Lower Esophageal Sphincter Is Achalasic in nNOS$^{-/-}$ and Hypotensive in W/W^{v} Mutant Mice

DIGAVALLI V. SIVARAO, HIROSHI L. MASHIMO, HEMANT S. THATTE, and RAJ K. GOYAL

Center for Swallowing and Motility Disorders, Department of Veterans Affairs Medical Center, West Roxbury, and Harvard Medical School, Boston, Massachusetts

Background & Aims: **It has been proposed that nitrergic nerves mediate lower esophageal sphincter (LES) relaxation with intramuscular interstitial cells of Cajal (ICC-IM) as an intermediary. Dysfunction of the nitrergic pathway has been shown to cause LES hypertension and impaired relaxation in achalasia. We determined whether mice with neuronal nitric oxide synthase gene disruption (nNOS$^{-/-}$) and W/W^{v} mice lacking ICC-IM have achalasia-like LES dysfunction.** ***Methods:*** **Intraluminal manometry using a customized micro-sized catheter assembly was performed in anesthetized mice. Basal LES pressure and swallow- and vagal-evoked LES relaxations were quantified in wild-type, *N*ω-nitro-L-arginine methyl ester HCl salt (L-NAME)–treated, nNOS$^{-/-}$, and W/W^{v} mice.** ***Results:*** **Wild-type mouse LES maintained a basal pressure (24 ± 3 mm Hg; N = 8) and relaxed normally to swallow (87% ± 3%; N = 8) and vagal stimulation (91% ± 4% mm Hg; N = 6). Pretreatment with L-NAME (100 mg/kg, intravenously) attenuated LES relaxation to both stimuli ($P < 0.05$). The LES in nNOS$^{-/-}$ was significantly hypertensive (36 ± 5 mm Hg; N = 10; $P < 0.05$) with a markedly impaired relaxation ($P < 0.05$). In contrast, W/W^{v} mouse LES was significantly hypotensive (11 ± 2 mm Hg; N = 6; $P < 0.05$) with normal relaxation that was blocked by L-NAME.** ***Conclusions:*** **nNOS$^{-/-}$ mice have LES hypertension with impaired relaxation resembling achalasia. In contrast, W/W^{v} mice have hypotensive LES with unimpaired relaxation, suggesting that ICC-IM do not play a role in nitrergic neurotransmission.**

Lower esophageal sphincter (LES) maintains a basal tone and relaxes with swallowing that is caused by activation of inhibitory nonadrenergic noncholinergic nerves.[1] Nitric oxide (NO) has been shown to be a major, if not the sole, inhibitory neurotransmitter of nonadrenergic noncholinergic nerves,[2] and intramuscular type of interstitial cells of Cajal (ICC-IM) have been proposed to be the conveyer of these inhibitory signals to the sphincteric smooth muscle cells.[3,4] Accordingly, chemical blockers of NO synthase (NOS),[5,6] genetically engineered deficiency of neuronal NOS (nNOS),[7] and genetic deficiency of ICC-IM[8] have all been reported to impair relaxation of LES muscle strips to transmural stimulation in vitro.

In the in vivo experimental condition, the role of NO in LES function is less clear. In intact animals, NOS blockers such as *N*ω-nitro-L-arginine methyl ester HCl salt (L-NAME) or *N*ω-nitro-L-arginine (L-NNA), or NO scavenger hemoglobin, have been reported either to increase or not affect the basal LES pressure while significantly suppressing swallow-induced LES relaxation.[9–12] In the rat, vagal stimulation–evoked LES relaxation was only partly affected by L-NAME.[13] In humans, NOS inhibitor N^{G}-momomethyl-L-arginine (L-NMMA) or NO scavenger hemoglobin has been reported to either increase or not change the basal LES pressure.[14–16] Inhibition of NOS was found to cause suppression of LES relaxation to swallow in 1 of the 2 published human studies.[14,16] Hirsch et al.[14] on the other hand, reported that L-NMMA does not affect basal pressure or swallow-induced relaxation of the LES, but that it primarily suppressed transient LES relaxation via a centrally mediated mechanism.

Thus, in vivo LES function associated with inhibition of NOS activity does not fully correlate with either the in vitro studies that show consistent and marked suppression of LES relaxation, or with the clinical studies that suggest that impaired nitrergic neurotransmission is responsible for LES hypertension and absence of swallow-induced LES relaxation in achalasia.[17,18] Moreover, LES tissue in achalasia has been shown to have deficiency of NOS[18] and abnormal ICC-IM.[17] The reason for these differences is not clear. They may be related to inadequate suppression of NOS activity by NOS inhibitors in

Abbreviations used in this paper: ICC-IM, intramuscular interstitial cells of Cajal; LES, lower esophageal sphincter; L-NAME, *N*ω-nitro-L-arginine methyl ester HCl salt; L-NMMA, N^{G}-momomethyl-L-arginine; L-NNA, *N*ω-nitro-L-arginine; MAP, mean arterial pressure; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; PBS, phosphate-buffered saline; WT, wild-type.

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vivo either because of insufficient dose or pharmacologic profile of the NOS inhibitor used. These problems could be overcome by investigating LES function in animals with genetic deficiency of nNOS or ICC-IM. However, such studies are currently unavailable, partly because the best-defined genetic models of nNOS and ICC-IM deficiency are strains of mice, and mouse has not been established as an animal model to study LES function in vivo.

The purpose of the present study was to: (1) examine the suitability of anesthetized mice for studying LES function in vivo; (2) examine basal tone, swallow-induced, and efferent vagal stimulation–induced LES relaxation in genetically engineered nNOS$^{-/-}$ mice and compare the results with those from L-NAME–treated wild-type (WT) mice; and (3) examine LES function in W/W^{v} mice and compare it with that in nNOS deficiency.

