Neural Mechanism of Bradycardiac Responses Elicited by Acupuncture-Like Stimulation to a Hind Limb in Anesthetized Rats

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Abstract: The effects of acupuncture-like stimulation of a hind limb on heart rate were examined in anesthetized rats. An acupuncture needle, having a diameter of either 160 or 340 μm , was inserted into the skin and underlying muscles at a depth of about 5 mm and twisted right and left twice every second for 1 min. Stimulation by a needle with a diameter of either 160 or 340 μm produced a decrease in heart rate. Severance of the femoral and sciatic nerves ipsilateral to the hind-limb stimulation completely abolished the bradycardiac response. Also, heart rate was significantly decreased by acupuncture-like stimulation of the hind-limb muscles alone, but was not significantly influenced

by the stimulation of the hind-limb skin alone. The bradycardiac response induced by acupuncture-like stimulation was not influenced by bilateral severance of the vagal nerves at the cervical level, but was abolished by bilateral stellectomy. Acupuncture-like stimulation of the hind limb induced a decrease in the activity of the cardiac sympathetic efferent nerve as well as a decrease in heart rate. These results indicate that the decrease in heart rate induced by acupuncture-like stimulation of a hind limb is a reflex response. The afferent pathway is composed of hind-limb muscle afferents, and the efferent pathway is composed of cardiac sympathetic nerves.

Key words: heart rate, acupuncture, cardiac sympathetic nerve, somatic afferent, rat.

Acupuncture has been used to improve visceral autonomic malfunctions [1–3]. Recent acupuncture studies in animals demonstrated that acupuncture-like stimulation performed on anesthetized animals produced reflex responses of various visceral functions, for example, gastric motility [4], bladder contraction [5], blood pressure [6], and adrenal medullary hormonal function [7]. These acupuncture-like stimulation-induced responses have been proven to be reflexes in which afferents are cutaneous and/or muscle somatic afferent nerves, and efferents are autonomic efferent nerves (see reviews by Sato *et al.* [8, 9]).

One of the numerous effects of acupuncture on various visceral functions is an improvement of cardiac dysfunction (e.g., tachycardia) [10–12]. There have been several reports that acupuncture produces a decrease in heart rate in healthy human subjects [13–17]. Concerning the efferent neural pathways of acupuncture-induced bradycardia in humans, the conclusions are inconsistent between reports, i.e., some reports suggested that bradycardia was caused by an activation of the cardiac vagal nerve [13, 18], but other reports suggested that bradycardia was caused by the reciprocal coordination of an increase in cardiac vagal activity and a decrease in cardiac sympathetic activity [14, 19]. In human subjects, it is difficult to

clarify the neural mechanisms of acupuncture-induced bradycardia, because various visceral functions including heart rate are generally influenced by emotions triggered by various kinds of stimuli, including acupuncture, and also experimental techniques are limited to indirect methods, such as the administration of autonomic blockades or the analysis of heart rate variability. To clarify the neural mechanisms of the bradycardiac response following acupuncture, we must use anesthetized animals in which emotional factors are eliminated. Kobayashi *et al.* [20] reported that in anesthetized rats, acupuncture produced variable responses in heart rate, i.e., tachycardia, bradycardia, or no response. Furthermore, by severing the somatic nerves and autonomic nerves, they suggested that the afferent pathway of these responses is the somatic afferent nerves, and the sympathetic nerves were involved in the efferent pathway. However, it was difficult to analyze the neural mechanisms of the acupuncture-induced heart rate responses because the responses were variable. It was reported that the fluctuations of acupuncture-induced changes in blood pressure were dependent on the depth of anesthesia [6]. Li *et al.* [21, 22] anesthetized rats by a continuous infusion of pentobarbital and established this anesthetic condition as a procedure suitable for studying the inhibitory cardiovascular responses following vis-

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ceral stimulation. Therefore in the present study we used their method to maintain the anesthetic condition and produced constant bradycardiac responses following acupuncture-like stimulation to the hind limb. We first examined the afferent pathway of the bradycardiac response, using techniques of surgical denervation of hind-limb somatic nerves, and further studied to determine the importance of skin and muscles as the afferent pathway of the acupuncture-like stimulation-induced bradycardia. Second, we examined the efferent pathway of the bradycardiac response using techniques of surgical denervation of autonomic nerves and/or a recording of autonomic efferent activities.

