Infantile hypertrophic pyloric stenosis (IHPS): A study of its pathophysiology utilizing the newborn *hph-1* mouse model of the disease

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Welsh C, Shifrin Y, Pan J, Belik J. Infantile hypertrophic pyloric stenosis (IHPS): A study of its pathophysiology utilizing the newborn *hph-1* mouse model of the disease. Am J Physiol Gastrointest Liver Physiol 307: G1198–G1206, 2014. First published October 30, 2014; doi:10.1152/ajpgi.00221.2014.—Infantile hypertrophic pyloric stenosis (IHPS) is a common disease of unknown etiology. The tetrahydrobiopterin (BH4)-deficient hyperphenylalaninemia-1 (*hph-1*) newborn mouse has a similar phenotype to the human condition. For *hph-1* and wild-type control animals, pyloric tissue agonist-induced contractile properties, reactive oxygen species (ROS) generation, cGMP, neuronal nitric oxide synthase (nNOS) content, and Rho-associated protein kinase 2 (ROCK-2) expression/activity were evaluated. Primary pyloric smooth muscle cells from wild-type newborn animals were utilized to evaluate the effect of BH4 deficiency. One-week-old *hph-1* mice exhibited a fourfold increase (*P* < 0.01) in the pyloric sphincter muscle contraction magnitude but similar relaxation values when compared with wild-type animals. The pyloric tissue nNOS expression and cGMP content were decreased, whereas the rate of nNOS uncoupling increased (*P* < 0.01) in 1-wk-old *hph-1* mice when compared with wild-type animals. These changes were associated with increased pyloric tissue ROS generation and elevated ROCK-2 expression/activity (*P* < 0.05). At 1–3 days of age and during adulthood, the gastric emptying rate of the *hph-1* mice was not altered, and there were no genotype differences in pyloric tissue ROS generation, nNOS expression, or ROCK-2 activity. BH4 inhibition in pyloric smooth muscle cells resulted in increased ROS generation (*P* < 0.01) and ROCK-2 activity (*P* < 0.05). Oxidative stress upregulated ROCK-2 activity in pyloric tissue, but no changes were observed in newborn fundal tissue in vitro. We conclude that ROS-induced upregulation of ROCK-2 expression accounts for the increased pyloric sphincter tone and nNOS downregulation in the newborn *hph-1* mice. The role of ROCK-2 activation in the pathogenesis of IHPS warrants further study.

Sphincter tone is determined by the balance between the neuronal nitric oxide (NO) synthase (nNOS) isoform and the Rho-associated protein kinase 2 (ROCK-2)-dependent contraction effects. nNOS is the most abundant enzyme in gastrointestinal smooth muscle (42), and its NO generation potential depends on the coupled dimeric state of the enzyme. When uncoupled, nNOS predominantly generates reactive oxygen species (ROS) (11, 37, 42).

Infantile hypertrophic pyloric stenosis (IHPS) is a relatively common disease affecting 2 in 1,000 live births (34) and manifesting clinically at 3–6 wk of age. The IHPS etiology and pathogenesis are unknown, but the increased sphincter tone is believed to be related to reduced pyloric tissue nNOS expression (2) and the resulting lower NO generation (13, 34, 45). Several experimental animal models of the condition have been studied, but the hyperphenylalaninemia-1 (*hph-1*) newborn mouse is the one that most closely reflects the human IHPS phenotype. These mice exhibit transient pyloric smooth muscle hypertrophy, gastric distension, and failure to gain weight that spontaneously resolves with development (15, 28).

The *hph-1* mouse has a congenital mutation in the gene coding for GTP cyclohydrolase (GTPCH-1), a rate-limiting enzyme in the production of tetrahydrobiopterin (BH4), an essential nNOS cofactor (15, 16, 29). BH4 deficiency results in nNOS uncoupling, leading to an increase in tissue superoxide content and tissue NO deficiency (11, 41, 47). The increased pyloric tone of the newborn *hph-1* mice has been attributed to the BH4-induced tissue NO deficiency (1), without any further consideration given to the potential role of ROS in the sphincter contraction. Poorly understood also is the mechanism accounting for the reduced pyloric tissue nNOS expression reported to be present in the 10-day-old *hph-1* mice (1).