Materials and Methods

General

Site-bred C57BL/6J mice of either sex (20–30 g) were used as control subjects. The site-bred nNOS$^{-/-}$ mice, whose origin was reported previously,[19] were of either sex and weighed between 23 and 26 g. Although these mice lack the membrane-associating nNOS α-isoform, they still contain soluble β and γ isoforms that account for some residual activity.[20] Adult W/W^{v} male mice weighing between 23 and 29 g were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were housed 5 animals per cage and allowed unlimited access to food and water and were maintained at 25°C in a 12-hour light/dark cycle. The mice were denied food but not water overnight before surgery.

Surgical

The Institutional Animal Use Review Committee of the Brockton/West Roxbury VA Medical Center approved all experimental procedures. Mice were anesthetized with pentobarbital (50 mg/kg, intraperitoneally [IP]), and core body temperature was maintained at 36 $\pm$ 1°C using a commercial heating pad. A midline incision was made below the mandible, and the left jugular vein was cannulated with PE10 tubing (Becton Dickinson, Parsippany, NJ) for intravenous (IV) infusions. The left carotid artery was cannulated using PE10 tubing connected to a BP-100 pressure transducer (CB Science, Dover, NH) for continuous mean arterial pressure (MAP) recording. In some animals, the left vagus that runs close to the left carotid artery was isolated and tied loosely with a silk thread and transected proximal to the knot such that the peripheral stump could be manipulated using the thread. The trachea was cannulated using a 1.5-cm-long PE50 tubing, and in general, animals breathed spontaneously.

Manometry

Intraluminal esophageal manometry was performed using a custom designed catheter assembly (Dent Sleeve, Adelaide, S. Australia) comprising a silicon tubing composed of 3 individual channels of 0.3-mm internal and 0.6-mm external diameter each. The length of the manometry probe was 6.2 cm. The pressure-sensing side openings were located 5, 13, and 21 mm from the tip of the catheter assembly. Each channel's in-flow port was connected to a flow-through pressure transducer (BP-100; CB Science) and was perfused with distilled water at a rate of 7 μL/min. The typical raise rate for each channel exceeded 400 mm Hg/s. At a flow rate of 7 μL/min, the compliance for each perfusion channel was found to be about 1.2 μL for the first 100 mm Hg pressure rise. The pressure transducers were connected to a Maclab data acquisition system (Maclab/8e; AD Instruments, Castle Hill, Australia) through a preamplifier (ETH-400; CB Sciences).

A midline incision into the abdomen was made and the fundus portion of the stomach was exteriorized, and a small, approximately 0.5-cm incision was made in the fundus along the greater curvature. A 10-cm-long PE50 tubing moistened in saline and carrying a piece of silk thread at one end was introduced into the esophagus through the stomach and was tunneled out of the oral cavity of the mouse. The other end of the silk thread was tied to the tip of the flexible esophageal manometry probe. The thread was then used to guide the flexible silicone tubing into the distal esophagus. Usually, the most distal of these ports was placed in the high-pressure zone, and the other 2 proximal ports were in the esophageal body. The thread was then anchored to the surgical platform to prevent the probe from slipping out of the high-pressure zone. Sometimes, to eliminate catheter migration entirely, it was sutured to the wall near the gastroesophageal junction. During experimentation, the catheter was visually inspected intermittently to isolate catheter movement artifacts.

Swallows were induced by injecting a bolus of 10–20 μL of water or saline using indwelling PE10 tubing placed in the pharyngeal cavity (approximately 1 cm from the incisors) of the anesthetized mouse. Under control conditions, this resulted in progressive esophageal body contractions and an LES relaxation. In some studies, onset of swallowing activity was monitored using a saline-filled pharyngeal silicone balloon (outside diameter, 1 mm; Length, 2 mm) attached to a BP-100 transducer. L-NAME (Sigma, St. Louis, MO) was prepared in normal saline (0.9% wt/vol). In experiments in which L-NAME was given, pharyngeal stimulus was presented every 4–5 minutes for over an hour. This duration was found sufficient to study both blockade and reversal of the effect of the antagonist. In control animals, such a protocol did not cause a progressive reduction in LES response, thus ruling out tachyphylaxis to repeated pharyngeal stimuli.

For electrical stimulation, the cut end of the vagus was placed on slightly elevated bipolar platinum electrodes mounted on a micromanipulator (Leica Microsystems, Bannockburn, IL) and connected to a Grass S11 stimulator (Grass

Instruments, Quincy, MA). The nerve was kept insulated and hydrated by submerging in a viscous mixture of mineral oil and petrolatum jelly. The elevated position of electrodes ensured that electrical contact was restricted to the nerve only. Stimuli applied were of 1-second duration square wave pulses at 8 V with a 0.5-millisecond pulse width at 20 Hz frequency. In preliminary studies, stimulation of the peripheral cut end of the vagus produced a frequency-, voltage-, and stimulus train–dependent relaxation of the LES. Based on this, the stimulus parameters (8 V, 20 Hz, 0.5 millisecond, 1-second train) were chosen because they produced maximal LES relaxation. A period of 3–5 minutes was allowed between successive electrical stimuli. Artifacts caused by direct stimulation of the skeletal muscle could be easily identified and isolated from normal responses by observing any contraction in the neck and thoracic muscles during the intrastimulus period.

