Gastric Stasis in Neuronal Nitric Oxide Synthase–Deficient Knockout Mice

HIROSHI MASHIMO,*,† ANN KJELLIN,§ and RAJ K. GOYAL*

*West Roxbury Veterans Affairs Medical Center, West Roxbury, Massachusetts; †Gastrointestinal Unit, Massachusetts General Hospital, Boston, Massachusetts; and §Karolinska Institute, Stockholm, Sweden

Background & Aims: **Nitric oxide (NO) is a major inhibitory neurotransmitter in the gut. This study aimed to identify the effect of chronic deprivation of NO derived from neuronal (nNOS) or endothelial (eNOS) nitric oxide synthase on gastric emptying.** ***Methods:*** **nNOS-deficient (knockout) mice were compared with wild-type mice for gastric size, fluoroscopic appearance after gavage of contrast, and histology of the pyloric sphincter. Wild-type mice treated with the NOS inhibitor N^{ω}-nitro L-arginine (L-NA) and eNOS-deficient mice were also compared with wild-type and nNOS-deficient mice for liquid and solid gastric emptying.** ***Results:*** **nNOS-deficient mice showed gastric dilation. Fluoroscopy showed delayed gastric emptying of radiologic contrast. There was no marked localized hypertrophy or luminal narrowing at the pyloric sphincter by histology of relaxed wild-type, nNOS-deficient, and eNOS-deficient tissues. Gastric emptying of both solids (28% ± 27%) and liquids (22% ± 18%) was significantly delayed in nNOS-deficient mice compared with control wild-type mice (82% ± 22% for solids; 48% ± 17% for liquids). eNOS-deficient mice showed no significant difference from wild-type mice (74% ± 28% for solids; 47% ± 23% for liquids). Wild-type mice treated acutely with L-NA showed delay in emptying of solids (43% ± 31%) but not liquids (39% ± 15%).** ***Conclusions:*** **Chronic depletion of NO from nNOS, but not eNOS, results in delayed gastric emptying of solids and liquids.**

Nitric oxide (NO) plays various roles in the physiology and pathophysiology of gastrointestinal motility. NO serves as an inhibitory neuromuscular neurotransmitter, an endothelium-derived relaxing factor, and an inflammatory mediator. In the stomach, both excessive and deficient NO production have been reported to produce gastric stasis. Exogenous NO donors relax gastric smooth muscle[1] and delay gastric emptying in humans.[2,3] Moreover, induction of large amounts of NO release by injection of endotoxin has been shown to delay gastric emptying in experimental animals. Treatment with inhibitors of nitric oxide synthase (NOS), such as aminoguanidine and N^{ω}-nitro L-arginine (L-NA), normalizes the relaxation and delayed gastric emptying in these animals.[4–6]

On the other hand, reduced NO production with the use of NOS inhibitors has been reported to cause suppression of gastric fundic relaxation and accommodation.[7] These abnormalities may lead to enhanced gastric emptying of liquids, as seen after vagotomy or with early diabetes.[8] However, NOS inhibitors have also been shown to delay gastric emptying in dogs[9,10] and rats,[11] in part from pyloric sphincter dysfunction.[12–14]

NO is produced by NOS from the amino acid L-arginine. Three isoforms of NOS derived from 3 different genes have been identified: neuronal (nNOS, or type I), endothelial (eNOS, or type III), and inducible (iNOS, or type II). iNOS is not present under physiologic conditions but is induced during inflammation and tissue injury. Neuronal and endothelial forms of NOS are constitutively present and play a role under physiologic conditions. nNOS is present primarily in nerves and is the source of NO that is involved in neurotransmission, whereas eNOS is mainly present in endothelial cells and produces NO that is involved in endothelial-dependent relaxing factor.[15] eNOS is also localized in the smooth muscles and has been proposed to play a role in neuromuscular transmission.[16]

Genetically engineered mice lacking nNOS were found to have gastric dilation that was thought to be caused by hypertrophic pyloric stenosis.[17] This was consistent with the finding of marked reduction of NOS staining in the myenteric neurons in the pylorus of infants with hypertrophic pyloric stenosis.[18] However, there are major differences in the gastric pathology of infantile hypertrophic pyloric stenosis and nNOS-deficient mice. In patients with infantile hypertrophic pylo-

Abbreviations used in this paper: eNOS, endothelial nitric oxide synthase; IJP, inhibitory junction potential; iNOS, inducible nitric oxide synthase; L-NA, N^{ω}-nitro L-arginine; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase.

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ric stenosis, NOS deficiency is localized to the pyloric area. In contrast, in the mutant mice, nNOS is absent throughout the stomach and the enzyme deficiency is not reversible. Our preliminary observations suggested that stomachs of mice lacking nNOS do not have the classic features of infantile hypertrophic pyloric stomachs.