There is accumulating evidence that ROS generation promotes RhoA/ROCK activation in vascular tissue. In the presence of xanthine/xanthine oxidase, rat aorta and pulmonary arteries generate superoxide, and this promotes ROCK activation, which is suppressed in the presence of a Rho kinase inhibitor (20, 23). In diabetic rats with erectile dysfunction, the increased cavernosal tissue superoxide content is associated with upregulation of RhoA/ROCK signaling (27). Superoxide is also known to activate ROCK expression and promote ductus arteriosus constriction through a redox-regulated, positive-feedback mechanism (21). Lastly, in subjects with chronic obstructive pulmonary disease, there is evidence of an interplay between pulmonary endothelial RhoA/ROCK signaling and endothelium NO (eNOS) expression, where upregulation of the former downregulates eNOS expression (5).

ROCK-2 plays an important role in the maintenance of sphincter tone in the lower esophagus and internal layer of anal muscle (35). The extent to which ROCK-2 contributes to pyloric sphincter tone has not been previously examined either physiologically or in subjects with IHPS.

As such, the main objective of this study was to comparatively evaluate the newborn *hph-1* and wild-type C57Bl/6 control animals’ pyloric sphincter tone, ROCK-2, and nNOS tissue expression, as well as ROS generation. We hypothesized that the increased pyloric sphincter tone of newborn *hph-1* mice results from ROS-induced ROCK-2 activation and the consequent reduced nNOS-dependent NO generation.
MATERIALS AND METHODS

Chemicals and reagents. All chemicals and reagents were obtained from Sigma Aldrich (Oakville, ON, Canada), unless otherwise indicated.

Animals. All procedures were conducted in agreement with the Canadian Animals for Research Act (1990) Canadian Council on Animal Care (CCAC) regulations, and the study protocol was approved by the Hospital for Sick Children’s Animal Care Committee. Adult hph-1 mice were bred in house and genotyped to confirm homozygous dominantly inherited GTPCH1 gene deficiency (data not shown). C57Bl/6 mice (Charles River, ON, Canada) were utilized as wild-type controls because this is the background of the hph-1 mice utilized in this study.

All animals were fed regular rodent pellets and housed under standard lighting and temperature conditions. Newborn (either 1–3 or 5–8 days of age) and adult (>21 days) wild-type and hph-1 mice of both sexes were studied. The animals were killed through cervical dislocation (newborn) or pentobarbital sodium (60 mg/kg ip, adult), and the pyloric tissue was quickly excised. Pyloric tissue samples obtained for Western blots were snap frozen in liquid nitrogen and stored for later processing.

Organ bath studies. The pyloric sphincter was quickly removed following death and maintained in ice-cold bubbled Krebs-Henseleit solution (in mM: 115 NaCl, 25 NaHCO3, 1.38 NaHPO4, 2.51 KCl, 2.46 MgSO4·7 H2O, 1.91 CaCl2, and 5.56 dextrose). Pyloric circular smooth muscle rings (average diameter 90–100 µm and length = 2 mm) were dissected free and mounted in a wire myograph (Danish Myo Technology, Aarhus, Denmark). Isometric changes were digitized and recorded (Myodaq; Danish Myo Technology). Tissues were bathed in Krebs-Henseleit buffer bubbled with 95% O2,5% CO2 and maintained at 37°C. After 1 h of equilibration, the optimal tissue resting tension was determined by repeated stimulation with 128 mM KCl until maximum active tension was reached. All subsequent force measurements were obtained at optimal resting tension.