Immunofluorescence Labeling

These studies were performed to examine morphologically the relationship between nitrergic nerve varicosities, ICC-IM, and circular smooth muscle cells in the LES. The mice were euthanized with an overdose of pentobarbital (100 mg/kg, IP) and perfused transcardially with 60 mL of phosphate-buffered saline (PBS; 0.9% wt/vol NaCl in 0.01 m sodium phosphate buffer, pH 7.4) followed by 60 mL of 4% wt/vol paraformaldehyde in PBS. The LESs containing 1 mm of adjacent tissue were removed and post fixed in the same fixative overnight at 4°C. After the fixative was removed with 3 washes in PBS, the tissues were stored in PBS containing 1% sodium azide and 30% sucrose for 24 hours at 4°C before sectioning. Frozen longitudinal sections 12-μm thick were cut and processed for immunolabeling.

The sections were immunolabeled for nNOS and c-KIT using rabbit anti-nNOS antibody (1:500; Transduction Laboratories, San Diego, CA), and a goat anti–c-KIT antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. The unbound antibodies were removed by 3 washes with PBS. The sections were then incubated with fluorescein isothiocyanate–conjugated donkey antirabbit IgG (1:200; Jackson Immunoresearch, West Grove, PA) and Texas Red–conjugated donkey antigoat IgG (1:200; Jackson Immunoresearch) for 2 hours at room temperature.

Microscopy

A BioRad MRC1024ES multi-photon imaging system (BioRad, Hercules, CA) coupled with a mode-locked titanium/sapphire laser (Spectra Physics, Mountain View, CA) operating at 82 MHz frequency, 80fs pulse duration, tuned to a wavelength of 790 nm, was used to image immunolabeled tissue samples. A Zeiss Axiovert S100 inverted microscope (Carl Zeiss, Inc, Thornwood, NY) was used to observe and image the samples at 40× magnification in the epifluorescence mode. The 512 × 512 pixel images at 30–50-μm depths were collected at a pixel resolution of 0.485 μm with a Kalman 10 collection filter. The images were reconstructed in pseudocolor using BioRad (Hercules, CA) lasersharp software.

Data Analysis

Sphincter pressures were recorded from the bottom of phasic changes. Mean LES pressure for each mouse was calculated by registering the pressure every 5 minutes for an hour, and these values were further averaged between mice to arrive at mean LES pressure for each strain. Maximal (100%) LES relaxation was determined by administering the NO species donor, sodium nitroprusside (1 mg/kg, IV) in each of the experiments. Duration of LES relaxation was calculated by recording the time between the onset of LES relaxation and the time it returned to the same level. A mean of 3–5 consecutive swallows or vagal stimulation–induced response events was calculated for each mouse, and these were averaged further to arrive at a mean percent relaxation for each strain or treatment group. To examine treatment significance, paired t test was used within each group. Comparison across groups was made using one-way analysis of variance with the difference assessed by Tukey post-test comparison; $P < 0.05$ was deemed statistically significant. Values are expressed as mean ± SEM with N indicating the number of animals per group.

Results

Responses in Wild-type Mice

Figure 1 shows representative examples of esophageal manometric responses to swallowing (*A*) and efferent vagal stimulation (*B*) in anesthetized mice.

The basal LES pressures in the wild-type control C57BL/6J mice were normally distributed with a mean of 24 ± 3 mm Hg (N = 8). Swallows elicited reproducible progressive peristaltic contractions in the pharynx and the esophageal body and relaxation of the LES. The swallow-induced LES relaxation resulted in an 87% ± 3% fall in baseline LES pressure. LES relaxation event was at times followed by an after-contraction that rapidly (<10 seconds) returned to baseline. The average duration of LES relaxation was 5.8 ± 0.8 seconds.

LES relaxation to vagal stimulation resulted in a 91% ± 4% fall in basal LES pressure that lasted 7.1 ± 0.7 seconds (N = 6). An example trace illustrating the effect of unilateral efferent vagal stimulation on LES is shown in Figure 1*B*. Vagal stimulation–induced LES relaxation was not associated with pharyngeal activity. Moreover, the esophageal body contractions in the 2 upper ports tended to be simultaneous rather than progressive. As with swallows, vagal stimulation–induced LES relaxation also was sometimes followed by an after-contraction.

Effect of L-NAME

A bolus infusion of L-NAME (100 mg/kg, IV) produced a marked increase in the MAP (72 ± 8 mm Hg before and 93 ± 10 mm Hg after L-NAME, N = 6; $P <$ 0.009), but had no significant effect on basal LES pres-