METHODS

Adult male Wistar rats weighing 300–330 g ($n = 15$) were housed in a climate-controlled environment with food and water *ad libitum*. All animal experiments were conducted according to the Guidelines for Animal Experimentation prepared by the Ethics Committee of Health Science University. The rats were anesthetized with 50 mg/kg of pentobarbital intraperitoneally for general operations, such as a cannulation of the trachea, artery, and vein. A microinjection pump was used to continually inject a 1.0% pentobarbital in saline solution at a rate of 0.5–0.8 ml/h [21, 22] through a cannula that was inserted into the right jugular vein. The animals were also intubated with a tracheal tube through which they were artificially ventilated (SN480-7, Shinano, Japan). End-tidal CO₂ concentrations, examined with a gas monitor (Microcap, Oridion Medical, Israel), were kept at about 3.0% by controlling respiratory rate and volume. Systemic blood pressure was continuously recorded from a cannula inserted into the common carotid artery with a strain gauge (P23XL-1, Becton Dickinson Critical Care Systems, USA). Body temperature was kept at 37–38°C using a thermostatically regulated DC current heating pad and an infrared lamp (ATB-1100, Nihon Kohden, Japan).

Measurements of the heart rate. Heart rate was measured with a pulse rate tachometer (AT601-G, Nihon Kohden, Tokyo) triggered by systolic blood pressure waves and recorded continuously.

Acupuncture-like stimulation. Acupuncture-like stimulation was performed on a hind limb using an acupuncture needle (Seirin, Shizuoka) with a diameter of 340 μ m (No. 10) or, occasionally, 160 μ m (No. 1). The needle was inserted through the skin to a depth of about 5 mm. The stimulation point was within an area of 1 cm² about 5 mm below and lateral to the anterior tubercle of the tibia of a hind limb. The anterior tibial muscle is below the skin in this area. The needle was twisted right and left with the fingers by 180 degrees at a rate of about twice every second for 1 min. In 4 rats, the skin was separated from the underlying muscles of the hind limb with the nerves kept

intact as much as possible. Following separation, the stimuli were delivered to either the skin or the underlying muscles.

Surgical severance of the sciatic and femoral nerves. In 4 rats, acupuncture-like stimulation was performed as described above after the sciatic and femoral nerves were cut in the femoral area ipsilateral to the stimulated hind limb.

Vagotomy and stellectomy. Heart rate responses following acupuncture-like stimulation in the vagotomized rats and stellectomized rats were compared with rats with intact nerves. A bilateral vagotomy was performed at the cervical level in 6 rats, and a subsequent bilateral stellectomy was carried out in 4 rats following removal of the second costal bone.

Recordings of the cardiac sympathetic nerve activity. With the rats in the prone position, the cardiac sympathetic nerves were visualized after the removal of the right second costal bone. The nerve branch from the stellate ganglion to the heart was cut as close to the heart as possible, and neuronal activity was recorded using bipolar platinum-iridium wire electrodes [23]. The vagus nerves were cut bilaterally at the cervical level to prevent vagal contamination when cardiac sympathetic nerve activity was recorded. Gallamine triethiodide (20 mg/kg, *i.v.*) was also used to stably record the nerve activity. Sympathetic efferent nerve activity was amplified using a preamplifier (time constant 0.01 s, S-0476, Nihon Kohden, Tokyo), and was displayed on an oscilloscope (TS-8500, Iwatsu, Tokyo). Neuronal activity was sampled using an analog-to-digital converter (PowerLab/8, AD Instruments, Australia) at a rate of 2,000 samples/s and was rectified and integrated every 5 s. The signal recorded at the end of the experiment (after the rat had died) was set as the background noise. For statistical analysis, the value of nerve discharge was calculated by subtracting the background noise from the recorded value [24].