The purpose of these studies was to examine the gastric pathology and gastric emptying abnormalities in *nNOS*$^{-/-}$ animals and to determine whether these gastric abnormalities were specific for nNOS deficiency by examining the gastric morphology and function in mice lacking eNOS. The studies show that *nNOS*$^{-/-}$ mice have gastric dilation and generalized gastric muscle thickening rather than purely hypertrophic pyloric stenosis. These animals also have delayed emptying of solids and liquids. Moreover, the gastric abnormalities are specific to nNOS deficiency because they are not seen in eNOS deficiency. These studies provide strong evidence that inhibitory nerves play an important role in gastric emptying and that their dysfunction leads to gastric stasis.

Materials and Methods

Animals

nNOS$^{-/-}$ and *eNOS*$^{-/-}$ mice were generated from 129/SV strain embryonic stem cells with targeted disruption by homologous recombination implanted into C57BL/6J blastocysts and confirmed to be lacking their respective genes on Southern blot and immunohistochemical stains, as described previously.[17,19] The *nNOS*$^{-/-}$ mice were generated by the replacement of exon 2 with the neo resistance gene cassette, which abolishes the ability to produce the α isoform of nNOS. The *eNOS*$^{-/-}$ were generated by replacement of the NADPH-binding domain by their respective targeting vectors. Age-matched adult male (C57Bl/6Jx129/J)F1 mice, representing the genetic background of the mutant mice, aged 8–10 weeks, and weighing between 25 and 30 g, were used as wild-type mice. These animals were bred on site.

For histologic studies, 3 mice each of wild-type, *nNOS*$^{-/-}$, and *eNOS*$^{-/-}$ were used to harvest the pyloric sphincter; 0.5 cm of adjacent tissue was resected, placed in Krebs solution containing 0.1 mmol/L isoproterenol for 5 minutes, pinned without stretching for orientation, and placed in 4% paraformaldehyde. This preparation was taken to increasing concentrations of ethanol, embedded in paraffin, and sectioned longitudinally in coronal planes of 6-μm thickness. Sections containing the maximum luminal diameter at the pyloric sphincter were used for H&E staining. Thickness of the muscularis of the pylorus, the width of the pyloric sphincter, and the maximal luminal aperture at the pyloric sphincter were measured by using a standard micrometer.

Stomach Weights and Volumes

The stomachs from 5 age-matched 4-month-old wild-type, *nNOS*$^{-/-}$, and *eNOS*$^{-/-}$ mice were cut 2 mm proximal to the lower esophageal sphincter and 2 mm distal to the pyloric sphincter, dissected out, and bathed in oxygenated physiologic Krebs solution. The stomach contents were emptied by flushing, blotted onto paper towel, and weighed. The stomach was ligated at the lower esophageal sphincter using silk thread, cannulated via the pylorus, and filled to gravity with normal saline at 10 and 20 cm of water column pressure.

Radiologic Studies

Three live wild-type and 3 *nNOS*$^{-/-}$ mice were fasted 36 hours for food and overnight for water. The stomach was outlined by administering 0.5 mL of Gastroview (Mallinckrodt, St. Louis, MO) via a purpose-built plastic gavage tube. Fluoroscopic examination was performed on unanesthetized mice using Philips 19150RF unit (Shelton, CT) set at 60 kV and 0.8 mA. Images were recorded on a Sony VCR sVHS (Irving, TX) and captured using Adobe Premiere software (San Jose, CA).

Western Blot Analysis

Whole stomachs from each mouse were homogenized separately in Tris-HCl (pH 7.4) buffer containing 0.5% Triton X-100 and protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN) using Polytron (Brinkman, Westbury, NY) for 20 seconds at medium speed, and centrifuged at 1000*g* for 15 minutes to remove debris. Protein concentration of supernatants was measured by BCA Protein Assay (Pierce, Rockford, IL). Protein (100 μg) was loaded per lane alongside protein molecular-weight standards (Kaleidoscope; Bio-Rad, Hercules, CA), separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions, and transferred onto Hybond-PVDF membranes (Amersham Inc., Arlington Heights, IL) by electroblotting. Membranes were blocked using 5% nonfat dry milk. nNOS, eNOS, and neuron-specific enolase were detected using polyclonal rabbit antibodies (catalog no. N31030-050 and N30030-50; Transduction Laboratories, Lexington, KY, and Immunotech, Westbrook, ME, respectively) at a dilution of 1:1000 overnight at 4°C, followed by goat anti-mouse secondary antibody conjugated to horseradish peroxidase, and determination of enhanced chemiluminescence by SuperSignal WestFemto (Pierce) using GS525 Molecular Imager System. Protein purification and blotting were performed in triplicate, starting with 3 separate mice of each genotype.