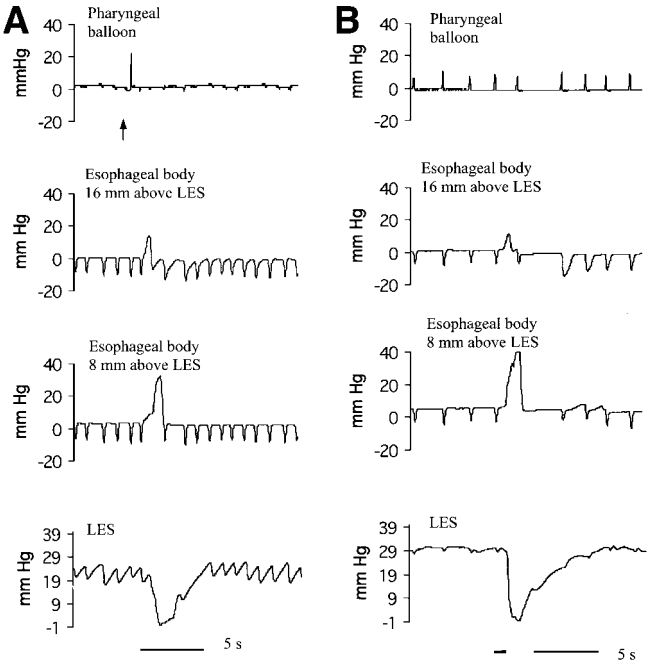

Figure 1. Esophageal manometry in anesthetized mice. Note that LES maintains basal tone. (*A*) Swallow-induced pharyngo-esophageal peristalsis and LES relaxation. Injection of water (10–20 μL) into the posterior part of oral cavity (*arrow*) elicits a swallow with a pharyngeal contraction and a peristaltic contraction in the esophageal body. LES relaxes transiently to swallow. This recording was made with custom designed catheter assembly of 3 catheters with side openings that are each 8 mm apart. Pharyngeal contraction was recorded with a specially designed balloon. (*B*) LES response to efferent vagal stimulation. Note that the discrete pharyngeal balloon contraction seen with swallow in (*A*) is absent here, whereas the esophageal body contractions occur almost simultaneously. Vagal stimulus parameters were 8 V, 20 Hz, 0.5 milliseconds pulse, 1-second train. *Short thick bar* indicates duration of vagal stimulation.

sure (24 ± 3 mm Hg before and 29 ± 5 mm Hg after L-NAME, N = 8; $P > 0.05$; Figure 2*A*).

L-NAME infusion decreased the magnitude of swallow-induced LES relaxation (Figure 2*B*). The peak inhibitory effect of L-NAME was seen between 10 and 20 minutes postinfusion. The relatively slow onset of action may be a result of the fact that L-NAME is a water-soluble ester that has to be hydrolyzed to L-NNA before it can compete for NOS.[21] Moreover, its peak effect in mice lasted only a short duration, presumably because of its rapid distribution/metabolism. At peak L-NAME effect, swallow-induced LES relaxation was reduced to a mean value of 36% ± 9%, which is significantly lower than the pretreatment value of 90% ± 3% (N = 6; $P < 0.01$). The duration of swallow-induced LES relaxation also significantly decreased from 7.5 ± 1.3 seconds to 2.3 ± 0.6 seconds ($P < 0.005$). The LES response was restored to control level within 60 minutes postinfusion with a relaxation amplitude of 80% ± 3% and a duration of 6.2 ± 0.6 seconds (N = 4; $P > 0.05$).

The stereospecific effect of L-NAME on swallow-induced LES relaxation was confirmed using L- and D-isomers of arginine in 2 animals each. Pretreatment with L-arginine, an endogenous NOS substrate, in a dose of 1000 mg/kg IV given 15 minutes before L-NAME, suppressed the latter's inhibitory effect on swallow-induced LES relaxation completely (83% ± 9% before and 78% ± 13% after L-NAME; $P > 0.05$). In contrast, D-arginine, an inactive enantiomer of L-arginine, did not affect L-NAME's inhibitory effect on LES relaxation (89% ± 4% before and 31% ± 10% after L-NAME; $P < 0.05$).

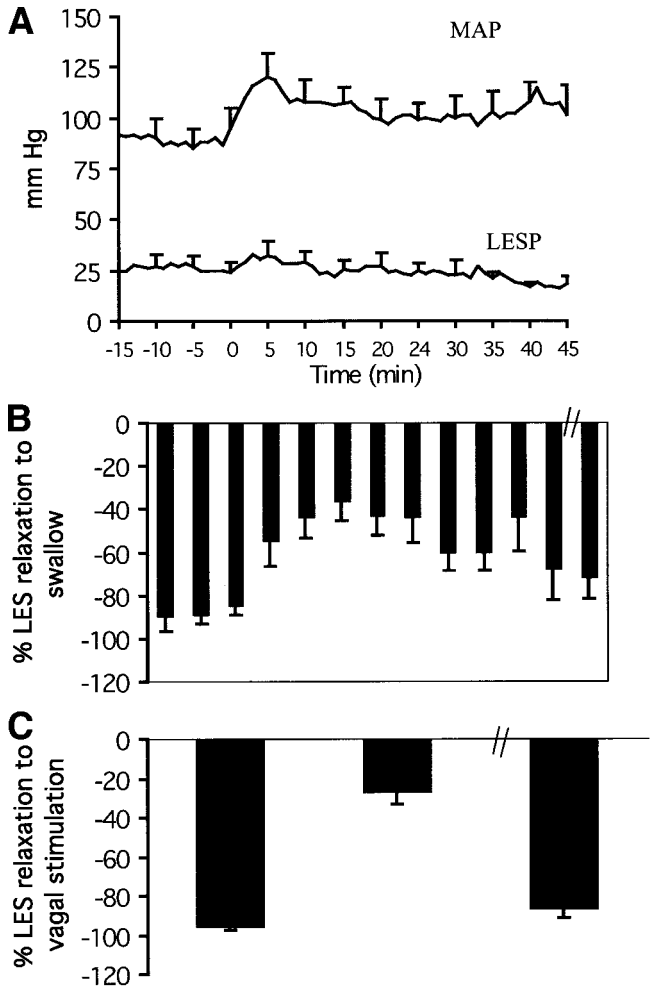

Figure 2. Effect of L-NAME on WT mice. (*A*) The effect of L-NAME (100 mg/kg) on basal LES pressure and MAP. A *solid bar* marks duration of L-NAME infusion. L-NAME produced a marked increase in MAP without affecting the LES pressure (LESP). (*B*) The effect of L-NAME on swallow-induced LES relaxation over time. Responses to swallow were pooled into 5-minute bins before and after L-NAME and averaged in each animal and then across animals (N = 8). Note that the peak inhibitory effect of L-NAME is between 10 and 20 minutes postinfusion and wanes thereafter. (*C*) The compiled data from efferent vagal stimulation–induced LES relaxation before, during the peak effect, and after recovery from L-NAME (N = 4–6).

