Distinct roles of nitric oxide synthases and interstitial cells of Cajal in rectoanal relaxation

Akiko Terauchi, Daisuke Kobayashi, and Hiroshi Mashimo

Center of Swallowing and Motility Disorders, Department of Veterans Affairs Medical Center, West Roxbury; and Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts

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Terauchi, Akiko, Daisuke Kobayashi, and Hiroshi Mashimo. Distinct roles of nitric oxide synthases and interstitial cells of cajal in rectoanal relaxation. Am J Physiol Gastrointest Liver Physiol 289: G291–G299, 2005. First published April 21, 2005; doi:10.1152/ajpgi.00005.2005.—Nitric oxide (NO) relaxes the internal anal sphincter (IAS), but its enzymatic source(s) remains unknown; neuronal (nNOS) and endothelial (eNOS) NO synthase (NOS) isoforms could be involved. Also, interstitial cells of Cajal (ICC) may be involved in IAS relaxation. We studied the relative roles of nNOS, eNOS, and c-Kit-expressing ICC for IAS relaxation using genetic murine models. The basal IAS tone and the rectoanal inhibitory reflex (RAIR) were assessed in vivo by a purpose-built solid-state manometric probe and by using wild-type, nNOS-deficient (nNOS^−/−), eNOS-deficient (eNOS^−/−), and W/Wv mice (lacking certain c-Kit-expressing ICC) with or without l-arginine or Nω-nitro-l-arginine methyl ester (l-NAME) treatment. Moreover, the basal tone and response to electrical field stimulation (EFS) were studied in organ bath using wild-type and mutant IAS. In vivo, the basal tone of eNOS^−/− was higher and W/Wv was lower than wild-type and nNOS^−/− mice. l-Arginine administered rectally, but not intravenously, decreased the basal tone in wild-type, nNOS^−/−, and W/Wv mice. However, neither l-arginine nor l-NAMe affected basal tone in eNOS^−/− mice. In vitro, l-arginine decreased basal tone in wild-type and nNOS^−/− IAS but not in eNOS^−/− or wild-type IAS without mucosa. The in vivo RAIR was intact in wild-type, eNOS^−/−, and W/Wv mice but absent in all nNOS^−/− mice. EFS-induced IAS relaxation was also reduced in nNOS^−/− IAS. Thus the basal IAS tone is largely controlled by eNOS in the mucosa, whereas the RAIR is controlled by nNOS. c-Kit-expressing ICC may not be essential for the RAIR.

basal internal anal sphincter tone; electrical field stimulation; internal anal sphincter; nitric oxide synthases; rectoanal inhibitory reflex

NITRIC OXIDE (NO) is a major inhibitory nonadrenergic/noncho- linergic (NANC) neurotransmitter in the gastrointestinal tract (1) and appears to play a role in internal anal sphincter (IAS) functions (28). In organ bath experiments, electrical field stimulation (EFS) of NANC neurons relaxes the IAS via NO release (30). Rectal administration of l-arginine, which is converted to NO by NO synthases (NOS), decreases IAS pressure in humans (10). In contrast, NOS inhibitors, such as Nω-nitro-l-arginine methyl ester (l-NAME), may reverse the IAS tone (12, 36). Besides mediating basal IAS tone, NO is an important mediator of the rectoanal inhibitory reflex (RAIR), i.e., relaxation of the IAS in response to rectal distension. The RAIR is absent in Hirschsprung’s disease, a condition associated with lack of local NOS (37), and it is abolished by rectal administration of NOS inhibitors (24, 36).

NO is synthesized from l-arginine by one of three NOS: inducible NOS (iNOS), neuronal NOS (nNOS), or endothelial NOS (eNOS) (25). nNOS is localized to NANC neurons in the myenteric plexus of the gastrointestinal tract. On the other hand, eNOS is diffusely distributed in the longitudinal and circular muscle layers, myenteric plexus, and mucosa (15). Although the relaxatory functions of NO are amply demonstrated in the gastrointestinal tract, the relative contributions of nNOS and eNOS to IAS functions are unclear. This investigation defines the differential roles of nNOS and eNOS in mediating basal IAS tone, the RAIR, and NANC responses induced by EFS in mice. These studies used mice genetically lacking eNOS (eNOS^−/−/− mouse) or nNOS (nNOS^−/−/− mouse), local and systemic administration of l-arginine to elicit NO production, and l-NAME to antagonize NO production. Our study demonstrates that nNOS and eNOS modulate different aspects of IAS function.