Solid and Liquid Gastric Emptying Studies

For initial studies, 30 wild-type mice were used to assess the kinetics of emptying of liquids and solids in our assays. Gastric emptying of indigestible solids was assessed by gavage of 30 glass beads (0.8 mm in diameter; Thomas Scientific, Swedesboro, NJ) to each conscious mouse using a purpose-built cannula. Animals were killed after 0.5, 1, 1.5, 2,

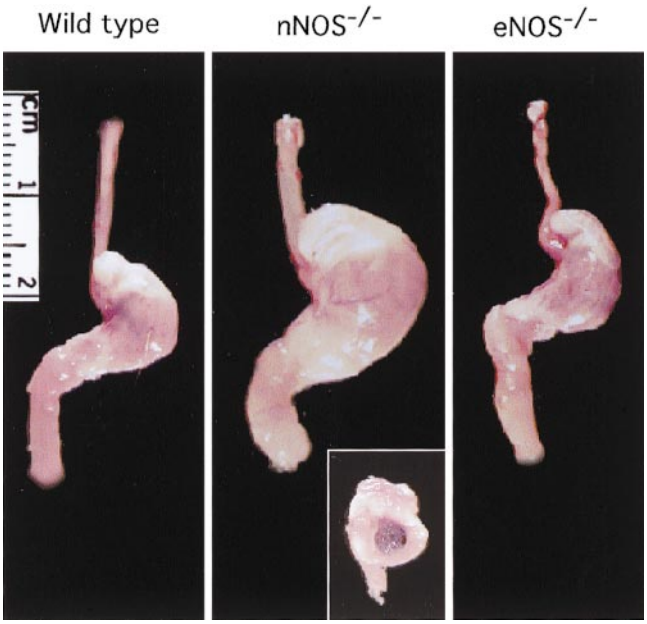

Figure 1. Comparison of stomachs from wild-type, *nNOS*$^{-/-}$, and *eNOS*$^{-/-}$ mice. There was diffuse enlargement of the esophagus, stomach, and duodenum in the *nNOS*$^{-/-}$ adult mice in comparison with *eNOS*$^{-/-}$ and wild-type mice. Frequently, bezoars were found in the stomach of *nNOS*$^{-/-}$ mice, despite 2 days fasting of food (*inset*).

2.5, and 3 hours (n = 5 each group). With the same mice, gastric emptying of liquid was assessed similar to protocols established in rats[20–22] by gavage of a 0.3 mL solution of 0.05% phenol red (Sigma, St. Louis, MO) administered 5, 10, 20, 30, and 40 minutes (n = 6 each group) before killing each mouse. Five additional wild-type animals were also killed immediately after intragastric administration of the test solution for baseline control. The stomach of each mouse was immediately tied off by fine silk thread at the pylorus and cardia, removed, and placed into 10 mL of 0.1N NaOH. Number of beads remaining in stomach were counted, and gastric emptying for solid beads was expressed as: (Number of Beads Administered − Number of Beads in Stomach)/Number of Beads Administered × 100%.

Stomach contents were then mixed thoroughly by Polytron for 15 seconds at medium speed, and centrifuged at 10,000*g* for 15 minutes to remove debris. The amount of phenol red was measured essentially according to the method of Scarpignato.[23] Supernatant (0.5 mL) was added to 0.05 mL of 20% acetoacetic acid. After centrifugation at 2800 rpm for 20 minutes, 0.4 mL of 0.5N NaOH was added to the supernatant. The absorbance of the sample was read at wavelength of 560 nm by a spectrophotometer. Gastric emptying for each mouse was calculated using the formula:

$$\text{Liquid Gastric Emptying (\%)} = \frac{1 - \text{Absorbance of Test Sample}}{\text{Absorbance of Baseline Control}} \times 100.$$

For comparative studies, 25 male wild-type, 10 male *nNOS*$^{-/-}$, and 10 male *eNOS*$^{-/-}$ mice were fasted as above. Five wild-type mice served as control for the liquid emptying test. Ten wild-type mice were injected intraperitoneally with 10 mg/kg L-NA 30 minutes before gavage. This dose is comparable with described doses in other animals including mice and has been shown in our laboratory to produce manometric increases in lower esophageal pressure in mice (personal observation, July 1998). All other mice were given sham intraperitoneal injections of normal saline. Gastric emptying of indigestible solids was assessed by glass beads, and gastric emptying of liquid was