After L-NAME infusion, as with swallows, efferent vagal stimuli resulted in a progressively smaller LES relaxation with peak decrement seen between 10 and 20 minutes postinfusion. During peak effect of L-NAME, LES relaxation was a markedly low 27% ± 3% in contrast to 96% ± 1% before treatment (N = 6; $P < 0.01$; Figure 2*C*). The duration of LES relaxation also decreased significantly from a mean of 7.2 ± 0.9 seconds to 2.6 ± 0.5 seconds ($P < 0.001$). The effect of L-NAME was reversed to pretreatment level within an hour. The post-treatment and pretreatment values of the amplitudes of LES relaxation were 87% ± 5% vs. 96% ± 1% and of the duration of relaxation were 6.2 ± 0.5 seconds vs. 7.2 ± 0.9 seconds (N = 4; $P > 0.05$).

Studies in nNOS$^{-/-}$ Mice

Mean resting LES pressure of 36 ± 5 mm Hg (N = 10) in nNOS$^{-/-}$ mice was significantly higher than the WT mean LES pressure of 24 ± 3 mm Hg ($P < 0.05$; Figure 3).

Swallow-induced LES relaxation was markedly but variably suppressed. The cumulative mean of swallow-induced LES relaxation in all the nNOS$^{-/-}$ mice was 26% ± 5% (N = 10; $P < 0.001$ vs. WT). Mean duration of relaxation also showed a significant attenuation as compared with WT response (3.0 ± 0.3 seconds; N = 10; $P < 0.001$). Four out of 10 mice tested showed a small percentage (25%) of sporadic and near complete LES relaxations (>75% relaxation) in response to pharyngeal stimulation. However, the duration of these otherwise complete relaxations was significantly less than that of WT mice (3.4 ± 0.6 seconds vs. 7.5 ± 1.3 seconds; N = 4; $P < 0.001$). The spectrum of responses observed in some nNOS$^{-/-}$ mice including no LES relaxation, partial relaxation, and a sporadic near complete relaxation, is shown in Figure 4. Partial and complete LES relaxation responses were sometimes followed by a brief after-contraction. LES relaxation response to swallow in nNOS$^{-/-}$ mice is summarized and compared with the other strains in Figure 5.

Efferent vagal stimulation yielded a highly attenuated LES relaxation response of 17% ± 8% (N = 8), a value significantly lower than the control response of 96% ± 1% in WT mice ($P < 0.01$). The duration of LES relaxation to efferent vagal stimulation was also highly attenuated compared with the WT response (2.0 ± 0.2

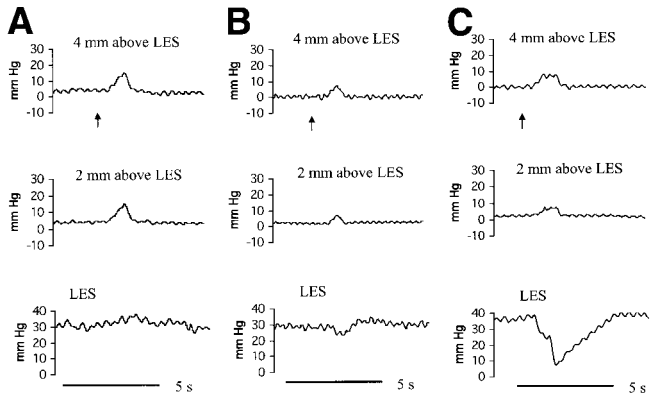

Figure 4. Spectrum of LES responses to swallow seen in a nNOS$^{-/-}$ mouse. (*A*) Esophageal body contraction with no LES relaxation. (*B*) An attenuated LES relaxation to pharyngeal water injection. (*C*) A complete relaxation. Note that the sphincter is hypertensive. Such near complete relaxations were elicited occasionally in 4 out of 10 nNOS$^{-/-}$ mice tested and showed a duration of relaxation that was less than that of WT mice. *Arrows* mark injection of fluid into the posterior pharynx.

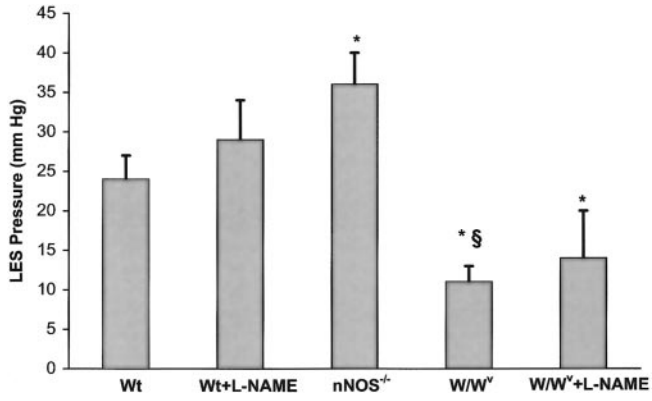

Figure 3. Basal LES tone in WT, nNOS$^{-/-}$, and W/W^{v} mice. Note that basal LESP is significantly higher in nNOS$^{-/-}$ mice and lower in the W/W^{v} mice as compared with that of WT. In WT and W/W^{v} mice, L-NAME treatment does not significantly affect resting LES tone. *Bars* represent mean ± SEM in 6–10 animals. *Statistically significant difference from WT mean ($P < 0.05$); §significant difference from nNOS$^{-/-}$ mean.