The muscle contraction potential was evaluated in response to either bethanechol (muscarinic receptor stimulant; 10^{-4} M) or U46619 (thromboxane A2 receptor agonist; 10^{-7} M; Cayman Chemical, Ann Arbor, MI). All force measurements were obtained in the presence of nitro-L-arginine methyl ester (L-NAME) (10^{-4} M), propranolol (10^{-5} M), and phentolamine (10^{-5} M) and normalized to the tissue cross-sectional area (length × diameter × 2), as previously reported for vascular tissue (10). Because of its phasic activity and inability to sustain a contraction in response to bethanechol, the pyloric muscle contraction response was measured by determining the area under the curve for force, as reported by others (9, 31, 33, 38, 44). The rate of contraction and relaxation were measured following agonist stimulation, as the slope of their respective phasic activity. Changes in force were analyzed digitally (LabChart 7; AD Instruments, Colorado Springs, CO).

![Fig. 1. Pyloric sphincter force response to 120 mM KCl (A; n = 5 per genotype; normalized to cross-sectional area) and bethanechol (B; n = 4 per genotype; expressed as area under the curve, AUC). The slope of phasic contraction/relaxation in response to bethanechol (C; n = 4 per genotype) and U46619 (D; n = 4 per genotype) and the frequency of contraction for both agonists (E; n = 4 per genotype) are shown. All data were obtained for the wild-type and hyperphenylalaninemia-1 (hph-1) newborn mice (5–8 days old) pyloric muscle. Inset: display of slope of phasic contraction/relaxation and representative tracings from newborn pyloric sphincter stimulated with either bethanechol or U46619 in both genotypes. **P < 0.01 compared with age-matched, wild-type samples by unpaired Student’s t-test.](http://ajpgi.physiology.org/doi/abs/10.1152/ajpgi.00221.2014)
Western blot analysis. Pyloric tissue frozen samples from both C57Bl/6 wild-type control and hph-1 mice were lysed in 10 mM Tris-HCl pH 7.4 lysis buffer containing 1% Triton X-100 and protease/phosphatase inhibitor cocktails (Thermo Fisher Scientific, Rockford, IL) and centrifuged at 14,000 g for 30 min. Protein concentrations were determined via the Bradford assay (Bio-Rad, Mississauga, ON, Canada). Equivalent amounts of lysate proteins in Laemmli buffer were fractionated on SDS-PAGE, transferred to polyvinylidene difluoride membranes, and blotted. Membranes were treated with 5% skim milk and exposed at 4°C overnight for the anti-nNOS (1:1,000; Invitrogen, Carlsbad, CA), anti-myosin phosphatase-targeting subunit 1 (MYPT-1) (Thr 852, the equivalent of the human Thr 853; 1:500; Santa Cruz Biotechnology, Santa Cruz, CA), MYPT-1 (1:1,000; BD Biosciences, Mississauga, ON, Canada), anti-ROCK-2 (1:1,000; Santa Cruz Biotechnology), and α-tubulin (1:10,000; Abcam, Cambridge, MA) antibodies. Appropriate IgGs conjugated with horseradish peroxidase were used as secondary antibodies. The enhanced chemiluminescence (Perkin Elmer, Shelton, CT) reagent was used for detection, and the band intensities were quantified by ImageJ software (National Institutes of Health, Bethesda, MD).

nNOS dimer/monomer ratio. To ascertain the presence of BH4 deficiency-induced nNOS uncoupling, newborn pyloric tissue was lysed in cold buffer containing protease inhibitors and separated on 4% lithium dodecyl sulfate-polyacrylamide gel electrophoresis constantly maintained at 4°C. Proteins were immunoblotted as above described, using nNOS antibody (Life Technologies, Carlsbad, CA). The dimer-over-monomer expression ratio was determined by measuring the respective band densities of Western blot using ImageJ software.

cGMP tissue content. cGMP pyloric tissue content was measured as a surrogate marker of NO generation to comparatively evaluate NOS activity. The commercially available Perkin-Elmer kit (AlphaScreen cGMP assay) was used for this purpose, following the manufacturer’s instructions.