Recent evidence suggests that interstitial cells of Cajal (ICC) are involved in NO-mediated inhibitory neuromuscular transmission (27) and also participate in pacemaker activity and electrical slow-wave propagation in the gastrointestinal tract (19). The c-Kit molecule acts as a molecular marker for ICC. A reduction in c-Kit-expressing ICC networks has been described in several motility disorders, including diabetes, chronic bowel obstruction, and Hirschsprung’s disease (9, 11, 22). W/Wv mice lack c-Kit-expressing ICC of the intramuscular (ICC-IM) and/or the myenteric plexus region (ICC-MY). NO-dependent inhibitory neurotransmission is greatly reduced in muscle strips from isolated fundus, lower esophageal sphincter (LES), and pyloric sphincters of these mice (5, 34). This suggests that c-Kit-expressing ICC are important for NO-dependent neurotransmissions in the gastrointestinal motility. To explore the role of c-Kit-expressing ICC in maintaining basal IAS tone and the RAIR, we also examined IAS function in W/Wv mice.

MATERIALS AND METHODS

Animal preparation. C57Bl/6Jx129/J F1 mice were used as wild-type mice, and nNOS^−/− and eNOS^−/− mice with targeted disruption by homologous recombination were used (13, 14). W/W^v mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Adult mice of either sex weighing 20–30 g were anesthetized by intraperitoneal injection of ketamine hydrochloride (22.5 mg/kg of body wt, Abbott Laboratories, N. Chicago, IL) and xylazine (2.5 mg/kg of body wt, Abbott Laboratories, N. Chicago, IL). Xylazine was injected by 10.220.33.5 on June 26, 2017 http://ajpgi.physiology.org/ Downloaded from
Ben Venue Laboratories, Bedford, OH). All animal procedures were performed in accordance with the guidelines of the Veterans Affairs Boston Healthcare Committee on Research Animal Care.

Drugs and administration. l-Arginine was purchased from Fisher Scientific (Fair Lawn, NJ), and l-N(3-arginine, d-arginine, and isoprote- nol were from Sigma (St. Louis, MO). For local in vivo administration of drugs, each drug was adjusted to pH 7.0 and dissolved in 25 μl of water-soluble jelly (Surgi-Lube, E. Fougera, Melville, NY) diluted 1:2 with water. Drugs (1.0 mg) were administrated intrarectally via an 18-gauge angiocatheter. For intravenous administration, l-arginine (3–6 mg/mouse) and l-NNAME (10–100 μg/mouse) dissolved in 10–60 μl normal saline were administered via the femoral vein. For organ bath experiments, NANC conditions in oxygenized modified Krebs solution were established as described previously (2) and l-NNAME (100–300 μM), l-arginine, d-arginine (5 mM), and isoproterenol (100 μM) were used. Each drug was prepared fresh on the day of the experiment. Tissues were treated with l-NNAME for 45 min before studying its effect.

IAS tension recording. In vitro muscle tension was recorded using standard organ bath techniques. A 1-cm segment of the anorectum was dissected from mice and incubated in the oxygenized modified Krebs solution for 30 min. In some of the preparations, the tissues were turned inside-out to remove the mucosa, and then reverted. Muscular rings of 0.3-mm width containing the IAS were dissected, suspended between a fixed glass tissue hook and a platinum hook attached to a force transducer (model FT03; Grass Instruments, Quincy, MA), then placed in 3-ml organ baths (Radnotti Glass Technology, Monrovia, CA) under NANC conditions. Tension was recorded by MacLab/8e digitizer (AD Instruments, Colorado Springs, CO) through an ETH-400 bridge amplifier (CB Sciences, Dover, NH). The tissues allowed to equilibrate for 45 min to establish spontaneous tone and were stretched to a initial uniform tension of 0.2 g. Preliminary studies showed that this preload gave maximal EFS-induced relaxation under NANC conditions (excepting nNOS−/− mice) and maximal relaxation to isoproteenol. Selective neural stimulation of the tissue was achieved using a pulse generator (model S11; Grass Instruments) to deliver 30-s duration trains of 15-V square-wave pulses, each of 2-ms pulse duration at 1–10 Hz through a simultaneous voltage splitter (Med-lab Stimu-Splitter II; Grass Instruments). For each parameter of stimulations, EFS was administered three times at 8-min intervals, and responses were averaged over the series. At the end of the experiments, isoproteenol was added to attain the maximal relaxation. Changes in basal IAS tone and EFS-induced NANC relaxation were expressed as a percentage of this maximal relaxation.