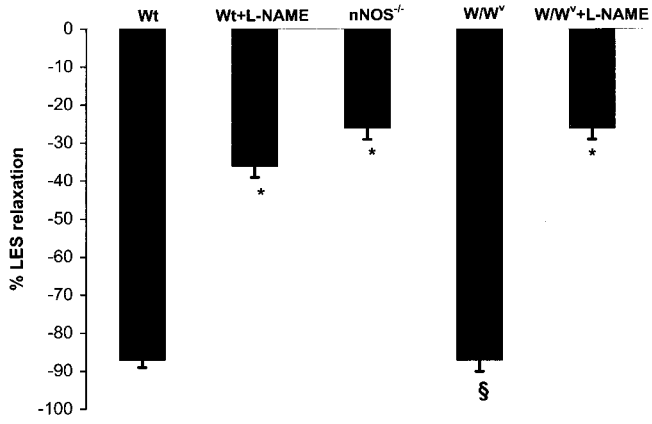

Figure 5. Swallow-induced LES relaxation in WT, nNOS$^{-/-}$, and W/W^{v} mice. LES relaxation is normal in WT and W/W^{v} mice, whereas it is significantly attenuated in the nNOS$^{-/-}$ mice. Moreover, pretreatment with L-NAME significantly blocks swallow-induced LES relaxation in WT and W/W^{v} mice. *Bars* represent mean ± SEM in 5 to 8 mice. *Statistically significant difference from WT mean; §significant difference from nNOS$^{-/-}$ mice ($P < 0.05$).

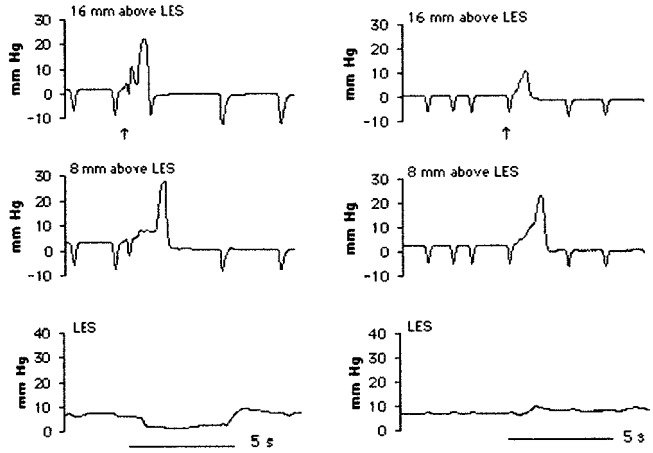

Figure 6. Swallow-induced LES relaxation in a W/W^{v} mouse before and after L-NAME treatment. Notice a remarkable LES hypotension and a clear LES relaxation followed by a brief after-contraction response to pharyngeal stimulus. The LES relaxation was markedly attenuated 15 minutes after L-NAME treatment. *Arrow* marks injection of 10–20 μL of water into the posterior pharyngeal cavity.

seconds; N = 7; $P < 0.001$). Moreover, only 1 out of 10 nNOS$^{-/-}$ mice tested showed a small percentage (29%) of 75% or greater LES relaxation.

Studies in W/W^{v} Mice

The resting LES pressure of 11 ± 2 mm Hg (N = 6) in W/W^{v} mice was significantly less than the basal tone of WT mice 24 ± 3 mm Hg as well as 36 ± 5 mm Hg in nNOS$^{-/-}$ mice ($P < 0.05$; analysis of variance; Figure 3). Quantitatively, LES relaxation to pharyngeal stimulation was 87% ± 5% (N = 6), a value that is not different from 87% ± 3% relaxation seen in WT mice ($P > 0.05$; Figure 5). Often, LES relaxation was followed by an after-contraction. L-NAME pretreatment caused an insignificant increase in resting LES pressure to 14 ± 6 mm Hg, while significantly diminishing swallow-evoked LES relaxation to 26% ± 5% (N = 5; $P < 0.05$). The duration of swallow-induced relaxation was also greatly diminished from 6.2 ± 0.7 seconds to 1.6 ± 0.5 seconds after L-NAME administration (N = 5; $P < 0.001$). The magnitude of reduction in LES relaxation and duration after L-NAME in W/W^{v} mice was similar to that of the WT mice. Sample traces of swallow-induced LES relaxation before and after L-NAME are shown in Figure 6.

Efferent vagal stimulation caused an 84% ± 5% (N = 5) relaxation in resting LES pressure. This value was not different from cumulative WT mean of 91% ± 1% ($P > 0.05$). Pretreatment with L-NAME temporarily attenuated this relaxation to 21% ± 5% (N = 5; $P < 0.01$). Along with the decreased LES relaxation, the duration of relaxation also was remarkably attenuated from 5.8 ± 0.9 seconds before L-NAME to 1.5 ± 0.5 seconds after L-NAME (N = 4; $P < 0.06$). The decrements in magnitude and duration of LES relaxations after L-NAME were not different from corresponding responses in WT mice ($P > 0.05$).