Statistics. Data were expressed as the mean $\pm$ standard error of the mean (SEM). Statistical comparisons were carried out using the paired *t*-test, unpaired *t*-test, or a repeated ANOVA (analysis of variance), followed by a Dunnett's multiple comparison test. A *p* value of <0.05 was considered to be statistically significant.

RESULTS

Resting conditions

Under resting conditions, heart rate and blood pressure were fairly stable. The resting heart rate and mean arterial pressure measured at the start of the experiment in rats with intact nerves were 430 ± 9.4 beats/min and 136 ± 2.6 mmHg, respectively ($n = 11$, mean $\pm$ SEM).

Heart rate response to acupuncture-like stimulation

Acupuncture-like stimulation of a hind limb with a

Fig. 1. Effects on the heart rate following acupuncture-like stimulation with a needle of either 160 μm (A, C) or 340 μm in diameter (B, D). A, B: Sample recordings of heart rate in the same animal. C, D: Summarizes the time course data of heart rate in response to acupuncture-like stimulation. The data are expressed as the mean $\pm$ SEM ($n = 7$ trials in 4 rats). * $p < 0.05$ compared with pre-stimulus control values using a one-way repeated ANOVA followed by Dunnett's multiple comparison test. Stimulation for 1 min was applied as indicated by the bottom bar and vertical dashed lines.

needle of either 160 μm or 340 μm diameter for 1 min produced decreases in heart rate (Fig. 1, A and B). Figure 1, C and D, summarizes the time courses of the responses of heart rate measured every 20 s following the acupuncture-like stimulation with needles of 160 μm and 340 μm diameter. Heart rate response induced by a needle of 340 μm was greater and lasted longer than the response induced by one of 160 μm . Following acupuncture-like stimulation with a needle of 340 μm , heart rate decreased within 20 s and reached a minimum at 60 s after the onset of stimulation, followed by a gradual return to the control level. Following acupuncture-like stimulation with a needle of 160 μm and one of 340 μm in diameter, heart rate decreased by 13.7 ± 5.1 and 30.4 ± 4.5 beats/min, respectively, at the end of stimulation (Fig. 1, C and D). Simultaneously, the mean arterial pressure decreased by 8.4 ± 2.1 and 33 ± 7.0 mmHg, respectively. Because the heart rate response elicited by stimulation with a 340- μm needle was greater than that elicited with a 160- μm needle, we used only one of 340 μm in diameter in all subsequent experiments.

Severance of the sciatic and femoral nerves

The bradycardiac response to acupuncture-like stimulation of a hind limb was completely abolished after severance of the sciatic and femoral nerves ipsilateral to the hind limb stimulated in all 4 rats that were examined.

Responses of heart rate to the stimulation of either the skin or underlying muscles

The effects of acupuncture-like stimulation on the hind limb of rats ($n = 4$) when skin and muscles were stimulated together are shown in Fig. 2A, and the effect on the skin alone or the underlying muscles alone is shown in Fig. 2, B and C, respectively. Although heart rate was not influenced by the stimulation of skin alone (Fig. 2B), it decreased significantly (33.4 ± 6.3 beats/min; the changes were measured at the end of stimulation) by stimulation of the underlying muscles alone (Fig. 2C). The response

Fig. 2. Responses of heart rate to acupuncture-like stimulation applied to the skin and muscles (A), skin alone (B), or underlying muscles alone (C) in a hind limb. The changes in heart rate at the end of stimulation were summarized (mean $\pm$ SEM, $n = 8$ trials in 4 rats). * $p < 0.05$ compared with prestimulus control values using a paired t -test.

evoked by stimulation of the muscles was nearly identical to that evoked by a stimulation of the skin and muscles together ($p > 0.05$).

Severance of the vagal nerve and the sympathetic nerve branches to the heart

In 5 rats, autonomic nerves innervating the heart were cut at the cervical level for the vagal nerves and at the stellate ganglia for the sympathetic nerves, bilaterally. Figure 3A shows a typical response, and Fig. 3D summarizes the changes in heart rate at the end of acupuncture-like stimulation with a 340- μm needle before severance of these autonomic nerves. These bradycardiac responses were not significantly affected by bilateral vagotomy (Fig. 3, B and E), and they were completely abolished by subsequent bilateral stellectomy (Fig. 3, C and F).