Anal canal pressure and basal IAS tone measurement. The anal canal pressure was recorded using a purpose-built catheter assembly consisting of a balloon catheter tied to a solid-state transducer (Fig. 1). The balloon was made of thin latex rubber and attached to the distal end of a polyvinyl chloride tubing (20-gauge, Boston Scientific-Meditec, Natick, MA). The recording catheter was a 2-Fr Micro-Tip catheter pressure transducer (Miller Instruments, Houston, TX). The solid-state transducer of the recording catheter was located ~1.8 mm from the distal end of the balloon catheter. The recording catheter was attached to an ETH-400 bridge amplifier (CB Sciences) and MacLab/8e digitizer (AD Instruments). The lubricated catheter assembly was inserted into the anal verge and clipped onto a micrometer (Mitutoyo, Aurora, IL). The transducer was initially placed 5.0 mm from the anal verge, because open dissection revealed the puborectalis sling was located ~8.0 mm from the anal verge. The pressure profile of the anal canal was initially obtained in three radial orientations by using a station pull-through technique, withdrawing 0.5 mm every 12 s. Pressures in the IAS consisted of rhythmic (phasic) contractions and more frequent respiratory variations superimposed on basal tone (Fig. 3A). Midrespiratory pressures were averaged over a 60-s period to define basal IAS tone.

Basal IAS tone was measured 15–30 min after the administration of drugs. Preliminary studies showed maximal drug effects ~10 min after drug treatment; these effects persisted for ~40 min (data not shown).

RAIR. With the transducer positioned in the IAS, threshold vol- umes for attaining the RAIR were determined by inflating the balloon to varying volumes (from 0 to 40 μl) over a period of 2.5 s. The balloon was deflated only after spontaneous recovery to basal pressures or after waiting a minimum of 6 s. At least 1 min of recovery was allowed before each reinflation.

Whole mount preparation and immunohistochemistry for c-Kit in IAS. Lengths of rectum (8.0 mm) were removed from wild-type, nNOS−/−, eNOS−/−, and W/Wv mice (9 wk old) of either sex. The tissues were removed longitudinally, and fecal matter was washed away using HEPES-buffered Hanks’ balanced salt solution (pH 7.35). Each rectal sheet was pinned with the mucosal side facing up. After we peeled away the mucosal layer, tissues were pinned to and stretched to ~120% of the original length and width. Tissues were then fixed in ice-cold absolute acetone for 10 min as described by others (4). After fixation, tissues were rinsed with PBS. Nonspecific binding of antibod- ies was blocked by preincubation in PBS containing 10% goat serum and 0.3% Triton X-100 for 30 min at room temperature (RT). Strips of muscle were then incubated overnight at 4°C with anti-c-Kit AKC2 antibody (eBioscience, San Diego, CA) diluted 1:200 in PBS containing 0.3% Triton X-100. The muscle strips were then washed in PBS containing 0.1% BSA and 0.3% Triton X-100. Next, tissues were fixed in ice-cold absolute acetone for 10 min as described by others (4). After fixation, tissues were rinsed with PBS. Nonspecific binding of antibod- ies was blocked by preincubation in PBS containing 10% goat serum and 0.3% Triton X-100 for 30 min at room temperature (RT). Strips of muscle were then incubated overnight at 4°C with anti-c-Kit AKC2 antibody (eBioscience, San Diego, CA) diluted 1:200 in PBS containing 0.3% Triton X-100. The muscle strips were then washed in PBS containing 0.1% BSA and 0.3% Triton X-100. Next, the immu- noreactivity was detected by incubating for 60 min at RT in fluorescein isothiocyanate-conjugated goat anti-rat IgG (Jackson Immuno Research Laboratories, West Grove, PA) diluted 1:200 in PBS containing 0.1% BSA and 0.3% Triton X-100. The IAS region was examined and photographed using a Nikon ECLIPSE TS-100 fluorescence microscope. Pictures were taken with a ×20 objective lens and ×10 eyepiece and SPOT RT-color digital camera (Diagnostic Instruments, Sterling Heights, MI).

