vessels, after administrating atropine, we performed MTS of the gastrocnemius muscle (Fig. 4). Mean blood pressure was not affected, and gastrocnemius MBF showed a significant increase in response (8/15, $n = 3$) or a response to return to the previous value (7/15) after the significant transient decrease in response.

After administrating phentolamine in order to block a sympathetic alpha receptor that innervates blood vessels, we performed MTS on the gastrocnemius muscle. Mean blood pressure before MTS was decreased under the influence of phentolamine administration, and a transient blood flow decrease of gastrocnemius MBF in the stimulated limbs disappeared in all the examples. However, the increase in response after the stimulation appeared in 45.5% (5/11, $n = 5$) of the examples.

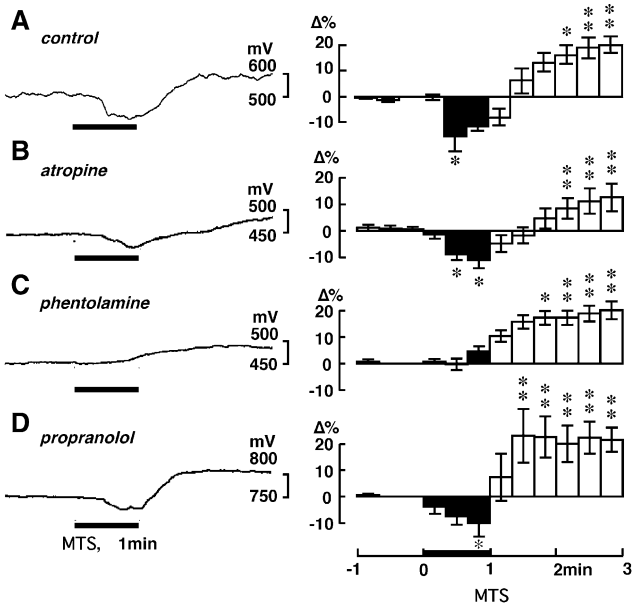

Fig. 4 Influence of vasomotor nerve block to an MBF. A typical example of MBF recorded every 20 s (*left column*) and mean ± SEM (*right column*). The *horizontal bar* and *black column* show the MTS period. *From top to bottom*: **a** control (4 rats: 9 trials); **b** atropine (2.5 mg 10 mg/kg, i.v.) (3 rats: 6 trials); **c** phentolamine (10 mg/kg, i.v.) (5 rats: 11 trials); **d** propranolol (3 mg/kg, i.v.) (5 rats: 9 trials). Statistical data details are the same as in Fig. 2

Also, after administrating propranolol in order to block a sympathetic beta receptor of blood vessels, we performed MTS of the gastrocnemius muscle. Gastrocnemius MBF of the stimulated limbs showed a significant increase in response (6/9, $n = 5$) or the response to return to the previous value (3/9) after the significant decrease in response (9/9).

Influence of vasodepressor material interception

In the local MBF response to MTS after intravenously administrating h-CGRP (8-37) (10-4 M/0.2 ml), which is a CGRP receptor antagonist, only the transient MBF decrease in response (8/8, $n = 4$) appeared, and the increase in response did not appear. For the response to MTS after intravenously administrating L-NAME (30 mg/kg) to inhibit the action of NO, which is a vasodepressor material derived from the vascular endothelium, both a transient MBF decrease in response (12/12, $n = 3$) and a subsequent increase in reaction (9/12) appeared (Fig. 5).

Discussion

This study demonstrated that local skeletal MBF produced by MTS resulted in an increase after a decrease. The MBF decrease in response was a transient phase that was induced when the stimulation started, and it continued for about 80 s. On the contrary, the significantly increased response was a long-lasting phase that continued for about 14 min or more. These results suggest that there is more than one action mechanism involved in localized MBF changes evoked by MTS.

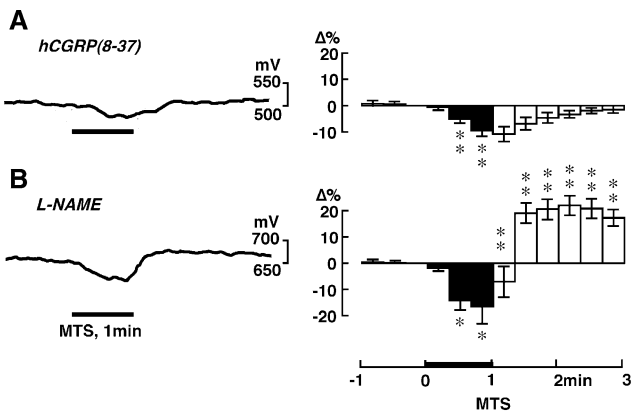

Fig. 5 Influence of vasodepressor material blocking. A typical example of MBF recorded every 20 s (*left column*) and mean ± SEM (*right column*). The *horizontal bar* and *black column* show the MTS period. *From top to bottom*: **a** hCGRP(8-37) (10-4 M/0.2 ml, i.v.) (4 rats: 8 trials); **b** L-NAME (3 mg/kg, i.v.) (3 rats: 12 trials). Statistical data details are the same as in Fig. 2

The vasomotor nerve was pharmacologically blocked by various blockers that were administered in order to examine the neural mechanism of the two-phase MBF response, and the effect was investigated.

