Nitric oxide modulates elicitation of reflex swallowing from the pharynx in rats

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Kijima, Hiroshi, Tomio Shingai, Yoshihiro Takahashi, Yuka Kajii, Shin-ichi Fukushima, Yo Taguchi, Tadashi Noda, and Yoshiaki Yamada. Nitric oxide modulates elicitation of reflex swallowing from the pharynx in rats. Am J Physiol Regul Integr Comp Physiol 291: R651–R656, 2006. First published April 6, 2006; doi:10.1152/ajpregu.00646.2005.—The pharynx is very important for elicitation of reflex swallowing. The region of the pharynx is innervated by the pharyngeal branch of the glossopharyngeal nerve (GPN-ph). Nitric oxide (NO) plays an important role in various physiological functions. The purpose of this study is to investigate the contribution of NO to reflex swallowing evoked by electrical stimulation of the GPN-ph. Swallowing was evoked in urethane-anesthetized rats by application of repetitive electrical stimulation (10- to 20-μA amplitude, 10- to 20-Hz frequency, 1.0-ms duration) to the central cut end of the GPN-ph or superior laryngeal nerve. Swallowing was identified by electromyographic activity of the mylohyoid muscle. Latency to the first swallow and the interval between swallows were measured. Intravenuous administration of Nω-nitro-L-arginine (L-NNA, 0.6 mg/kg), a nonselective inhibitor of NO synthase (NOS), extremely prolonged latency to the first swallow and the interval between swallows evoked by the GPN-ph. Intraperitoneal administration of 7-nitroindazole (5.0 mg/kg), a selective inhibitor of neuronal NOS, significantly prolonged latency to the first swallow and the interval between swallows evoked by the GPN-ph. Administration of L-arginine (an NO doner, 500 mg/kg) and sodium nitroprusside (an NO releaser, 0.6 mg/kg) restored the suppression of swallowing induced by the NOS inhibitor. Superior laryngeal nerve-evoked swallowing was suppressed by administration of a higher dose of L-NNA (6.0 mg/kg). Swallowing evoked by water stimulation of the pharynx was also suppressed by L-NNA (0.6 mg/kg). These results suggest that NO plays an important role in signal processing for initiation of reflex swallowing from the pharynx.

nitric oxide synthase; pharyngeal branch; glossopharyngeal nerve; superior laryngeal nerve; L-arginine

THE PHARYNX IS VERY IMPORTANT for elicitation of reflex swallowing. The regions of the pharynx involved in initiation of reflex swallowing have been analyzed in many studies (5–7, 15, 17, 21, 24–28). One study showed that, in the cat, the posterior pillars are the most reflexogenic to mechanical stimulation, and the posterior pharyngeal wall is slightly less sensitive (27). Recently, Kitagawa et al. (17) examined the receptive regions for swallowing in the rat and reported that the palatopharyngeal arches, the posterior pharyngeal wall, the edge of the soft palate, the epiglottis, and the aryepiglottic fold are the most sensitive areas for reflex swallowing. On the basis of a study by Pomerenke (24), the receptive regions for swallowing in humans are considered to be essentially similar to those in animals.

The effective regions for elicitation of reflex swallowing are innervated by the glossopharyngeal nerve (GPN) and the superior laryngeal nerve (SLN) (6, 14, 20, 21, 26, 28, 30). The SLN has been well known as the most important afferent nerve for initiation of reflex swallowing from the laryngeal region, i.e., the epiglottis and arytenoid region (21). On the other hand, the role of the GPN in reflex swallowing has not been demonstrated, because it is much more difficult to evoke swallowing by stimulation of the GPN than by stimulation of the SLN (6, 28). Recently, Kitagawa et al. (17) clearly demonstrated that the pharyngeal, not the lingual, branch of the GPN plays a major role in the initiation of reflex swallowing from the pharynx, i.e., the palatopharyngeal arches, the posterior pharyngeal wall, and the edge of the soft palate.

El-Haddad et al. (8) showed that inhibition of central nitric oxide (NO) reduces spontaneous fetal swallowing. They speculated that NO acts as a neuromodulator of fetal swallowing. NO, a free radical gas produced by L-arginine (L-Arg) in the presence of NO synthase (NOS) in many tissues and cells, including those of the central nervous system, plays an important role in various physiological functions (1, 2, 11). Furthermore, histochemical studies have provided evidence in cats (19, 22), rats (31, 32), rabbits (10), and humans (18) that NOS is localized in the nucleus of the solitary tract (NTS), the nucleus ambiguus, and the dorsal motor nucleus of the vagus, which are believed to be responsible for the central control of swallowing (12, 13).