Frozen section and immunohistochemistry for nNOS and eNOS in IAS. Five millimeters of rectum were removed from wild-type, nNOS−/−, eNOS−/−, and W/Wv mice (12 wk old) of either sex. The lumens were further flushed with 25% sucrose in PBS before embed- ding the tissues in optimum cutting temperature compound (Triangle

Fig. 1. Schematic representation of the purpose-built catheter assembly, de-
Biomedical Sciences, Durham, NC). Samples were frozen in dry ice/acetone bath. Longitudinal sections (14 μm thick) were placed on precleaned microscope slides, dried, and frozen at −80°C. Sections were fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 10 min at RT and rinsed with PBS. Tissues were then incubated with 1:500 dilution of rabbit anti-nNOS/NOS type I antibody (BD Transduction Laboratories, Lexington, KY) in PBS containing 0.5% BSA and 0.3% Triton X-100 overnight at RT in a humid chamber. On the following day, sections were rinsed in PBS and incubated with FITC-conjugated donkey anti-rabbit IgG (Jackson Immuno Research Laboratories) diluted 1:100 in PBS containing 1% BSA and 0.3% Triton X-100. The slides were incubated for 90 min at RT, rinsed in PBS, and mounted using anti-fading GEL/MOUNT (Biomeda). These sections were then examined and photographed as described above.

Statistical analysis. Radial pressure profiles were measured from each radial position in five mice. Basal IAS tone, the RAIR, and EFS-induced responses were measured three times in each of these mice. In organ bath experiments involving changes in basal tension, measurements were obtained in five mice. Results were expressed as means ± SE. Paired Student’s t-test and one-factor ANOVA were used to compare the treated and untreated groups and to compare results of the different mice. A two-tailed P value of <0.05 was considered statistically significant.

RESULTS

Location and pressure of IAS in wild-type mice. A station pull-through maneuver along each radial orientation revealed a 2.5-mm region of elevated pressure between 1.0 and 3.5 mm from the anal verge with a similar profile in all three orientations (Fig. 2). Histopathology also revealed a prominent region bordered by a thickened circular smooth muscle at the same location. From histological comparisons, there were no significant differences in the length (~1.1 mm) or muscular width (~0.1 mm) of the IAS among the various mice (data not shown). The highest pressures, defined as the region of IAS, were attained ~2.0 mm from the anal verge and averaged 12.8 ± 0.7 mmHg in all three vectors. In the ventral orientation, the basal IAS pressure of wild-type mice was 12.8 ± 0.4 mmHg. There was no significant difference in maximal IAS pressures among these three radial vectors. Subsequently, we used pressures recorded by ventral sensors to compare basal IAS tone in various mice.

![Fig. 2. Basal pressures along the anal canal using a ventrally oriented sensor catheter assembly. Pressure profiles in three radial positions (ventral sensor, 120 degree, and 240 degree) are similar. Basal IAS tone was expressed as mmHg.](http://ajpgi.physiology.org/)

Changes in basal IAS tone of nNOS−/−, eNOS−/−, and W/Wv mice. In comparison to wild-type mice, basal IAS tone was similar in nNOS−/− mice (12.8 ± 0.8 mmHg), significantly higher (P < 0.01) in eNOS−/− mice (25.9 ± 1.0 mmHg), and significantly lower (P < 0.01) in W/Wv mice (9.5 ± 0.4 mmHg; Fig. 3, A and B).

Changes in basal IAS tone after rectal administration of l-arginine or l-NAME for wild-type, nNOS−/−, eNOS−/− and W/Wv mice. In wild-type mice, IAS tone decreased after rectal administration of l-arginine (4.5 ± 0.6 mmHg, i.e., 64.8% relaxation) and increased after rectal administration of l-NAME (24.7 ± 1.4 mmHg, i.e., 19-fold increase over basal IAS tone). The specificity of this nitrergic pathway was confirmed using the enantiomer d-arginine, which did not alter the basal IAS tone (13.1 ± 0.8 mmHg, P = 0.7). l-Arginine also reduced IAS tone in nNOS−/− mice (3.1 ± 0.3 mmHg, i.e., 75.8%) and in W/Wv mice (4.0 ± 0.6 mmHg i.e., 57.9%), whereas l-NAME increased basal IAS tone in nNOS−/− mice (26.9 ± 2.3 mmHg) and in W/Wv mice (20.0 ± 1.2 mmHg). In contrast, l-arginine and l-NAME did not affect IAS tone in all eNOS−/− mice; IAS tone was 26.5 ± 1.4 mmHg with l-arginine and 26.3 ± 1.4 mmHg with l-NAME. Consequently, after l-NAME, basal IAS tone was not significantly different between wild-type (P = 0.2) and nNOS−/− (P = 0.6) nor eNOS−/− (P = 0.5) mice (Fig. 3, A and B).