Pyloric H2O2 content. The pyloric tissue H2O2 content was measured as a superoxide generation surrogate marker. For this, pyloric tissue was lysed in cold lysis buffer containing 10 mmol/l Tris-HCl pH 7.4, 1% Triton X-100, and protease/phosphatase inhibitors (Roche Diagnostics Canada, Laval, Quebec, Canada) followed by centrifugation at 14,000 g for 30 min. The lysate H2O2 content was determined by the Amplex Red hydrogen peroxide/peroxidase assay kit (Invitrogen) according to the manufacturer’s protocol. Absorbance was measured at ~560 nm using PolarStar Omega microplate reader (BMG Labtech, Ortenberg, Germany). Tissue total protein was determined by the Bradford method, using Bio-Rad protein assay, and the values were used for H2O2 content data normalization.

Tissue superoxide dismutase-dependent ROS generation. Pyloric tissue was homogenized in 50 mM phosphate buffer containing 1 mM EGTA, pH 7.0, 150 mM sucrose, and protease inhibitors (10 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.75 μg/ml pepstatin, and 0.5 mM PMSF). Protein content was measured by Bradford reagent. Tissue homogenates were divided into two groups. Lucigenin (Invitrogen) was added to the first group of samples to a final concentration of 5 mM and 50 ml of the homogenate-lucigenin mixture was transferred to individual wells of an opaque white 384-well plate. To further confirm the specificity of the measurement for superoxide determination, the s group of tissue homogenates was preincubated with polyethylene glycol-superoxide dismutase (SOD; Sigma) (250 U/ml) for 30 min at 37°C in the dark before we added lucigenin. The reaction was started by the addition of 100 mM NADPH. Chemiluminescence readings were recorded every 3 min over a period of 30 min in a PolarStar Omega microplate reader (BMG Labtech, Cary, NC). Background signals from buffer were subtracted from homogenate signals, and the resulting value was normalized for SOD-inhibitable control signal and for protein concentration.

Tissue oxidative stress via hypoxanthine + xanthine oxidase. Pylorus and fundus tissues from wild-type newborn mice were dissected,
and the mucosa and submucosa layers were carefully removed by sharp dissection. Tissues were preincubated in Krebs-Henseleit solution (115 mM NaCl, 25 mM NaHCO₃, 1.38 mM NaHPO₄, 2.51 mM KCl, 2.46 mM MgSO₄·7 H₂O, 1.91 mM CaCl₂, and 5.56 mM dextrose), bubbled with 95% O₂-5% CO₂ for 1 h at 37°C, and subsequently stimulated with a mixture of 1 mM hypoxanthine and 10 mU/ml xanthine oxidase (Sigma) for 10 min. Immediately after, tissues were immersed in ice-cold mixture of acetone, 10% trichloroacetic acid, and 10 mM dithiothreitol for 2 min, transferred into microcentrifuge tubes, and snap frozen in liquid nitrogen.

Pyloric smooth muscle primary cell culture. The newborn wild-type mice pyloric sphincter smooth muscle cell primary cell culture was obtained as follows. The excised pyloric tissue was incubated in 10% antibiotic-antimycotic solution in PBS for 30 min at room temperature. Tissues were then digested overnight with 1 mg/ml collagenase in DMEM supplemented with 10% FBS and 10% antibiotics/antimycotics medium, serum, and antibiotic/antimycotic solutions (Wisent, St-Bruno, Quebec, Canada). Cell suspension was pelleted by centrifuging at 400 g for 5 min, and the pellet was resuspended in DMEM, containing 10% FBS and 10% antibiotics/antimycotics. Cells were cultured on six-well tissue culture plates until confluency, serum starved for 1 h in serum-free DMEM, and incubated with 2,4-diamino-6-hydroxy-pyrimidine (DAHP) (10⁻⁴ M) or L-NAME (10⁻⁴ M) at 37°C, 5% CO₂. After 48 h, cell proteins were extracted and analyzed by Western blotting as described above.