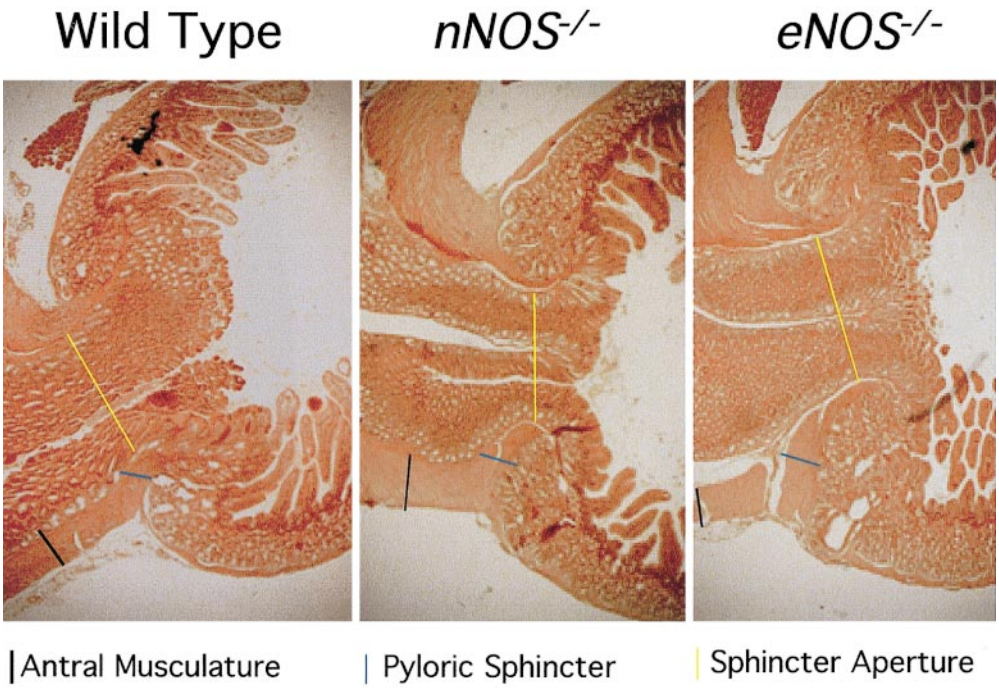

Figure 4. Comparison of pylorus histologic cross sections from wild-type, *nNOS*$^{-/-}$, and *eNOS*$^{-/-}$ mice. There was marked thickening of the circular muscle layer of the antrum in *nNOS*$^{-/-}$ mice compared with wild-type or *eNOS*$^{-/-}$ mice (*black lines*). However, the thickness of the pyloric sphincter muscle (*blue lines*) and lumen of the sphincter (*yellow lines*) was similar in the 3 mice. Stomachs were oriented with antrum to the left and duodenum to the right. Measurements (see Materials and Methods) were performed on parallel processed tissues from 3 mice of each genotype.

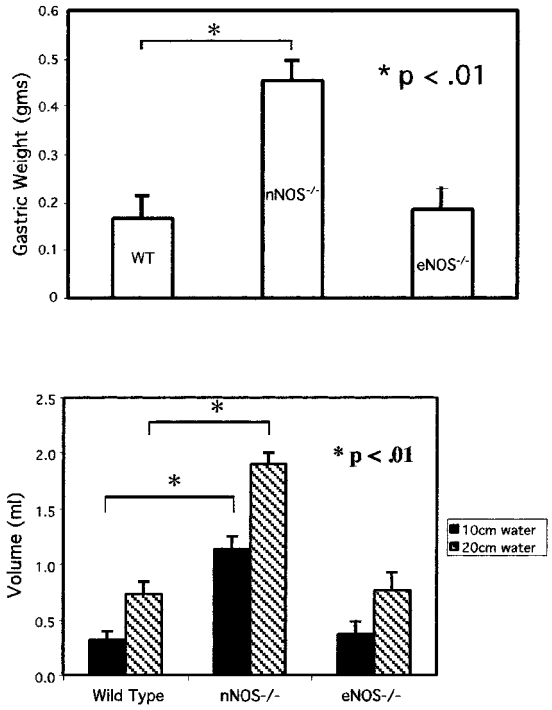

Figure 2. Stomach weight and volume comparisons of wild-type and *nNOS*$^{-/-}$ mice. There was marked increase in the mass and volume of the stomach in age-matched *nNOS*$^{-/-}$ mice compared with wild-type (WT) and *eNOS*$^{-/-}$ mice. Volumes were recorded for 10 and 20 cm of water column pressure used to inflate the stomach.

assessed by phenol red as above. All manipulations in mice have been performed in accordance with institutional review and approval.

Statistics

Data are expressed as mean ± SEM for wild-type, *eNOS*$^{-/-}$, and *nNOS*$^{-/-}$ tissues. Differences in the data were evaluated by the Student *t* test (nonparametric analysis), and Bonferroni correction was applied to account for the use of 5 mice to derive the baseline control for liquid gastric emptying. *P* values of <0.05 were considered statistically significant. The n values represent the number of animals used for each protocol.