Routes of l-arginine and l-NAME administration on IAS tone of wild-type mice. In wild-type mice, neither 3 (13.5 ± 0.8 mmHg, P = 0.4) nor 6 mg (13.0 ± 0.3 mmHg, P = 0.7) of intravenous l-arginine affected IAS tone. This amount (3 mg) of l-arginine was sufficient to prevent l-NAME-induced inhibition of RAIR. Administered rectally, 1 mg of l-arginine decreased basal IAS tone (4.0 ± 0.4 mmHg, P < 0.01, 68.5% relaxation) despite concomitant intravenous administration of l-NAME (6 mg). Intra-rectal (100 µg or 6 mg) but not intravenous l-NAME increased basal IAS tone in wild-type mice. The differential effects of these agents on basal IAS tone are summarized in Table 1.

Effect of l-arginine on basal IAS tension in organ bath studies. The effects of l-arginine on basal IAS tone in organ bath experiments were evaluated using wild-type, nNOS−/−, and eNOS−/− IAS (Fig. 4A). Twenty minutes after administration, l-arginine decreased IAS tone in wild-type (51.0 ± 7.6%) and nNOS−/− (52.6 ± 4.4%) mice; this relaxation persisted well beyond 30 min (data not shown). The specificity for the NO pathway was supported by the lack of relaxation with d-arginine. In contrast, l-arginine failed to relax the IAS in all eNOS−/− preparations. Moreover, l-arginine did not cause IAS relaxation in the wild-type IAS, which was stripped of mucosa (Fig. 4B).

Influence of NO derived from NOS, l-arginine, and l-NAME on the RAIR. In wild-type mice, 25 μl of inflation consistently induced an RAIR, i.e., basal IAS tone decreased by 80% (Fig. 5). Subsequently, the balloon was inflated with 25 μl for each experiment. This corresponded to a balloon diameter of ~4.0 mm. Figure 6, A and B, demonstrate an RAIR of comparable magnitude in eNOS−/− mice (87.8 ± 2.1%), wild-type mice (90.0 ± 1.4%, P = 0.4), and W/Wv mice (66.8 ± 4.2%). In contrast, the RAIR was completely absent in all nNOS−/− mice. The RAIR was also completely blocked by l-NAME in wild-type, eNOS−/−, and W/Wv mice. The RAIR was not
EFS-induced relaxation of wild-type IAS and influence of L-NAME. In vitro experiments demonstrated a frequency-dependent relaxation of wild-type IAS. The degree of relaxation increased over the range of 0.5–1 Hz and plateaued over the range of 2–10 Hz with >80% relaxation. The relaxatory response was completely blocked by tetrodotoxin (data not shown). L-NAME significantly decreased the EFS-induced relaxation in a concentration-dependent manner (Fig. 7A). For example, 5 Hz EFS produced 83.3 ± 2.4% relaxation. L-NAME at 100 and 300 μM reduced this relaxation to 39.4 ± 3.0 and 28.8 ± 3.3%, respectively (P < 0.05).

Effect of EFS on relaxation of wild-type, nNOS−/− and eNOS−/− IAS. In vitro experiments showed similar EFS-induced relaxations in wild-type (83.3 ± 2.4%) and eNOS−/−
(81.7 ± 1.5%, P = 0.6) IAS. l-NAME similarly reduced this relaxation in both wild-type (28.8 ± 3.3%, P < 0.05) and eNOS−/− (26.6 ± 4.6%, P < 0.05) IAS. In contrast, EFS relaxed the IAS by only 23.5 ± 1.7% in nNOS−/− mice. This EFS-induced IAS relaxation was comparable in nNOS−/− mice to wild-type and eNOS−/− mice treated with L-NAME (Fig. 7B).