Statistical methods. Data were first evaluated to determine Gaussian distribution by Skewness, Kurtosis, and Omnibus testing. Normally distributed data were analyzed by parametric data. Genotype and age differences were statistically evaluated by one-way ANOVA with multiple comparisons obtained by the Tukey-Kramer test, or unpaired Student’s t-test, when appropriate. The Mann-Whitney U-test was utilized for nonparametric data. Statistical significance was determined at P < 0.05. All statistical analyses were performed with the Number Cruncher Statistical System software (NCSS, Kaysville, UT). Data are presented as means ± SE.

RESULTS

Pyloric muscle contraction potential is increased in the 1-wk-old hph-1 mice. We first comparatively evaluated the pyloric muscle mechanical properties of 1-wk-old mice of both

Fig. 3. 1-wk-old wild-type and hph-1 (n = 3 per genotype and age) pyloric tissue reactive oxygen species measured as H₂O₂ content (A) and lucigenin-dependent chemiluminescence (B). Data from both assays were normalized to tissue protein content. **P < 0.01, compared with age-matched, wild-type animal samples, by unpaired Student’s t-test.

Fig. 4. 1-wk-old wild-type and hph-1 (n = 3 per genotype and age) pyloric muscle Rho-associated protein kinase 2 (ROCK-2) protein expression (A) and ROCK-2 activity (B) assessed through phospho-myosin phosphatase-targeting subunit 1 (pMYPT-1) content normalized to total MYPT-1 expression (n = 3 per genotype and age) in wild-type and hph-1 mice. Insets: representative Western blots. *P < 0.05. **P < 0.01, compared with age-matched, wild-type control animals, by unpaired Student’s t-test.
genotypes. The pyloric muscle contraction response to KCl and bethanechol stimulation was higher (P < 0.01; Fig. 1, A and B, respectively), and the slope of the agonist-stimulated force development increased (P < 0.01; Fig. 1, C and D) in hph-1 mice, compared with wild-type animals. No genotype difference in the pyloric muscle relaxation rate was observed (Fig. 1, C and D). The frequency of phasic contraction, following bethanechol and U46619 stimulation, was higher in the hph-1 animals, when compared with wild-type control pups (P < 0.01; Fig. 1E).

BH4 deficiency-induced pyloric tissue nNOS changes and ROS generation. In an attempt to determine the mechanism accounting for the greater force development documented in the hph-1 mice, we evaluated the pyloric tissue nNOS expression, enzyme-coupling state, and cGMP generation. As shown in Fig. 2, the 1-wk-old hph-1 mice pyloric tissue nNOS expression, the dimer/monomer ratio, and the cGMP content were all significantly reduced (P < 0.01; Fig. 2, A–C, respectively), when compared with similarly aged wild-type animals. These nNOS changes were associated with a significant increase in pyloric tissue superoxide generation, as determined by the higher H2O2 content and lucigenin-dependent chemiluminescence (Fig. 3, A and B).

The newborn hph-1 pyloric tissue ROCK-2 expression/activity is increased. Given the known interplay between ROCK activity and nNOS expression in vascular tissue (20, 23), we proceeded to evaluate the genotype differences in pyloric tissue ROCK-2 expression and activity. ROCK-2 protein expression (Fig. 4A) and activity (Fig. 4B) were significantly increased (P < 0.05) in 1-wk-old hph-1 mice, when compared with age-matched controls.

To determine whether the hph-1 pyloric tissue ROCK-2 activity upregulation is causally related to the ROS increase, we induced O2•− generation in newborn wild-type pyloric and fundal tissues in vitro by incubating them with hypoxanthine and xanthine oxidase. ROS exposure markedly increased pyloric tissue ROCK-2 activity (Fig. 5). The newborn hph-1 pyloric tissue ROCK-2 expression/activity is increased.