With muscarinic cholinergic receptor antagonist and sympathetic beta receptor blocker administration, the decrease and increase in response to MBF were not influenced by MTS. However, only the transient blood flow decrease disappeared with the administration of a sympathetic alpha receptor blocker. The transient MBF decrease in response to MTS was considered to be a vasomotor response via a sympathetic alpha receptor.

Furthermore, with the pharmacologic blocking of CGRP, which is a vasodepressor substance involved in axon reflex, only the MBF increase in response to MTS was abolished. Also, the administration of L-NAME, which inhibits the action of NO and is a vasodepressor substance derived from the vascular endothelium, did not influence the MBF increase response.

Since a report by Lewis and Marvin [9], it has been well known that flare occurs around the stimulation site because of noxious stimulation of the skin. Because this flare disappears when a skin nerve is severed, it is thought that the flare is produced by axon reflex that is not via the central nerve system. This response is vasodilation that occurs by antidromic stimulation of the afferent nerve and is controlled by a CGRP antagonist [10].

The mechanism of the axon reflex-induced vasodilatation in the skin has been well clarified by Zimmermann [11], Holzer [12], and Koltzenburg and Handwerker [13].

Excitation of cutaneous afferent fibers originating in a restricted skin area can be conducted to other branches of the same afferents innervating the cutaneous blood vessels. It has been suggested that some spinal afferent nerves may have dichotomizing branches to two different nerves innervating two different tissues, for example, the sciatic and pudendal nerves [14], the intercostal and splanchnic nerves [15, 16], the intervertebral disc and groin skin [17], and the gastric mucosa and the abdominal wall [18].

Furthermore, Sato et al. [6] reported that antidromic stimulation of the dorsal root nerve causes the blood flow increase via CGRP in the dominated muscles. Hotta et al. [19] reported that the increase in the sciatic nerve blood flow, which disappears with a CGRP antagonist, occurred because of afferent stimulation of the sural nerve.

Therefore, our results delivered repetitive MTS of the cutaneous afferents innervating the skin of the gastrocnemius region and could elicit a vasodilative response with increased local blood flow in the gastrocnemius muscle.

Uchida et al. report that a reflectivity reaction of cerebral blood flow occurs in real moxibustion stimulation at 146°C.

However, our study suggests that the local MBF stimulated by moxibustion responded even to low temperature noxious stimulation of 40–50°C. Kuffman's report, changing the heart rate by heat stimulation of more than 45°C, suggested a nociceptor. Therefore, it was considered that our cutaneous heat stimulation by MTS was nociception.

Loaiza et al. [7] reported that NO is involved in the increase in local blood flow caused by electro-acupuncture applied for 30 min. On the other hand, in the present study, L-NAME did not influence the MBF response induced by MTS for a few minutes. From the above, the MBF increase in response caused by MTS observed in this study is considered to be induced via the axon reflex-like response with involvement of CGRP.

In the present study, two notable results were demonstrated in the experiments involving surgical neural treatment. One is that the surgical sciatic nerve section did not influence the transient decrease in responses, but abolished only the continual increase in responses, and the other is that pithing of the thoracic-lumbar spine did not influence the MBF response.

These results suggest that the increase in response is a reaction occurring through axon reflex that has a reflex arc below the spinal cord, and the decrease in response is a reaction induced by direct stimulation of muscle postganglionic sympathetic fibers.

For the organ blood flow change caused by acupuncture stimulation, in a previous study [4] using anesthetized rats we reported on the skeletal MBF increase in response that depended on a change of the internal organ's blood flow caused by electro-acupuncture. This increase in response in blood flow was a systemic response accompanying the increase of blood pressure resulting from the decrease in response in renal blood flow via a sympathetic alpha receptor. Uchida et al. [20] observed the increase in the cerebral cortex blood flow caused by acupuncture stimulation and moxibustion stimulation [8] to the face, forepaw, upper arm, and hindpaw, and reported that this response occurs in reflex with a cholinergic nerve of the Mynert nucleus within the brain as a centrifugal pathway. As mentioned above, it has been clarified that the neural mechanism of change in organ blood flow that occurred with MTS as a local response came via the central nervous system, and the secondary systemic response was associated with blood pressure change.

However, this study did not show any systemic blood pressure change with moxibustion-like stimulation of a gastrocnemius muscle. It was a limited response in that there was no MBF response at the region 10 mm from the stimulation site. Moreover, pithing the spinal cord that innervates the gastrocnemius muscle did not influence this MBF response, which indicated that there was no involvement of the central nervous system.

Uchida did not observe a significant blood pressure increase with MTS other than in the forepaw and hindpaw in a previous study [8]. Similarly, this MTS of the gastrocnemius muscle did not reveal a systemic blood pressure change.

It has long been known that noxious thermal stimulation excited postganglionic muscle sympathetic fibers in anesthetized cats [21]. From the above, it is inferred that the MTS of the gastrocnemius muscle region in this experiment caused a decrease in response that did not depend on a systemic blood pressure change, but caused the local MBF at the stimulation site via excitation of postganglionic sympathetic fibers. Since the somatic afferent nerve and postganglionic muscle sympathetic fibers at the stimulation site were simultaneously blocked by lidocaine, all the MBF responses caused by MTS, equivalent to noxious thermal stimulation, disappeared.

Conclusion

This experimental result proved for the first time that there is a local MBF response at the moxibustion stimulation site without mediation from the central nervous system that is not dependent on a blood pressure response and is a part of its mechanism.

Acknowledgment This work was supported by the research fund from the National University Corporation of Tsukuba University of Technology.

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