Responses of cardiac sympathetic efferent nerve activity

The efferent nerve activity of cardiac sympathetic nerves was recorded in 4 rats. The spontaneous efferent activity of the cardiac sympathetic nerves, integrated for 5 s, was 3.16 ± 0.5 $\mu\text{V}\cdot\text{s}$ ($n = 4$). The cardiac sympathetic

Fig. 3. Effects of acupuncture-like stimulation on heart rate before (**A**, **D**) and after cutting the vagus nerves (**B**, **E**) and stellectomy (**C**, **F**). **A–C**: Sample recordings of heart rate. **D–F**: Summarizes the changes in heart rate at the end of the acupuncture-like stimulation. The data are expressed as the mean $\pm$ SEM ($n = 9$ trials in 5 rats). * $p < 0.05$ compared with prestimulus control values using a paired t -test.

Fig. 4. Sample recordings of cardiac sympathetic nerve activity (**A**, **B**) and heart rate (**C**) to acupuncture-like stimulation in one rat. **A**: Nerve activity before (**a**) and during (**b**) acupuncture-like stimulation. **B**, **C**: Simultaneous recordings of integrated nerve activity (**B**) and heart rate (**C**).

nerve activity and the heart rate were simultaneously inhibited following acupuncture-like stimulation (Fig. 4). Figure 5 summarizes the results of the time course data of cardiac sympathetic nerve activity following acupuncture-like stimulation. Cardiac sympathetic nerve activity decreased significantly within 20 s after the onset of stim-

Fig. 5. Time course of responses of cardiac sympathetic nerve activity to acupuncture-like stimulation ($n = 8$ trials in 4 rats). * $p < 0.05$ compared with prestimulus control values using a one-way repeated ANOVA followed by Dunnett's multiple comparison test. Other details are the same as in Fig. 1.

ulation, reached minimum around the end of stimulation, and gradually returned to control levels within 3 minutes thereafter (Fig. 5).

DISCUSSION

The present study is the first report proving that acupuncture-like stimulation of a hind limb produces a decrease in heart rate as a reflex response, independent of emotional responses, in anesthetized rats. This response was proven to be a reflex in which the afferent pathways consist of somatic muscle afferents and the efferent pathways consist of cardiac sympathetic nerves.

Kobayashi *et al.* [20] reported that in anesthetized rats, acupuncture produced variable responses in heart rate, i.e., tachycardia (18%), bradycardia (55%), and no response (27%). In the present study, we anesthetized rats by continuous infusion of pentobarbital [21, 22] and produced constant bradycardiac responses following acupuncture-like stimulation to the hind limb.

It was reported that acupuncture stimulation caused bradycardia in conscious humans [13–17]. Relative to the neural mechanisms of acupuncture-induced bradycardia, Sugiyama *et al.* [13] demonstrated that acupuncture decreases heart rate and increases muscle sympathetic nerve activity in conscious humans. From these results, they speculated that acupuncture also increases cardiac sympathetic nerve activity. They further speculated that acupuncture increases cardiac vagal nerve activity, and the vagal nerve plays a major role in the bradycardiac response. On the other hand, the acupuncture-induced bradycardia appears to be a result of a reciprocal coordination of an increase in cardiac vagal activity and a decrease in cardiac sympathetic activity, because the response was attenuated by the administration of atropine (muscarinic acetylcholine receptor blocker) and propranolol (β -adrenergic receptor blocker) [14]. It has been very difficult to prove true reflex heart rate responses following acupuncture in conscious humans because somatic sensory stimulation by acupuncture produces various emotional responses that are difficult to distinguish from true reflex responses; also, experimental techniques are limited to indirect methods such as the administration of autonomic blockers or analysis of heart rate variability. In the present study, we demonstrated for the first time that in anesthetized rats, cardiac sympathetic nerves mediate the efferent pathway of the acupuncture-like stimulation-induced bradycardiac response because (1) the bradycardiac response following acupuncture-like stimulation was not influenced by vagotomy, but abolished by stellectomy as reported before [20], and (2) the bradycardiac response was accompanied by a decrease in the activity of the cardiac sympathetic efferent nerves. In the present study, the vagal nerve did not appear to contribute to this bradycardia following acupuncture stimulation. From the present results, it can be inferred that acupuncture stimulation reduces the cardiac sympathetic nerve activity and produces bradycardia also in conscious humans. However, we could not overrule the possibility that anesthesia masked the vagal contribution because cardiac vagal efferent activity is strongly depressed by anesthesia [25].

