INCREASED BLOOD FLOW IN SALIVARY glands plays an important role in salivary secretion, because salivary fluid is a mixture of water and ions created from blood plasma (20). Electrical stimulation of sensory nerves in the orofacial area induces salivary secretion, causing cholinergic and noncholinergic parasympathetic vasodilation in cat and rat submandibular glands (1, 10, 16). Vasoactive intestinal peptide (VIP) is thought to be a potent neurotransmitter of parasympathetic noncholinergic vasodilator fibers (9). Although blood vessels in the salivary glands are innervated by both parasympathetic and sympathetic nerves, it is the parasympathetic vasoactive nerve fibers that predominantly contribute to vasodilation under reflex conditions, whereas the sympathetic vasoactive nerve fibers are in a state of tonic control in these glands (20).

The submandibular and sublingual glands are major salivary glands located beneath the lower jaw and inferior to the tongue, respectively. The sublingual gland may differ from the submandibular gland in terms of control of hemodynamics because the submandibular gland mainly consists of serous acini, while the sublingual gland mainly consists of mucous acini (3). However, little is known about the involvement of parasympathetic nerves with the hemodynamics of the sublingual gland.

In the present study, we simultaneously analyzed blood flow in the submandibular gland (SMGBF) and sublingual gland (SLGBF) during electrical stimulation of the central cut end of the lingual nerve (LN) in deeply urethane-anesthetized, artificially ventilated, and cervically vago-sympathectomized rats using a laser speckle imaging flow meter.

MATERIALS AND METHODS

Animal preparation. The experiments were performed on male Wistar rats aged between 11 and 16 wk and weighing 325–490 g. Rats were kept in a 12:12-h light-dark cycle and allowed free access to food and water. After induction with ether inhalation, urethane (1 g/kg) was subcutaneously injected into the backs of the animals. A femoral vein and artery were cannulated to allow drug injection and monitoring of systemic arterial blood pressure (SAPB) and heart rate (HR), respectively. Rats were intubated, paralyzed by an intravenous injection of pancuronium bromide (Mioblock; Organon Teknika, Netherlands; 0.6 mg/kg initially, supplemented with 0.4 mg/kg every hour), and artificially ventilated with a 50:50 mixture of air and O2 via a tracheal cannula. The ventilator (model SN-480-7; Shinano, Tokyo, Japan) was set with a tidal volume of 5.0–7.5 cm³/kg at a rate of 18–20 breaths/min, and the end-tidal concentration of CO₂ was determined by an infrared analyzer (Capnomac Ultima; Datex, Helsinki, Finland), as previously described (7). Rectal temperature was maintained at 37°C with a heating pad. We checked that the depth of anesthesia was adequate by monitoring the absence of a flexion response to a noxious stimulus, such as pinching the digit for ~2 s. If the depth of anesthesia was considered inadequate, additional urethane (100 mg/kg iv) was administered. The skin covering the submandibular and sublingual glands was carefully removed to avoid injury to the nerve and vessels. At the end of the experiment, all rats were killed with an overdose (~100 mg) of pentobarbital sodium. The experimental protocol conformed to the Guidelines for the Care and Use of Laboratory Animals at the Health Sciences University of Hokkaido and was approved by the Animal Ethics and Research Committee (no. 074). All animals were cared for in accordance with the recommendations in the current National Research Council guide.