Immunohistochemistry

In the WT LES circular muscle, nNOS-positive neuronal varicosities (*green*) and c-KIT–positive ICC-IM (*red*) were clearly visualized (Figure 7*A*). As reported earlier,[3,8] varicosities were often associated closely with the ICC-IM (*arrows*). However, there were also many examples of nNOS-positive varicosities being independent of the ICC-IM and vice versa (*arrowheads*). In the LES circular muscle of nNOS$^{-/-}$ mice, there was no demonstrable nNOS immunoreactivity, whereas there was an ample presence of ICC-IM (Figure 7*B*). Conversely, ICC-IM were absent in the W/W^{v} mouse LES, whereas nNOS immunoreactivity was present in the myenteric varicosities (Figure 7*C*). Quantitative analysis of numbers of ICC-IM in the LES of WT and W/W^{v} mutant mice was performed by counting the number of c-KIT–positive ICC-IM–like cells in 512 pixel (220 μm) × 512 pixel (220 μm) images that imaged tissues at an average depth of 40 μm. The average number of ICC-IM were 12.3+ per 2 mm^{3} of the tissue in the WT mice. No ICC-IM were observed in W/W^{v} mutant mice.

Discussion

These studies show that anesthetized mice serve as an excellent model for the study of LES function in vivo. The most important finding of this study is that LES in nNOS$^{-/-}$ mice is hypertensive and relaxes poorly to swallowing and efferent vagal stimulation. In contrast, LES in W/W^{v} mice is hypotensive and relaxes normally to swallow and efferent vagal activation.

Hypertensive and nonrelaxing LES in nNOS$^{-/-}$ mice is very similar to findings in human achalasia and is consistent with the fact that in achalasia, nitrergic nerves are deficient because of their destruction by various pathologic processes.[22] Hypertensive LES in achalasia is thought to result from a combination of the lack of tonic inhibitory nitrergic influence and an unopposed cholinergic activity. Experimental chemical destruction of nitrergic innervation in opossum LES also produces increased LES tone as a result of unopposed cholinergic excitation.[23,24] A similar phenomenon may explain LES basal hypertension in nNOS$^{-/-}$ mice.

The most characteristic finding in achalasia is impaired LES relaxation to swallow. Similar impairment in LES relaxation was found in nNOS$^{-/-}$ mice pointing to the role of nitrergic inhibitory neurotransmission in

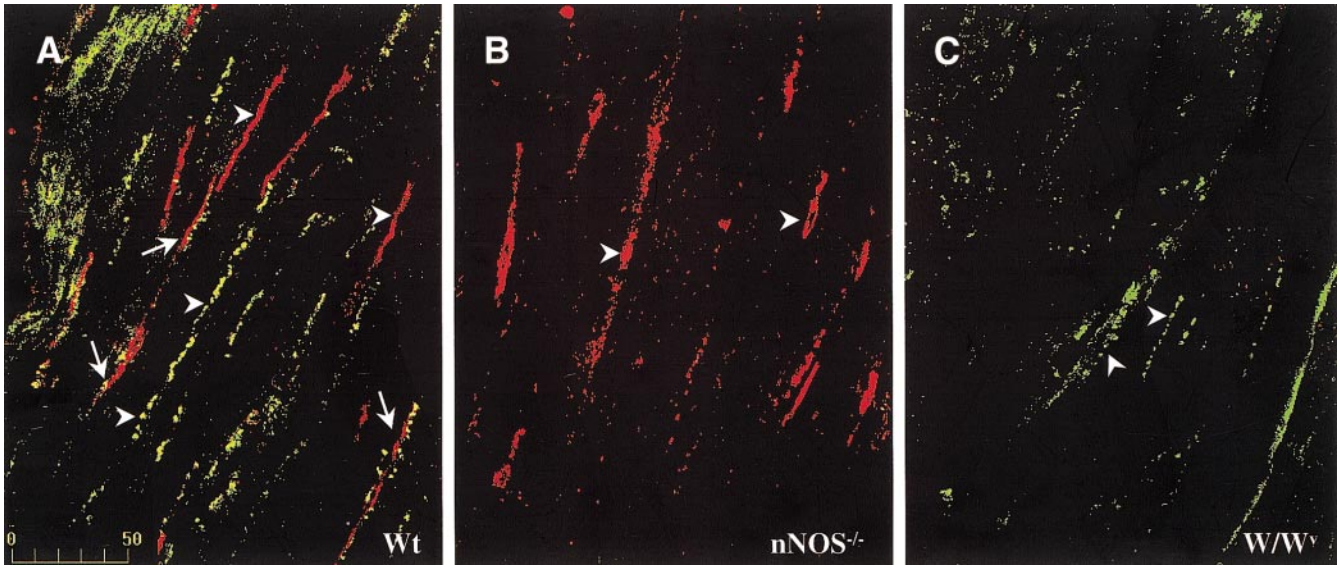

Figure 7. Immunofluorescence labeling for nNOS and c-KIT in the LES circular muscle of 3 strains of mice. In the WT LES (*A*), examples of close association between nNOS-immunoreactive varicosities (*green*) and c-KIT–positive ICC are shown by *arrows*. *Arrowheads* in all panels indicate ICC and nNOS-immunoreactive varicosities that are not associated with each other. Tissues from $nNOS^{-/-}$ mice (*B*) did not react for nNOS, whereas LES samples from W/W^{v} mice (*C*) did not label ICC.

swallow-evoked and vagally evoked LES relaxation. However, loss of LES relaxation to swallows was not complete, as approximately 25% residual relaxation to swallows and 18% to efferent vagal stimulation persisted in these mice. The cause of residual relaxation is unclear. It was not affected by L-NAME treatment, suggesting that it was not caused by another isoform or splice variant of NOS that may persist in $nNOS^{-/-}$ mice. The residual relaxation may be a result of other inhibitory neurotransmitters such as vasoactive intestinal polypeptide or adenosine triphosphate.[25,26]

Apart from the residual LES relaxation, 4 of 10 $nNOS^{-/-}$ mice also showed occasional normal amplitude LES relaxation to swallow. However, their duration of relaxation was significantly smaller than the WT mice. These normal amplitude but short duration relaxations occurred randomly with up to 25% of swallows. This observation is similar to that reported in patients with achalasia, by Katz et al.[27] These authors reported that a subgroup of achalasia patients showed near normal amplitude but short duration LES relaxations.[22,27] Interestingly, in the $nNOS^{-/-}$ mice, such random complete LES relaxations were not seen after treatment with L-NAME, suggesting that they are mediated by NOS activity remaining in some $nNOS^{-/-}$ mice. Recently, it has been shown that alternately spliced messenger RNA transcripts that lack exon 2 can encode partially active nNOS isoforms.[28,29] It is possible that these partially active isoforms may mediate the sporadic complete LES relaxations seen in some $nNOS^{-/-}$ mice. Further studies are needed to test this possibility.