To reveal the contribution of NO to initiation of reflex swallowing from the pharynx, the present study was designed to examine the effects of NOS inhibition and NO application on reflex swallowing evoked by electrical stimulation of the pharyngeal branch of the GPN (GPN-ph). We present a new finding: NO plays an important role in signal processing for the initiation of reflex swallowing from the pharynx.

METHODS

The experimental protocols were approved by the Intramural Animal Care and Veterinary Science Committee of Niigata University. Fifty-four male Wistar rats (Charles River Japan, Yokohama, Japan; 250–400 g body wt) were anesthetized with urethane (1.0 g/kg ip; Sigma, St. Louis, MO) and fixed in the supine position. Body temperature was maintained at 37°C with a heating pad. A longitudi-
dinal midline incision was made in the ventral surface of the neck. The trachea was cannulated to maintain respiration. A catheter was placed into the femoral vein for administration of drugs. Paired unipolar enamel-nichrome wire electromyographic (EMG) electrodes were placed in the mylohyoid muscle to record EMG activity. The muscle was recognized as an “obligate muscle” involved in swallowing (7). Swallowing was identified by EMG activity of the mylohyoid muscle and by visual observation of laryngeal movement. After these procedures were performed, the following two experiments (electrical stimulation and water stimulation) were carried out.

**Experiment 1: electrical stimulation of the nerves.** In 48 rats, SLNs were bilaterally exposed by blunt dissection of the sternothyroid muscle. GPNs were bilaterally exposed by removal of the digastric muscles and posterior horn of the hyoid bones. GPNs were then dissected free from the surrounding tissue bilaterally. The lingual and pharyngeal branches of the GPN were carefully bilaterally isolated. Figure 1A shows a schematic diagram of our experiment. After the GPN-ph and SLN were bilaterally severed, bipolar platinum wire electrodes were unilaterally fitted onto the central cut end of the GPN-ph or SLN to stimulate these nerves. The nerves were stimulated by repetitive electrical impulses with a rectangular pulse (10- to 20-μA intensity, 10- to 20-Hz frequency, 1.0-ms duration) to evoke reflex swallowing. The stimulus intensity and frequency were adjusted so that swallowing was induced five or six times during the stimulation period. Latency to the first swallow was defined as the time required to evoke the first swallow from the onset of electrical stimulation. The interval between the first and third swallows was also measured. This interval divided by 2 was considered to be the mean interval between swallows (Fig. 1B).

The following drugs were used: Nω-nitro-L-arginine (l-NNA; Cayman Chemical, Ann Arbor, MI), a nonselective inhibitor of NOS; 7-nitroindazole (7-NI; Sigma), a selective inhibitor of neuronal NOS (nNOS); l-Arg (Nacalai Tesque, Kyoto, Japan), an NO donor; and sodium nitroprusside (SNP; ICN Biomedicals, Aurora, OH), an exogenous NO releaser. 7-NI was suspended in PBS solution with a few drops of DMSO and administered intraperitoneally. The other drugs were dissolved in saline and administered intravenously.

In 28 rats, l-NNA (0.6 mg/kg, n = 14) or 7-NI (5.0 mg/kg, n = 14) was administered, and electrical stimulations were applied to the GPN-ph and SLN every 5 min for 30 min. Then l-Arg (500 mg/kg, n = 16) or SNP (0.6 mg/kg, n = 12) was administered, and the GPN-ph was stimulated again. In 12 rats, saline (n = 6) or PBS solution (n = 6) was administered, and electrical stimulations were applied, as described above. Latency to the first swallow and the mean intervals between evoked swallows for the GPN-ph and SLN after administration of each drug were compared with those before administration of each drug. Effects of NOS inhibitors and their solvents were also compared.

In eight rats, l-NNA (6.0 mg/kg) was administered, and electrical stimulation was applied to the SLN to evoke reflex swallowing. The effect of l-NNA was investigated, as described above.