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Fig. 5. 1-wk-old pyloric (A) and fundal (B) tissue ROCK-2 activity assessed as pMYPT-1 levels normalized to total MYPT-1 levels (n = 4 and 3 per group, respectively) in the absence and presence of hypoxanthine/xanthine oxidase (HX/XO). **P < 0.01 when compared with untreated (control) tissue by unpaired Student’s t-test. Newborn primary pyloric smooth muscle cells H2O2 content (C, normalized to tissue protein content; n = 3 per group) and ROCK-2 activity (D, n = 3 per group) in the absence and presence of 2,4-diamino-6-hydroxy-pyrimidine (DAHP) or nitro-L-arginine methyl ester (l-NAME). Representative Western blots are shown. *P < 0.05, **P < 0.01 compared with untreated control cells by 1-way ANOVA and Tukey-Kramer multiple-comparison testing.

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Fig. 6. Stomach content/body weight ratio in mice 1–3 days old (n = 7 for both genotypes), 6–7 days old (n = 11 and 15), and adults (n = 8 and 4) wild-type and hph-1 mice, respectively. Adult data here shown were previously reported by our research group (47). **P < 0.01 compared with age-matched, wild-type controls by Mann-Whitney U-test.
loric tissue ROCK-2 activity, whereas no changes were observed in similarly aged fundal tissue (Fig. 5, A and B).

Primary wild-type newborn mice pyloric smooth muscle cells were then utilized to determine the BH4 and NO deficiency on ROS generation and ROCK-2 activity. Near-confluency cells incubated with DAHP (GTPCH-1 inhibitor) to induce BH4 deficiency resulted in significantly higher H₂O₂ generation (Fig. 5C) and increased ROCK-2 activity (Fig. 5D), when compared with the untreated group. NO deficiency induced via L-NAME-induced nNOS inhibition had no effect on the cell H₂O₂ generation but equally increased ROCK-2 activity (Fig. 5D). These results strongly suggest that BH4 deficiency upregulates pyloric muscle ROCK-2 activity via ROS generation and reduced nNOS-derived cGMP.

**Pyloric tissue age-dependent changes.** Lastly, we conducted age-dependent comparative studies to address the mechanism accounting for the transient nature of gastric content stasis in *hph-1* mice. As we have previously reported, the stomach content/body weight ratio is no longer increased in adult *hph-1* mice, when compared with wild-type control animals, indicating that aging normalizes the increased pyloric sphincter tone (47). The *hph-1* mice are BH4 deficient from birth and thus expected to show increased pyloric sphincter tone in the immediate postnatal period. However, when the stomach content/body weight ratio was comparatively measured between 1–3 days of age, no genotype differences were found, as opposed to the higher *hph-1* mice values documented for pups 6–7 days old, when compared with age-matched, wild-type control animals (Fig. 6). The higher stomach content/body weight ratio of *hph-1* pups 6–7 days old, when compared with age-matched, wild-type pups, was independent of the animal’s sex (female 0.025 ± 0.005, *n* = 6; male 0.025 ± 0.005, *n* = 8).

To further evaluate the factors accounting for the apparently normal gastric emptying immediately after birth and adulthood, we comparatively measured nNOS expression and ROCK-2 activity in pyloric tissue from mice 1–3 days old and 10–12 months old. As we have previously reported (47), in newborn *hph-1* mice, reduced nNOS expression (Fig. 7A and B) and increased ROCK-2 activity (Fig. 7C and D) were observed. The expression and activity of ROCK-2 are also increased in adult *hph-1* mice (Fig. 7A and B), whereas no changes were observed in similarly aged fundal tissue (Fig. 5A and B).

**Fig. 7.** Newborn mice 1–3 days old and adult wild-type and *hph-1* pyloric tissue nNOS content (A and B, respectively; normalized to tissue tubulin expression), ROCK-2 activity (C and D) measured as pMYPT-1 total MYPT-1 content ratio (*n* = 3 per genotype and age), and H₂O₂ content (E and F) (*n* = 3 per genotype; normalized to tissue protein content). Representative Western blots are shown.
adult mice of both genotypes. No significant genotype differences for both parameters were documented at either age (Fig. 7, A and B, and C and D, respectively). To ascertain whether the lack of genotype differences for these parameters related to reduced or absent nNOS uncoupling in hph-1 mice, we comparatively measured the tissue H$_2$O$_2$ content. As shown in Fig. 7, E and F, the hph-1 pyloric tissue H$_2$O$_2$ content was not statistically different at both ages, when compared with wild-type control values.