Results

Gastric Stasis in *nNOS*$^{-/-}$ Mice

By adulthood, mutant mice lacking nNOS developed diffusely enlarged stomachs on gross examination compared with age-matched wild-type and eNOS-deficient mice (Figure 1). Frequently, the stomachs of *nNOS*$^{-/-}$ mice contained bezoars, even after 2 days of fasting (Figure 1, inset). This gross gastric abnormality is specific to nNOS deficiency because eNOS-deficient mice are indistinguishable from wild-type mice. The weights and volumes of the *nNOS*$^{-/-}$ stomachs were also significantly greater than the age-matched wild-type stomachs and did not represent mere dilation (Figure 2). Wild-type mice stomachs had an average stomach weight of 0.17 ± 0.04 g. In comparison, the *nNOS*$^{-/-}$ mice stomachs had an average weight of 0.45 ± 0.09 g. There was no significant difference in the overall weight of these mice. The radiographs showed persistently retained food in the dilated stomach of the *nNOS*$^{-/-}$ mice, even after 36 hours of fasting (Figure 3), consistent with a gastric stasis syndrome.

Morphologic Features of *nNOS*$^{-/-}$ Stomach

Comparative examination (Figure 4) revealed that *nNOS*$^{-/-}$ mice have muscular thickening throughout the stomach. The antral muscular layers, for example, measured 112 ± 3 μm in thickness for *nNOS*$^{-/-}$ mice compared with 70 ± 2 μm for wild-type and 75 ± 3 μm for *eNOS*$^{-/-}$ mice, representing an approximately 60% increase in thickness of the muscular layers in the *nNOS*$^{-/-}$ mice. The pyloric sphincter thickness, measured at the base of the muscle, was 17% thicker compared with wild-type tissue ($P < 0.05$), i.e., 77 ± 2 μm in thickness for *nNOS*$^{-/-}$ mice compared with 65 ± 3 μm for wild-type and 70 ± 3 μm for *eNOS*$^{-/-}$ mice. The luminal aperture at the pyloric sphincter of the relaxed

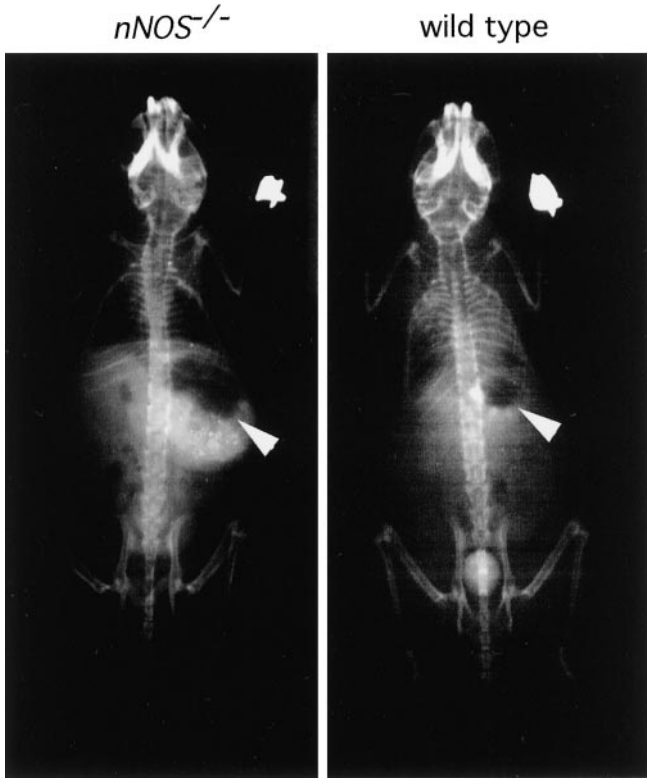

Figure 3. Fluoroscopic comparison of wild-type and *nNOS*$^{-/-}$ mice. There was marked enlargement of the stomach of *nNOS*$^{-/-}$ mice compared with wild-type mice in vivo (*arrowhead*). A large gastric bubble and retained food were noted in the *nNOS*$^{-/-}$ mice.