**Experiment 2: water stimulation of the pharynx.** Experiments were performed on six male rats as described previously by Kajit et al. (15). Briefly, after the SLNs were bilaterally exposed and cut, stimulating solution was applied to the pharyngeal region through the infusion tube, the tip of which was placed precisely flush to the end of the soft palate. Distilled water was applied to the pharynx at a flow rate of 3.0 μl/s by an infusion pump. After l-NNA (0.6 mg/kg) was administered intravenously, the number of swallows evoked in 10 s was compared with the number evoked before administration of l-NNA.

The paired t-test and Student’s t-test were used for statistical analysis. In the water stimulation experiment, P < 0.05 was considered to be a statistically significant difference. In the electrical stimulation experiment, P < 0.025 indicated statistical significance after multiple comparisons using Bonferroni’s correction.

**RESULTS**

**Experiment 1: electrical stimulation of the nerves.** Figure 2 shows the effect of l-NNA (0.6 mg/kg) on reflex swallowing elicited by stimulation of the GPN-ph and SLN. Before l-NNA administration, reflex swallowing evoked by stimulation of the GPN-ph was characterized by sequential swallowing and by almost regular intervals between swallows. After the stimulation was stopped, no swallowing occurred. Latency to the first swallow for the GPN-ph stimulation was 0.85 s, and the mean interval between swallows was 1.06 s. The pattern of sequential swallowing elicited by stimulation of the SLN was similar to that elicited by stimulation of the GPN-ph: latency to the first swallow was 0.71 s and the mean interval between swallows was 0.86 s. However, after l-NNA administration, for the GPN-ph stimulation, latency to the first swallow increased and the mean interval between swallows increased with the passage of time. Latency to the first swallow shifted to 1.46 s and the interval between swallows shifted to 1.95 s at 15 min after l-NNA administration. At 30 min after l-NNA administration, latency to the first swallow was increased and the interval between swallows was extremely prolonged. Latency to the first swallow reached 3.30 s (∼4 times preadministration latency), and the interval between swallows reached 3.68 s (∼3.5 times preadministration value). The regularity of elicitation of swallowing disappeared when the suppressive effect of l-NNA on swallowing was exerted. On the other hand, l-NNA did not influence reflex swallowing evoked by stimulation of the SLN even 30 min after administration.

Figure 3 shows the effects of l-NNA (0.6 mg/kg) on latency to the first swallow and the mean interval between swallows evoked by electrical stimulation of the GPN-ph 30 min after administration. Latency to the first swallow increased signifi-
cantly: from 0.84 s before to 3.10 s after L-NNA. On the other hand, latency to the first swallow for saline, a solvent of L-NNA, did not change: 0.76 s before and 0.86 s at 30 min after administration. The mean interval between swallows for the GPN-ph 30 min after administration of L-NNA was significantly prolonged (from 1.02 to 3.27 s), whereas the interval between swallows for saline was not varied (0.95 s before and 1.22 s at 30 min after administration). L-NNA had a significant effect on latency to the first swallow and the mean interval between swallows compared with saline.

Because L-NNA is a nonselective inhibitor of NOS, it could be supposed that nNOS and/or endothelial NOS was involved in this suppression of reflex swallowing. To investigate this possible involvement, a selective inhibitor of nNOS, 7-NI, was used.

Figure 4 shows the effects of 7-NI on latency to the first swallow and the mean interval between swallows evoked by electrical stimulation of the GPN-ph 30 min after administration. Latency to the first swallow for the GPN-ph was significantly increased 30 min after administration of 7-NI: from 0.84 to 2.66 s. On the other hand, latency to the first swallow for PBS, a solvent of 7-NI, was not changed: 0.80 s before and 1.01 s at 30 min after administration. The mean interval between swallows 30 min after administration of 7-NI was also significantly prolonged: from 1.21 to 3.25 s. On the other hand, the mean interval between swallows for PBS was not altered 1.12 s before and 1.34 s at 30 min after administration. 7-NI significantly affected latency to the first swallow and the mean interval between swallows compared with PBS.

Figure 5 shows the effects of L-NNA (0.6 mg/kg) and 7-NI on reflex swallowing elicited by electrical stimulation of the SLN. Latency to the first swallow was not altered by L-NNA or 7-NI. Latency to the first swallow 30 min after administration of L-NNA shifted only slightly (from 0.64 to 0.83 s) and that for 7-NI barely shifted (from 0.72 to 0.83 s). The mean interval between swallows before and 30 min after administration of L-NNA or 7-NI was not changed. The mean values shifted only slightly from 0.93 to 1.13 s by administration of L-NNA and from 0.85 to 1.05 s by 7-NI.