Laser speckle blood flow imaging. SMGBF and SLGBF on both sides were monitored using a laser speckle flow meter (Omegazone; Omegawave, Tokyo, Japan), which provides high-resolution two-dimensional blood flow images in a matter of seconds, as described previously (8, 18). As shown in Fig. 1A, the anesthetized animals were placed in a horizontal position, and the submandibular and sublingual glands were mounted on a black urethane pad and diffusely illuminated by a semiconductor laser (780 nm). The scattered light was filtered and detected by a charge-coupled device camera positioned using a laser speckle imaging flow meter.
Fig. 1. The effects of electrical stimulation of the central cut end of the left lingual nerve (LN) on blood flow of the submandibular gland (SMG) and the sublingual gland (SLG) on both sides. A: photograph showing the SMG and SLG on both sides mounted on a black urethane pad and a typical time series of speckle images at prestimulation (rest), 12 s, and 20 s after the start of LN stimulation. Regions of interest (ROIs) are shown as open squares on the left and right SMG and SLG. B: typical examples of the changes in blood flow (au, arbitrary units) and vascular conductance (VC) of SMG and SLG on both sides, and systemic arterial blood pressure (SABP). The LN was stimulated for 20 s with a supramaximal voltage of 20 V at 20 Hz using 2-ms pulses (LN stim.). C: mean data (± SE) of changes in VC of SLG (solid bars) and SMG (open bars) on both sides (n = 8). The statistical significance of the differences between changes in VC was assessed by ANOVA followed by Tukey’s test. *P < 0.01.
above the neck. The raw speckle images that corresponds to the number and velocity of moving red blood cells, were recorded and transferred to a computer for analysis. A color-coded blood flow image was obtained in the high-resolution mode (638×480 pixels; one image). One blood flow image was generated by averaging the numbers obtained from 20 consecutive raw speckle images. Averaged signals in the submandibular and sublingual glands in the region of interest (ROI) were obtained using the pallet software installed in the Omega3 imaging system (Omega3, Tokyo, Japan). The signals obtained by this method represent the blood flow in the superficial vessels of the submandibular and sublingual glands by demonstrating relative changes in blood flow; technical details and evaluation of the laser speckle imaging have been described in the study Basak et al. (2). Vascular conductance (VC) in the submandibular and sublingual glands was obtained by dividing the averaged signals from the ROI by the mean SABP.

**Electrical stimulation of the lingual nerve.** A central cut end of the left LN was electrically stimulated using a bipolar silver electrode attached to an electrical stimulator (model SEN-7103; Nihon Kohden, Tokyo, Japan). The LN was sectioned under a binocular microscope and unilaterally stimulated with various voltages (1–30 V) at various frequencies (1–30 Hz) for 20 s using 2-ms pulse durations at 15-min intervals in each rat. In all experiments, the cervical vagus and cervical sympathetic trunk were cut in the neck bilaterally. This ensured that only nonvagal parasympathetic effects were examined.

**Pharmacological agents.** All drugs were dissolved in sterile saline. To examine whether the increases in SMGBF and SLGBF evoked by electrical stimulation of LN were mediated via the autonomic nervous system and/or activation of muscarinic receptors and VIP receptors, the following substances were intravenously administered: hexamethonium bromide (autonomic ganglion cholinergic blockade), atropine sulfate (muscarinic cholinergic blockade), and the VIP receptor antagonist. The hexamethonium bromide (10 mg/kg; Sigma, St. Louis, MO) was injected in volumes of 0.05 ml. The administration of a similar volume of saline alone had no effect on SMGBF, SLGBF, or SABP (data not shown). The response evoked by LN stimulation was determined at least 5 min after administration of hexamethonium because changes in SMGBF, SLGBF, and SABP had reached a steady state at this time point. Atropine sulfate (0.1 mg/ml; Mitsubishi Tanabe, Osaka, Japan) and VIP receptor antagonist (0.2 mg/ml, AnyGen, Gwangju, Korea) were infused at a flow rate of 2 ml/h for 15 min using a syringe pump (Pump 11 Elite; Harvard, Holliston, MA). The responses evoked by LN stimulation were determined at least 10 min after the start of the infusion. The magnitude of the response evoked by LN stimulation after administration of each agent was expressed as a percentage of the VC of the control response recorded before its administration. The dose of hexamethonium chosen for the present study was 10 mg/kg because this dose showed a marked inhibition of SMGBF increase in a previous study (16). The continuous infusion of atropine sulfate (0.1 mg/ml, 2 ml/h) and VIP receptor antagonist (0.2 mg/ml, 2 ml/h) was sufficiently potent to inhibit the SMGBF and SLGBF increase evoked by LN stimulation via muscarinic and VIP receptors. This choice of dose was based on data from our preliminary experiment (data not shown) showing that the vasodilator system in the sublingual gland, time course analysis was performed. In the SLGBF increase, the time taken from peak to basal blood flow level was designated as the recovery time. The SLGBF increase was evoked by electrical stimulation of the central cut end of the left LN for 20 s with 20 V at 20 Hz using 2-ms pulses, and intravenous administration of ACh bromide (100 ng/kg; Sigma-Aldrich) or VIP (10 ng/kg; AnyGen, Gwangju, Korea). Atropine sulfate (1–100 μg/kg; Mitsubishi Tanabe, Osaka, Japan) was intravenously administered in volumes of 0.1 ml. The administration of a similar volume of saline alone had no effect on SMGBF, SLGBF, or SABP (data not shown). The recovery time of the SLGBF increase evoked by LN stimulation in the presence of atropine and together with administration of ACh bromide or VIP was compared with the control response evoked by LN stimulation alone.