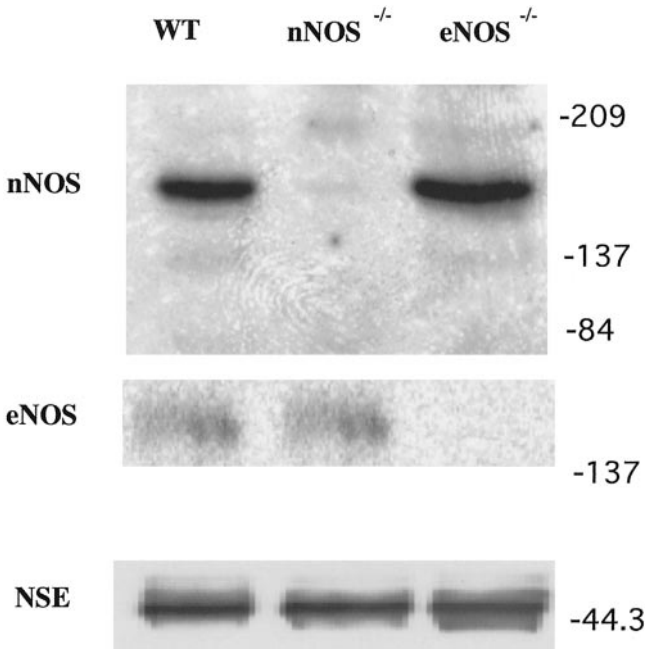

Figure 5. Western blot analysis of stomachs. Western blot analysis, using antibodies that distinguish the NOS isoforms, confirm the absence of nNOS in the $nNOS^{-/-}$ and absence of eNOS in the $eNOS^{-/-}$ whole stomach protein preparations. Total neurons are likely to be comparable in these mice because there was no overt difference in detectable neuron-specific enolase (NSE) in these preparations. Molecular-weight markers (in kilodaltons) are indicated on the right.

tissues was 273 ± 4 μm for $nNOS^{-/-}$ mice compared with 287 ± 5 μm for wild-type and 302 ± 3 μm for $eNOS^{-/-}$ mice; the differences in these measurements were not statistically significant. This diffuse muscular thickening in the $nNOS^{-/-}$ mice is in contrast to the human condition of idiopathic hypertrophic pyloric stenosis, in which the muscle hypertrophy is reportedly limited to the pylorus. Moreover, there was no fixed stricture as evidenced from the luminal aperture at the pyloric sphincter of these relaxed tissues, suggesting rather a functional impairment in the $nNOS^{-/-}$ mice. Sections of $eNOS^{-/-}$ mice were grossly indistinguishable from those of wild-type mice, and measurements described were not statistically significant from wild-type measurements. Thus the gastric dilation and stasis in $nNOS^{-/-}$ mice were not caused primarily by a fixed structural abnormality of the pyloric sphincter, but likely represent a functional motility problem.

Western Blot Analysis of Gastric Tissue for nNOS and eNOS

To ascertain that the respective NOS was completely abolished in the knockout animals, Western blot analysis was performed using total protein preparation of whole stomachs and antibodies specific for the NOS enzymes. There was no apparent nNOS in the $nNOS^{-/-}$ whole stomach throughout the range of molecular weight 140–170 kilodaltons, which span the region where alternatively spliced products of nNOS have been described.[24] The antibodies used were polyclonal and raised against an immunogen that could detect the described splice products of *nNOS*. Similarly, there was no eNOS in the $eNOS^{-/-}$ tissues (Figure 5). Amounts of neuron-specific enolase were unaltered in these mice tissues, suggesting that there were no evident reduction of neurons in the stomachs of the knockout animals.

Gastric Emptying of Solids

Wild-type mice showed increasing emptying of indigestible solid glass beads over time after gavage, from 0.5 to 2.5 hours. The cycle lengths of migrating motor complexes are particularly short (about 15 minutes) in rodents,[25] yet may add to the variability seen in this simplified measurement of emptying particularly between 1.0 and 2.0 hours after gavage (Figure 6*A*). A time point of 2 hours after gavage was chosen from these studies to compare gastric emptying of the solid beads in the various mouse groups.

The percent gastric emptying of glass beads 2 hours after gavage in unanesthetized wild-type mice was 82% ± 22%. In comparison, mice administered L-NA showed

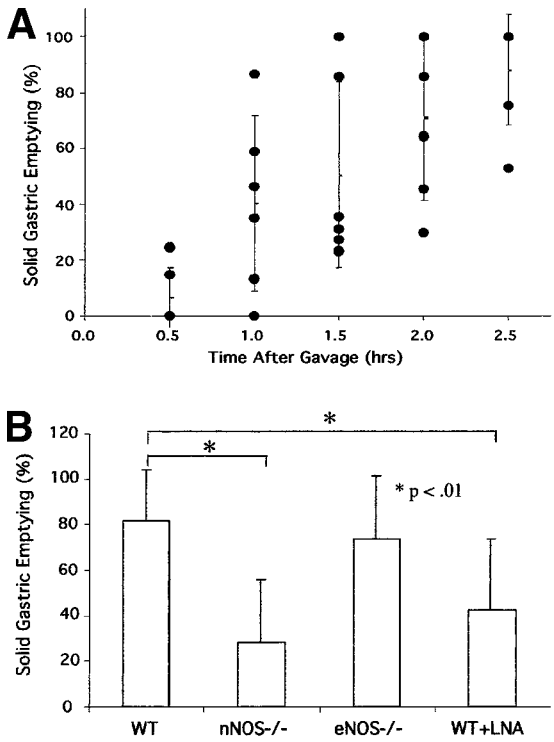

Figure 6. Gastric emptying of solids. (*A*) Time course of gastric emptying of solids at 0.5, 1, 1.5, 2, and 2.5 hours after gavage of glass beads in wild-type mice. Bars indicate standard error for 6 mice. (*B*) Gastric emptying of glass beads at 2 hours after gavage in various mice. *Bars* indicate standard error for 5 mice.

a significant delay in solid gastric emptying, of 43% ± 31% ($P < 0.01$). Mice deficient in nNOS showed a further decrease in gastric emptying, of 28% ± 27% ($P < 0.01$). In contrast, eNOS-deficient mice showed no significant decrease in gastric emptying (74% ± 28%, $P = 0.41$) (Figure 6*B*).