To investigate whether NO facilitates swallowing, despite suppression by an NOS inhibitor, L-Arg or SNP was administered 30 min after administration of the NOS inhibitor.

Table 1 shows the effects of L-Arg and SNP on the suppression of swallowing induced by L-NNA and 7-NI. Reduction of latency to the first swallow and/or shortening of the mean...
interval between swallows were considered facilitation of swallowing. When L-Arg was administered under the condition of suppressed reflex swallowing for the GPN-ph, elicitation of reflex swallowing was facilitated in almost all cases. Facilitation was observed in six of eight rats treated with L-NNA and in seven of eight rats treated with 7-NI. In some cases, latency to the first swallow and the interval between swallows after administration of L-Arg were restored to values almost equal to those before administration of L-NNA or 7-NI. The facilitative effects of L-Arg were exerted within a few minutes after administration and continued for~10 min. In the other three rats, no change was observed in reflex swallowing after administration of L-Arg.

SNP was administered in the same manner as L-Arg. When SNP was used after L-NNA was administered, latency to the first reflex swallow and the interval between reflex swallows for the GPN-ph were reduced in all six trials. This effect was exerted immediately and disappeared instantly. Similarly, administration of SNP after 7-NI shortened the latency to the first reflex swallow and the interval between reflex swallows in all six trials.

Although neither NOS inhibitor had an effect on swallowing evoked by the SLN, it was unclear whether those responses were dose dependent. To confirm the effectiveness of NOS inhibitors in SLN-evoked reflex swallowing, we used a higher dose of an NOS inhibitor.

Figure 6 compares latency to the first swallow and the interval between swallows for the SLN before and 30 min after administration of L-NNA (6.0 mg/kg). Latency to the first swallow for the SLN was significantly increased 30

Fig. 4. Effect of 7-nitroindazole (7-NI, 5.0 mg/kg) on latency to the 1st swallow (top) and mean time interval between swallows (bottom) evoked by electrical stimulation of GPN-ph 30 min after administration. Values are means ± SE (n = 6 for PBS, n = 14 for 7-NI). *P < 0.025 vs. before 7-NI (by paired t-test). #P < 0.025 vs. PBS (by Student’s t-test).

Fig. 5. Effects of L-NNA (0.6 mg/kg) and 7-NI (5.0 mg/kg) on latency to the 1st swallow and mean interval between swallows evoked by electrical stimulation of SLN before and 30 min after administration. Values are means ± SE (n = 14).

Fig. 6. Effects of L-NNA (6.0 mg/kg) on latency to 1st swallow and mean interval between swallows evoked by electrical stimulation of SLN 30 min after administration. Values are means ± SE (n = 8). *P < 0.025 vs. before L-NNA.

Table 1. Effects of L-Arg and SNP on suppression of swallowing induced by L-NNA and 7-NI

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Values represent number of trials. Swallowing was evoked by electrical stimulation of the pharyngeal branch of the glossopharyngeal nerve (GPN-ph). Facilitation, trial in which latency to swallowing was reduced and/or mean interval between swallows was shortened. L-NNA, N⁶-nitro-L-arginine; 7-NI, 7-nitroindazole; SNP, sodium nitroprusoide (0.6 mg/kg); L-Arg, L-arginine (500 mg/kg).
elicited by water stimulation for 10 s before and after administration of L-NNA or 7-NI. These results indicate that reflex swallowing is suppressed by the inhibitory effect of L-NNA or 7-NI. Although no clinical data on the contribution of NO to reflex swallowing in humans have been reported, we expect that further investigation based on the present results may finally lead to an elucidation of the mechanism underlying swallowing disorders.

In conclusion, this study demonstrates that inhibition of NOS suppresses the elicitation of reflex swallowing evoked by electrical stimulation of the GPN-ph, suggesting that NO plays a significant role in the nervous system of reflex swallowing.
an important role in the initiation of reflex swallowing from the pharynx. The mechanism underlying this action of the NO system is unclear but may be associated with NMDA-NO pathways within the NTS.

GRANTS
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REFERENCES