**Statistical analysis.** All numerical data are presented as means ± SE. The statistical significance of changes in response was assessed by a paired/unpaired t-test or one/two-way ANOVA followed by Tukey’s test. Differences were considered significant at P < 0.05. Data were analyzed using a Windows computer with SPSS ver. 19.

**RESULTS**

**Effect of LN stimulation on SMGBF, SLGBF, VC, and SABP.** Figure 1 shows the effects of electrical stimulation of the left LN on blood flow (in arbitrary units, au) and VC of submandibular and sublingual glands on both sides, and SABP. When the left LN was electrically stimulated for 20 s with 20 V at 20 Hz using 2-ms pulses, increases in SMGBF and SLGBF were observed predominantly on the left side (Fig. 1, A and B). The changes in VC on the left and right sides of the submandibular gland were 0.38 ± 0.04 and 0.03 ± 0.01, and of the sublingual gland were 0.23 ± 0.03 and 0.05 ± 0.02, respectively (Fig. 1C). Statistically significant differences in the increases in SMGBF and SLGBF were observed between the left and right sides and also between the left side before and after left LN stimulation [F (3, 28) = 18.6, n = 8 in each group, P < 0.01; Fig. 1C]. Mean SABP increased significantly from 95.2 ± 3.3 before LN stimulation to 134.4 ± 5.9 mmHg during LN stimulation (n = 8 in each group, P < 0.01, paired t-test). HR increased only slightly from 383 ± 13/min before LN stimulation to 386 ± 11/min during stimulation; this change was not significant (n = 8 in each group, NS, paired t-test).

Figure 2 shows the mean data (±SE) of the changes in the VC of the submandibular and sublingual glands on the left side following left LN stimulation at various intensities (1–30 V; Fig. 2A) and frequencies (1–30 Hz; Fig. 2B) in each rat undergoing nonpharmacological treatment. Significant changes in the VC of the submandibular and sublingual glands took place above 5 V [for submandibular gland, F (5, 29) = 118.7, n = 6 in each group, P < 0.01; for sublingual gland, F (5, 30) = 11.8, n = 6 in each group, P < 0.01; Fig. 2A]. A statistically significant difference was revealed between changes in the VC of the submandibular and sublingual glands above 5 V (n = 6 in each group, P < 0.01; Fig. 2A). Left LN stimulation at 20 V exerted a significant effect on changes in the VC of the submandibular and sublingual glands on the left side above 5 and 10 Hz, respectively [for submandibular gland, F (6, 35) = 47.8, n = 6 in each group, P < 0.01; for sublingual gland, F (6, 35) = 4.1, n = 6 in each group, P < 0.05; Fig. 2B]. A statistically significant difference was revealed between changes in the VC of the submandibular and sublingual glands above 10 Hz (n = 6 in each group, P < 0.01; Fig. 2B).

**Effect of chemical reagents on the SMGBF and SLGBF increase evoked by LN stimulation.** Figure 3 shows the effects of intravenous administration of hexamethonium, atropine, and VIP receptor antagonist on the SMGBF and SLGBF increase evoked by electrical stimulation of the left LN for 20 s with 20 V at 20 Hz using 2-ms pulses. LN stimulation induced increases in SMGBF and SLGBF. The changes in the VC of the
submandibular gland after administration of hexamethonium and infusion of atropine in the absence or presence of VIP receptor antagonist, expressed as a percentage of the control response, were 13.5 ± 3.0, 5.5 ± 5.6, and 7.9 ± 4.7%, respectively (Fig. 3, A, B, and E) and were revealed to be significant [F (4, 30) = 86.5, n = 5 in each group, P < 0.01; Fig. 3E]. Infusion of VIP receptor antagonist alone had no effect on SMGBF increase (109.6 ± 8.2%; Fig. 3, C, D, and E). On the other hand, the changes in the VC of the sublingual gland after administration of hexamethonium and after infusion of atropine in the absence or presence of VIP receptor antagonist, expressed as a percentage of the control response, were 29.4 ± 4.2, 58.6 ± 5.9, and 24.1 ± 9.2%, respectively (Fig. 3, A, B, and E). Infusion of VIP receptor antagonist alone had no effect on the SLGBF increase (110.8 ± 7.2%; Fig. 3, C, D, and E).