Comparison of c-Kit-expressing ICC and nNOS distribution by immunohistochemistry. To assess whether physiological difference in basal IAS tone and the RAIR in the various mice were attributable to differences in the distribution or morphology of the c-Kit-expressing ICC, the tunica muscularis of rectum including IAS were dissected and stained using antibody to c-Kit (Fig. 8). In wild-type tissues, c-Kit-expressing ICC were found within ICC-IM and formed interconnecting networks within the muscularis in IAS. c-Kit-expressing ICC were also found within the ICC-MY. The distribution and density of c-Kit staining of eNOS−/− and nNOS−/− tissues were not different from those of wild-type tissues. In contrast, W/Wv tissues failed to stain for c-Kit.

To assess whether the RAIR in various mice were attributable to differences in the distribution of nNOS, cryosections of IAS from wild-type, eNOS−/−, nNOS−/−, and W/Wv mice were compared (Fig. 9). nNOS was present in the tunica muscularis in wild-type mice, and the distribution of nNOS-containing neurons in eNOS−/− and nNOS−/− tissues were not different from those of wild-type tissues. In contrast, W/Wv tissues failed to stain for c-Kit.

DISCUSSION

In the absence of highly specific NOS isoform antagonists, genetic animal models are necessary to understand how NOS isoforms mediate IAS functions. In this study, we demonstrated that NOS isoforms mediate distinct facets of IAS functions. Specifically, nNOS was the major mediator of the RAIR and EFS-induced IAS relaxation, but eNOS was the major mediator contributing to basal IAS tone. The specificity of these findings for the NO pathway was evidenced by administration of l-arginine, which induced basal IAS relaxation by 10.220.33.5 on June 26, 2017 http://ajpgi.physiology.org/ Downloaded from by 10.220.33.5 on June 26, 2017
In vivo measurements revealed that basal IAS tone was twofold higher in eNOS\(^{+/+}\) mice compared with wild-type mice. In nNOS\(^{-/-}\) mice, basal IAS tone was similar, and in W/W\(^{+}\) mice, basal tone was slightly lower (0.7-fold) compared with wild-type mice. L-Arginine decreased basal IAS tone in wild-type, nNOS\(^{+/+}\), and W/W\(^{+}\) mice but not in eNOS\(^{-/-}\) mice. In contrast, intrarectal L-NAME increased IAS tone in wild-type, nNOS\(^{-/-}\), and W/W\(^{+}\) mice. However, this treatment did not affect the already elevated basal IAS tone in eNOS\(^{-/-}\) mice. Taken together, this supports the notion that eNOS, rather than nNOS, iNOS, or c-Kit-expressing ICC, is responsible for maintaining basal IAS tone. These findings contrast with the increased LES pressure in the nNOS\(^{-/-}\) mice (18, 29). However, our findings suggest that NO, generated by nNOS is necessary for the RAIR, consistent with previous studies suggesting a role for NANC neurons in the RAIR (24, 36).

Fig. 6. The effect of rectal balloon inflation on basal IAS tone of wild-type, nNOS\(^{+/+}\), eNOS\(^{+/+}\), and W/W\(^{+}\) mice with or without drugs. The RAIR is absent in nNOS\(^{-/-}\) mice but preserved in eNOS\(^{-/-}\) and W/W\(^{+}\) mice. L-Arginine does not change the RAIR, but L-NAME inhibits the RAIR in wild-type, eNOS\(^{-/-}\), and W/W\(^{+}\) mice. A: representative recordings of the RAIR induced by the rectal balloon inflation on IAS. The start of inflation (arrow 1), end of inflation (arrow 2) and deflation (arrow 3) are shown in each figure. B: changes in IAS pressure are expressed as %decrease from basal IAS tone. The groups without drugs (n = 9), with L-arginine (n = 9), and with L-NAME (n = 9) were shown for each strain of mice.
Contrary to our results, Jones et al. (17) observed that the RAIR was present in all wild-type mice and absent in three of seven nNOS−/− mice in their study. Perhaps, this discrepancy may be explained, in part, by technical differences, including the transducer type, the balloon inflation volume, and the depth of catheter insertion between studies. Jones et al. (17) used perfusion manometry wherein large flow rates may introduce volume artifacts within the small rectal lumen. Otherwise, an extremely noncompliant tubing and large-flow impedance were required to maintain adequate rise and fall rates. Recognizing the limitations of using a water perfusion catheter in small animals, we switched to a solid-state transducer in this study. Moreover, we found that rectal balloon inflation to 40 ml caused outward movement of the entire catheter assembly and created an artifactual drop in IAS pressures. Therefore, the larger inflation volume (500 ml) used by Jones et al. may have caused catheter migration and a spurious drop in pressure. We minimized the possibility of catheter migration by using a fixed micrometer and deflated the balloon only after the entire RAIR. The diameter of our thin latex balloon filled with 25 ml of water was ~4.0 mm, which is comparable with the diameter of murine feces (i.e., ~2.0–3.0 mm). Lastly, Jones et al. measured the IAS pressures at ~5.0 mm from the anal verge, which is near the puborectalis sling and above the anal canal, located at ~2.0 mm from the anal verge by manometry and histology.