When used in maximally effective doses, L-NAME also produced a 70% reduction in swallow-induced LES relaxation that is similar to that in $nNOS^{-/-}$ mice, indicating no difference in the degree of impairment of swallow-induced LES relaxation with acute or chronic nNOS deficiency. This observation suggests that lifelong deficiency in nNOS is not associated with any compensatory changes to normalize LES function. However, unlike $nNOS^{-/-}$ mice, L-NAME–treated WT mice did not show LES hypertension. This may be a result of the fact that L-NAME, apart from suppressing NOS activity, has antimuscarinic effects that could interfere with any concomitant cholinergic activity.[30]

The differences in reported results of studies on the contribution of NO in LES function are dependent on many factors, including the differences in the type of NOS inhibitor used, dose, and the experimental conditions. Whereas L-NAME and L-NNA are equipotent on a molar basis, L-NMMA is between 5–50-fold less potent than either of the above in suppressing NOS activity.[31] Thus, the lack of effect of L-NMMA on LES relaxation to swallowing may be a result of suboptimal suppression of nNOS.[14] Kawahara et al.,[13] on the other hand, may have underestimated the role of NO in rat LES by not examining the effect of L-NAME within the 10–20-minute time frame after L-NAME bolus.[13] Of the 2 LES functions, relaxation seems to be affected before the basal pressure by NOS inhibitors or NO scavengers.[9,12,32,33]

In contrast to the effect of chemical suppression or genetic deficiency of nNOS, W/W^{v} mice did not reveal LES basal hypertension or impaired relaxation

to swallowing. Instead, LES in W/W^{v} mice was hypotensive and relaxed normally to swallows and electrical stimulation of the vagus. W/W^{v} mutant mice have a genetic abnormality that results in partial deficiency of c-KIT and associated lack of ICC-IM.[3] Because ICC-IM have been proposed to mediate nitrergic signals from nerves to smooth muscles, loss of ICC-IM should result in LES dysfunction similar to that in nNOS$^{-/-}$ mice or achalasia in humans, but this was not what we found.

To investigate the reason for differences between our results and those previously reported by others,[3,8] we carefully examined the morphologic relationship between ICC-IM and nitrergic nerves in the LES of WT, nNOS$^{-/-}$, and W/W^{v} mice. We found, as described before, that in WT mice, many nNOS immunoreactive nerve terminals made baskets around c-KIT immunoreactive ICC-IM. However, a large number of nNOS-positive nerve endings directly innervated the smooth muscle bundles. NOS immunoreactive nerve terminals were lacking in nNOS$^{-/-}$ mice. In W/W^{v} mice, c-KIT–positive ICC-IM were lacking, but nNOS-positive nerve endings were preserved. These observations are consistent with our finding of impaired swallow-induced LES relaxation in nNOS$^{-/-}$ and normal relaxation in W/W^{v} mutant mouse. Although LES relaxation was normal in W/W^{v} mice, these animals had hypotensive LES. The genesis of LES hypotension in W/W^{v} mice is unclear. It has been suggested that cholinergic neural influences, like the nitrergic inhibitory influences on smooth muscles, may be exerted via ICC-IM, and deficiency of ICC-IM in W/W^{v} mutant mice may result in the loss of tonic cholinergic influence on the LES resulting in LES hypotension. However, this is unlikely because of the following reasons: (1) because ICC-IM mediates both inhibitory and excitatory neural inputs to the smooth muscle, it is difficult to see how there could be selective deficiency of cholinergic excitatory neural pathways that could explain LES hypotension in W/W^{v} mutant mice[34,35]; (2) the concept that ICC-IM is an important mediator of excitatory and inhibitory neural influences onto the smooth muscle cells is not supported by the current data; and (3) it has been reported that stomachs of W/W^{v} mice lack basal tone and are more compliant than those of WT mice treated with atropine. These observations are consistent with the view that the smooth muscle itself in W/W^{v} mice may be defective. Further studies are needed to resolve this issue. The physiologic significance of hypotensive LES in W/W^{v} mice is unclear. Studies are also needed to determine if hypotensive LES in mice is associated with incompetent LES and gastroesophageal reflux disease.

In conclusion, it is feasible to study LES function in anesthetized mice. The mouse LES behaves in the same manner in swallowing as other established experimental models of LES function. NO is a principal, but not the sole mediator of LES relaxation. Genetic loss of nitrergic innervation in mice leads to a phenotype of hypertensive and poorly relaxing LES that is reminiscent of clinical achalasia. On the other hand, LES in W/W^{v} mice is hypotensive and may be used as a model to study the pathogenesis of LES hypotension.

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Address requests for reprints to: Raj K. Goyal, M.D., Research and Development Service (151), West Roxbury VA Medical Center, 1400 VFW Parkway, West Roxbury, Massachusetts 02132. e-mail: raj_goyal@hms.harvard.edu.

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