Significant changes in VC after administration of hexamethonium and after administration of atropine in the absence or presence of VIP receptor antagonist were revealed [F (4, 30) = 49.2, n = 5 in each group, P < 0.01; Fig. 3E]. The inhibitory effect on the SLGBF increase was greater for atropine in the presence of VIP receptor antagonist than for atropine alone (P < 0.05, ANOVA followed by a Tukey’s test; Fig. 3E).

Additionally, the inhibitory effect of hexamethonium or atropine was greater on the SMGBF increase than on the SLGBF increase (110.8 ± 11.9, n = 6 in each group, P < 0.01; Fig. 4B).

**FIG. 4** shows the time course analysis of blood flow increase in the sublingual gland evoked by electrical stimulation of the left LN for 20 s with 20 V at 20 Hz using 2-ms pulses, and intravenous administration of ACh or VIP. The administration of ACh (100 ng/kg) or VIP (10 ng/kg) induced increases in SLGBF (Fig. 4A). The changes in the VC after administration of ACh or VIP, expressed as a percentage of the response evoked by LN stimulation, were 98.4 ± 7.8% and 108.2 ± 16.2%, respectively; however, these changes were not significant (n = 6 in each group, ANOVA). The recovery time to the basal blood flow level was shorter for ACh (69.4 ± 7.5 s) and longer for VIP (338.3 ± 43.3 s) than it was for LN stimulation (139.7 ± 14.9 s; Fig. 4B).

The recovery times after LN stimulation with administration of 1, 10, and 100 µg/kg atropine were 198.9 ± 28.2, 242.6 ± 32.8, 292.8 ± 20.6 s, respectively (Fig. 4, B). There were significant differences in recovery time after administration of ACh, VIP, and LN stimulation in the presence of atropine (100 µg/kg), compared with LN stimulation alone [F (5, 35) = 11.9, n = 6 in each group, P < 0.01; Fig. 4B].

**DISCUSSION**

Lingual nerve stimulation elicited intensity- and frequency-dependent increases in SMGBF and SLGBF in all of the animals examined in the present study (Figs. 1 and 2). Although there was a significant increase in SABP during LN stimulation, the increases in SMGBF and SLGBF observed in the present study appeared not to be secondary to changes in the SABP, because the increases in SMGBF and SLGBF on the ipsilateral side were significantly larger than those on the contralateral side (Fig. 1). Furthermore, there were no significant differences between HR before and during LN stimulation. These results suggest that the increases in SMGBF and SLGBF caused by LN stimulation is not a passive result of any evoked blood pressure change and that it is justified to refer to it as “vasodilation”.

Reflex saliva is secreted by various sensory inputs from the orofacial region during feeding, and contributes to chewing, swallowing, and taste (4). The significant increases in SMGBF and SLGBF in the present study elicited by the electrical stimulation of LN, which supplies sensory innervation to the tongue, including taste, suggest its importance in salivary secretion during feeding.

The increase in SMGBF was higher than that of SLGBF when LN was stimulated at high intensities or frequencies (Figs. 1 and 2). Larger amounts of saliva were secreted by the submandibular glands compared with the sublingual glands during periods of acidic or mechanical stimulation (6). Electrical stimulation of sensory nerve in the orofacial region elicits intensity- and frequency-dependent salivary secretion, as well as blood flow increase in the salivary gland (10, 22). Thus, although the precise reasons for the difference in the vasodilator activity in response to LN stimulation between the submandibular and sublingual glands are unclear, it seems likely to involve differences in the mechanism of salivary secretion between the two glands.