Gastric Emptying of Liquids

Wild-type mice showed increasing gastric emptying of liquids with time after gavage from 5 to 25 minutes, with a plateau of approximately 70% at 25 and 30 minutes (Figure 7*A*). This correlated with the observation that Gastrografin was almost entirely passed from the stomach by approximately 30 minutes under fluoroscopic examination. This is comparable with observations in rats showing that enumeration of emptying did not reach 100%, perhaps because of dye adherent to the mucosa, as described in the investigators' initial study.[20] The residual dye noted in wild-type mice is consistent with their data. Alternatively, there may be some immediate dye loss into the small intestine within seconds of entry into the stomach.

A time point of 20 minutes after gavage was chosen for comparison of liquid emptying among wild-type, *nNOS*$^{-/-}$, *eNOS*$^{-/-}$, and L-NA–treated wild-type mice.

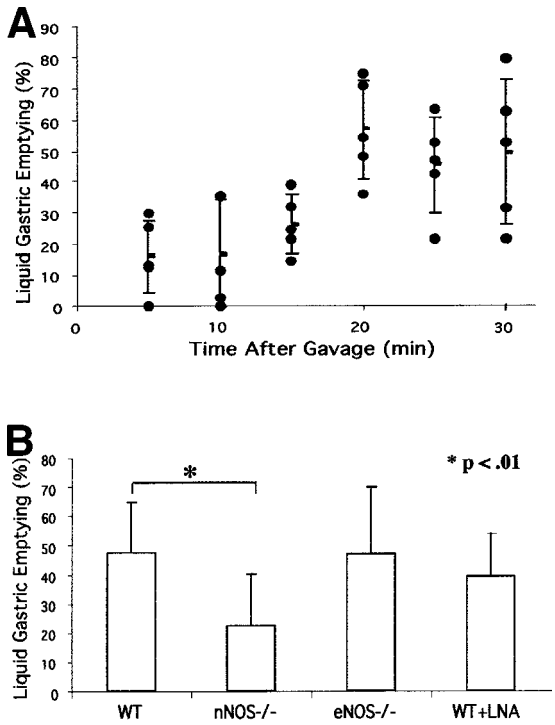

Figure 7. Gastric emptying of liquids. (*A*) Time course of gastric emptying of liquids at 5, 10, 15, 20, and 25 minutes after gavage of dye in wild-type mice. *Bars* indicate standard error for 6 mice. (*B*) Gastric emptying of liquid at 20 minutes after gavage in various mice. *Bars* indicate standard error for 5 mice.

There was marked delay of gastric emptying for liquids in nNOS(−) (22% ± 18%, $P < 0.01$) compared with sham-treated wild-type mice (82% ± 22%). However, there was no significant delay in liquid gastric emptying compared with wild-type mice in the *eNOS*$^{-/-}$ mice (47% ± 23%, $P = 0.93$) or L-NA–treated wild-type mice (39% ± 15%, $P = 0.23$) (Figure 7*B*).

Discussion

These studies show that mice chronically lacking nNOS develop gastric dilation and diffuse muscle thickening that is associated with delayed gastric emptying of both solids and liquids. These abnormalities are not caused by a hypertrophic pyloric stenosis alone because the gastric muscle thickening noted in this study is generalized to the entire stomach and is not limited to the pylorus. Infantile hypertrophic pyloric stenosis is characterized by marked and regional hypertrophy of pyloric sphincter that can be appreciated as an olive on abdominal examination and on abdominal ultrasonography. Such marked hypertrophy is not observed in these mutant mice. Upper gastrointestinal barium studies show characteristic narrowing of the pyloric channel that is responsible for pyloric stenosis and gastric outlet obstruction leading to gastric stasis and dilation. Such marked hypertrophy is not observed in these mutant mice.

nNOS has been shown to localize to the intrinsic gastric inhibitory motor nerve endings, intrinsic interneurons, preganglionic motor neurons in the caudal dorsal motor nucleus of the vagus that project to the stomach, and the nucleus tractus solitarius neurons that are premotor to dorsal motor nucleus of the vagus neurons.[26,27] nNOS deficiency would disrupt NO neurotransmission at all these sites and result in selective but total loss of nitrergic inhibitory neurotransmission throughout the body, including the stomach.