Both NO and carbon monoxide (CO) have been implicated in NANC relaxation of the IAS. Lack of EFS-induced relaxation in the aganglionic IAS of lethal spotted mutant was associated specifically with lack of nitrergic neurotransmission (6), but the selective heme oxygenase (HO) inhibitor SnPP-IX caused no significant attenuation of EFS-induced NANC relaxation of wild-type IAS (23). In this present study, we also showed that nNOS is important in EFS-induced IAS relaxation, because L-NAME reduced this relaxation and nNOS−/− IAS had...
a significant decrease in EFS-induced relaxation. EFS parameters are specifically chosen to stimulate enteric nerves rather than muscle. Thus nNOS appears to coordinate neurogenic responses including RAIR, but eNOS and HO are not involved in this pathway. However, Watkins et al. (35) showed that in similar in vitro experiments, EFS-induced IAS relaxation did not differ between nNOS−/− and wild-type IAS. This discrepancy could be explained by differences in experimental procedures, such as their use of indomethacin in the bath solution, their EFS parameters (not fully described), and possibly their initial loading tension.

Analogous to the LES (29), basal IAS tone was reduced in W/Wv mice. Decreased tone may be due to reduction of excitatory mediators or changes in musculature (26, 33). Our results suggest that c-Kit-expressing ICC do not have a critical role in the RAIR. Moreover, in these mice, the immunohistochemical distribution of nNOS, which was found essential for the RAIR, was similar to those in the anorectal regions of wild-type and eNOS−/− mice. Also, it is reported that ICC-IM in human intestine and ICC-DMP in small intestine of rats and human do not normally contain nNOS (27, 32). In contrast, there are reports suggesting the importance of c-Kit-containing ICC in the inhibitory role of NO. For example, others have described that ICC-MY in colon, ileum, and anorectum contain nNOS (2, 21 31), and in gastric muscle strips of W/Wv mice that lack ICC-IM, NANC relaxation to EFS was absent (5, 34).

Despite of these reports, several possibilities may explain why W/Wv mice have intact RAIR: 1) W/Wv mice, despite lack of c-Kit in the anorectal region, may have functional ICC (16); 2) nNOS-positive nerve fibers may not need c-Kit for ICC function. For example, ICC-DMP of human intestine, which normally lack c-Kit, formed synapselike junctions with cholinergic and nitrergic nerves and formed gap junctions onto smooth muscle cells (32); 3) there may also be differences in NANC pathways in the rectum and IAS compared with the foregut. For example, there may be other mediators such as CO, vasoactive intestinal polypeptide, pituitary adenylate cyclase-activating peptide, and other peptides involved in relaxation (3, 7, 8).

The route of administration influenced the effects of L-arginine and L-NAME on basal IAS tone but not on the RAIR. Similar to the opossum, intravenous administration of L-arginine did not affect basal IAS tone (24). However, intrarectal L-arginine relaxed the IAS, similar to previous studies in humans and rats (10, 12). Moreover, intrarectal but not intravenous L-NAME increased basal IAS tone in wild-type mice. These results suggest that systemic administration of these nitrergic compounds does not influence basal production of NO in the IAS. Furthermore, our studies in organ bath experiments showed that mucosectomy abolished L-arginine-induced IAS relaxation in wild-type mice. Thus eNOS, notably in the mucosa, maintains the basal IAS tone. Local delivery of
l-arginine may be effective for treatment of dyssynergia, rectal spasm, and Hirschsprung’s disease in the future.

Our results have potential implications for understanding anorectal disorders in humans. Thus the loss of RAIR in mice lacking nNOS is consistent with a selective loss of nNOS and RAIR in Hirschsprung’s disease. These studies also provide the rationale for using topical administration of nitroglycerin (NTG) for treating chronic anal fissures in humans (20). Intrarectal l-arginine may be preferable to NTG, because it reduced anal pressure without causing side effects (e.g., headache) associated with NTG (10).

REFERENCES