The SMGBF and SLGBF increases were significantly inhibited by intravenous administration of the autonomic cholinergic ganglion blocker hexamethonium (Fig. 3, A, B, and E). The antimuscarinic agent atropine markedly inhibited the...
Fig. 3. The effect of intravenous administration of atropine (Atr), vasoactive intestinal peptide antagonist (VIPa), and hexamethonium (C6) on blood flow increase of SMG and SLG evoked by electrical stimulation of the central cut end of the left LN. A: typical speckle images of the SMG and SLG on the left side at the maximal phase of blood flow increase evoked by LN stimulation with and without continuous infusion of Atr (0.1 mg/ml, 2 ml/h) and VIPa (0.2 mg/ml, 2 ml/h). The ROIs are shown as open squares on the SMG and SLG. B: typical examples of changes in blood flow (au, arbitrary units) and, VC of the SMG and SLG on the left side, and SABP. The LN was stimulated for 20 s with a supramaximal voltage of 20 V at 20 Hz using 2-ms pulses. C: typical speckle images of the SMG and SLG on the left side at the maximal phase of blood flow increase evoked by LN stimulation with and without continuous infusion of VIPa (0.2 mg/ml, 2 ml/h). D: typical examples of changes in blood flow and VC of the SMG and SLG on the left side, and SABP. The LN was stimulated for 20 s with a supramaximal voltage of 20 V at 20 Hz using 2-ms pulses. E: mean data (± SE) of changes in the VC of the SLG (solid bars) and SMG (open bars) (n = 5). The changes in VC of the SLG and SMG evoked by LN stimulation (control) were taken as 100%. The statistical significance of the differences was assessed by ANOVA followed by Tukey’s test. *P < 0.05, vs. control. **, †P < 0.01, vs. control.
SMGBF increase and partly inhibited the SLGBF increase (Fig. 3, A, B, and E). Our results indicate that 1) the increases in SMGBF and SLGBF caused by LN stimulation is predominantly evoked via parasympathetic nerves; and 2) the SMGBF increase is mainly evoked by cholinergic vasomotor fibers, while the SLGBF increase is evoked by cholinergic and non-cholinergic vasomotor fibers. The atropine-resistant SLGBF increase was significantly inhibited by infusion of VIP receptor antagonist in the presence of atropine, although administration of VIP receptor antagonist alone had no effect (Fig. 3). This suggests that a noncholinergic SLGBF increase is evoked via VIP receptors when the muscarinic receptors are blocked. Furthermore, it is possible that there is another pathway apart from the autonomic nerves involved in the SMGBF and SLGBF increase caused by LN stimulation, because the blood flow increase caused by LN stimulation was not completely inhibited, especially in the sublingual gland (Fig. 3, A, B, and E). In the time course analysis of blood flow increase in the sublingual gland, the recovery time after LN stimulation was significantly delayed by administration of atropine in a dose-dependent manner to the same level as the SLGBF increase evoked by administration of VIP (Fig. 4, A and B). Because ACh and VIP produce vasodilation via different underlying mechanisms (15, 21), the recovery time in the blood flow increase evoked by administration of ACh and VIP may differ, although the precise mechanisms are still unclear. Thus, our data suggest that the mechanism of the SLGBF increase evoked by LN stimulation changes from cholinergic to VIP-

**Fig. 4.** Time course analysis of blood flow increase in the SLG evoked by electrical stimulation of the central cut end of the left LN. A: typical examples of changes in blood flow in the sublingual gland (SLGBF; au), vascular conductance (VC), and SABP after LN stimulation with and without intravenous administration of atropine (Atr) (1–100 µg/kg), and intravenous administration of ACh (100 ng/kg) or VIP (10 ng/kg). The LN was stimulated for 20 s with a supramaximal voltage of 20 V at 20 Hz using 2-ms pulses. B: mean data (± SE) of recovery time in LN stimulation (solid bar), ACh administration (open bar), LN stimulation with atropine treatment (hatched bar), and VIP administration (shaded bar) (n = 7). Statistical significance of the differences was assessed by ANOVA followed by Tukey’s test. *P < 0.05, **P < 0.01, vs. control.
MACHINERY TO CONTROL HEMODYNAMICS IN SALIVARY GLANDS

**AUTHOR CONTRIBUTIONS**

Author contributions: T.S. and H.I. conception and design of research; T.S. performed experiments; T.S. analyzed data; T.S. and H.I. interpreted results of experiments; T.S. prepared figures; T.S. drafted manuscript; T.S. and H.I. approved final version of manuscript; H.I. edited and revised manuscript.

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