The loss of nitrergic inhibitory neurotransmission exerts several effects on the stomach. Suppression of NO neurotransmission by blockers of NOS causes loss of receptive relaxation and a gastric accommodation reflex that may result in clinical symptoms of early satiety and may be associated with accelerated gastric emptying of liquids.[7,28] Suppression of NO transmission in dogs and rats causes disruption of migrating motor complexes that play a key role in gastric emptying of indigestible solids.[29,30] Therefore, disruption of the migrating motor complex activity in *nNOS*$^{-/-}$ could explain delayed gastric emptying of solids. Loss of NO transmission in the pylorus has been shown to cause increase in pyloric tone and impaired pyloric relaxation.[12] These pyloric abnor-

malities would also lead to gastric stasis of both solids and liquids. It is interesting that L-NA treatment caused significant delay in gastric emptying of solids but the liquid emptying remained normal. The normal liquid emptying caused by active NOS inhibition could be caused by a summation of 2 opposing effects: accelerating liquid emptying because of loss of inhibition in the gastric fundus and delaying effect because of pyloric sphincter dysfunction. In contrast, with chronic lifelong deficiency of nitrergic inhibitory neurotransmission in *nNOS*$^{-/-}$ mice, gastric emptying of both solids and liquids is impaired.

There is also mounting evidence for the importance of interstitial cells of Cajal in electrical pacemaking and motor neurotransmission in the stomach,[31] and these cells were closely associated with neurons containing NOS.[32] Moreover, mice lacking these interstitial cells had abnormal NO-dependent neurotransmission in the pyloric sphincter.[33] The exact relationship between NO-containing neurons and the interstitial cells of Cajal in mediating gastric emptying needs to be studied further.

The generalized gastric smooth muscle thickening in *nNOS*$^{-/-}$ animals may be caused by several factors: NO is known to inhibit smooth muscle proliferation, thus deficiency of NO may lead to muscle hypertrophy; and the muscle thickening may be secondary to functional pyloric obstruction and may represent work hypertrophy. Further studies are needed to resolve this issue.

It is now believed that the vagus nerve conveys parallel cholinergic excitatory and inhibitory projections to the stomach. Therefore, gastric stasis observed in patients after bilateral vagotomy could result from the loss of both excitatory and inhibitory influences.[27,34] After vagotomy, there may be reduced gastric contractions and fundic relaxation, pylorospasm, and impaired pyloric sphincter relaxation.[35–37] Decreased gastric fundic accommodation and impaired pyloric relaxation may be attributed to the loss of inhibitory innervation. The inhibitory nerves may use several inhibitory neurotransmitters, including NO, and therefore the importance of nitrergic inhibitory innervation in gastric stasis remained unclear. This model of nNOS deficiency unequivocally establishes that chronic and selective loss of inhibitory NO neurotransmission can lead to gastric stasis and dilation.

Diabetic gastroparesis involves multiple defects in the muscle, circulating local hormones, and the nerves. Diabetic vagal neuropathy may involve deficiency of both cholinergic excitatory and nitrergic inhibitory neurotransmission. The role of nitrergic inhibition is supported by the following observations: (1) experimental diabetic neuropathy is associated with decreased expression of nNOS in gastric myenteric neurons[8]; (2) functional studies in diabetic animals and diabetic patients show enhanced gastric emptying that is consistent with loss of inhibitory neurotransmission in the gastric fundus[38–40]; and (3) patients with diabetic gastroparesis have been found to have pylorospasm and dysfunction that is consistent with loss of nitrergic neurotransmission.[41]

A large number of patients with gastroparesis are classified as idiopathic because no etiologic factors for gastroparesis are found. A syndrome of gastroparesis associated with genetic deficiency of nNOS caused by loss or mutation of the nNOS gene is not currently known. However, proper studies that look for defects in the nNOS gene in idiopathic gastroparesis would be of interest.

nNOS is the enzymatic source of NO for nitrergic neurotransmission throughout the body including the entire gut. However, the obvious phenotype in the *nNOS*$^{-/-}$ mouse is only the enlarged and dilated stomach. This may be caused by redundancy associated with parallel mechanism or compensatory factors in other organs. However, the stomach showed no redundant inhibitory mechanisms or appearance of compensatory mechanisms. Wild-type mice showed 2 overlapping inhibitory junction potentials (IJPs),the fast IJP and the slow IJP. In the *nNOS*$^{-/-}$ mouse, the fast IJP was unchanged but the slow IJP was lacking.[42] These studies show that the nitrergic slow IJP is independently important and its loss is associated with significant functional problems in the stomach. Detailed functional studies of the other parts of the gut in the *nNOS*$^{-/-}$ mouse have not been performed. Our studies also show that eNOS does not play a major role in regulation of gastric motor function.

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Address requests for reprints to: Hiroshi Mashimo, M.D., Ph.D., Research and Development Service (151), Veterans Affairs Medical Center, 1400 VFW Parkway, West Roxbury, Massachusetts 02132. e-mail: mashimo@helix.mgh.harvard.edu; fax: (617) 363-5592.

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