In the present study, blood pressure was reduced following acupuncture-like stimulation, confirming a previous report [6]. Therefore it is clear that the bradycardiac response induced by acupuncture-like stimulation could not be a secondary effect of pressor response (i.e., baroreflex).

The present bradycardiac response following acupuncture-like stimulation of the hind limb was completely abolished by a severance of the femoral and sciatic nerves of the stimulated hind limb. These results confirmed those reported by Kobayashi *et al.* [20] and suggests that this bradycardiac response was elicited by hind limb somatic afferent nerve excitation. Furthermore, the acupuncture-like stimulation of the hind limb applied to the muscles

alone induced bradycardia, but stimulation to the skin alone was ineffective. Therefore muscle afferents have an important role in the afferent pathway of this bradycardiac response induced by acupuncture-like stimulation of the hind limb. Kobayashi *et al.* [20] reported that acupuncture-like stimulation produced bradycardiac responses in 55% of cases when both skin and muscle were stimulated, and in 22% and 70% when only skin and only muscle were stimulated, respectively. Their results that muscle stimulation tended to produce higher rates of bradycardia than cutaneous stimulation are consistent with our present results. In the present experiments, we could not observe significant responses following acupuncture-like stimulation to the skin alone. These differences may depend on the experimental anesthetic conditions. The importance of muscle afferent nerves for acupuncture-like stimulation was also reported by Ohsawa *et al.* [6] using anesthetized rats. They demonstrated that acupuncture-like stimulation to the muscles produced a depressor response, but stimulation to the skin did not. On the other hand, it was reported that both cutaneous and muscle afferents were important for producing reflex responses of gastric [4] and vesical motility [5] to acupuncture-like stimulation in anesthetized rats, though muscle afferents played a more dominant role. In the case of acupuncture-like stimulation of skin alone, a very small area of skin (equal to the thickness of an acupuncture needle) was stimulated. Therefore acupuncture-like stimulation to only skin excited a very small number of afferent nerve fibers and produced small responses or no response.

It has been recently reported that manual acupuncture needle stimulation to the hind limb activates afferent nerve fibers of all four groups (groups I–IV) of afferents in rats [26]. Thus it is thought that all groups of afferents were activated by acupuncture-like stimulation in our animals. In anesthetized animals, the electrical stimulation of groups I and II muscle nerves were ineffective [27–31], but the additional stimulation of group III nerves induced tachycardia or bradycardia [31, 32]. The tachycardiac response, which was induced by the stimulation of group III nerves, was changed to a small bradycardiac response by larger doses of anesthetic [31]. The additional stimulation of group IV muscle nerves of a hind limb always produced tachycardia [30, 31, 33]. From these facts, group III fibers of muscles seem to be responsible for the present bradycardiac responses following acupuncture-like stimulation in rats.

In traditional acupuncture, spots or points of acupuncture have been emphasized as important for effectiveness or responsiveness. However, some animal experiments show that the areas of stimulation effective in producing visceral or vascular responses are rather diffuse and not strictly localized in small spots [4, 5, 34]. Therefore it can be inferred that the stimulated point is not important in the present acupuncture-like stimulation-induced bradycardiac response.

In conclusion, the reflex decrease in the efferent discharge activity of the cardiac sympathetic nerve following acupuncture-like stimulation of hind limb is responsible for the bradycardiac response in anesthetized rats. The afferent pathway of present bradycardiac response is composed of hind-limb muscle afferent nerves.

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