**DISCUSSION**

In the present study, we confirmed that nNOS expression is downregulated in the newborn hph-1 mice pyloric tissue, as documented by others in these animals at 10 days of age (1). However, novel data derived from this study indicate that the hph-1 pyloric tissue nNOS downregulation is a secondary phenomenon and not primarily related to the gastric stasis observed in these animals. Our findings indicate that both a reduction in nNOS-derived NO and ROS-induced ROCK-2 upregulation account for the newborn hph-1 mice increased pyloric sphincter muscle tone. The proposed signaling pathway involved in these changes is illustrated in Fig. 8.

The hph-1 newborn mouse is the most suitable animal model for the study of the IHPS pathobiology because these animals exhibit the transient manifestations of pyloric stenosis present in the human condition (1, 47), except for lack of sex predilection and absent vomiting. However, rodents lack the vomiting reflex, and this precludes them from getting as maldonated and dehydrated as seen in human infants with this condition (12, 43).

The mechanism accounting for the age-dependent pyloric changes in hph-1 mice is unclear but likely related to the degree of nNOS uncoupling and tissue BH4 content. In this present study, we showed that, in the immediate postnatal period (1–3 days of age), there is no evidence of pyloric tissue nNOS uncoupling in the hph-1 mice. Such conclusion is based on the lack of genotype differences in pyloric tissue H$_2$O$_2$ content, nNOS, and ROCK-2 expression in mice 1–3 days old.

We propose that the lack of changes in these parameters accounts for the absence of gastric stasis immediately after birth, in hph-1 mice. However, despite being BH4 deficient from birth, the hph-1 pyloric tissue increased H$_2$O$_2$ content is not present throughout life. The factors accounting for the absent nNOS uncoupling during the first 3 days of life in hph-1 mice are presently unclear but possibly related to either age-dependent changes in enzyme activity or tissue BH4 content. Arrigoni et al. (4) have shown that the porcine lung eNOS activity increases with age during the first week of life, and a similar developmental pattern may exist for nNOS activity. A lower nNOS activity early in life in hph-1 mice pylorus may limit ROS generation, thus preventing ROCK-2 upregulation. Alternatively, the absence of nNOS uncoupling in the hph-1 mice immediate postnatal period is related to the fact that BH4 can cross the placenta (25, 32), thus precluding BH4 deficiency during fetal and early neonatal life. As the animals age, the hph-1 mice tissue BH4 content is known to increase to 50% of wild-type control values (16). This age-related increase in pyloric tissue BH4 content explains the normalization of their delayed gastric emptying because ROS generation is no longer increased in hph-1 mice, when compared with wild-type control animals.

To date, the pyloric sphincter ROCK-2 expression and activity have not been investigated. Compared with other non-sphincter gastrointestinal tissue, the sphincter muscles exhibit both phasic and tonic activity, and the maintenance of tone is mostly dependent on ROCK-2 and 17-kDa PKC-potentiated protein phosphatase 1 inhibitor activity (6, 7). In adult rat (36) and human (39) lower esophageal sphincters, ROCK inhibitors abolish both spontaneous basal and nerve-evoked contractions. Recently, we reported a similar response to ROCK-2 inhibition in the newborn rat lower esophageal sphincter (48). In the present study, we documented a direct relationship between pyloric tissue ROCK-2 expression/activity and the magnitude of agonist-induced muscle contraction in newborn mice.

On the basis of its involvement in the lower esophageal and internal anal sphincter tone modulation, we postulated and confirmed that ROCK-2 plays an equally important role in the pyloric muscle. It is known that ROS (in particular superoxide) through a redox and RhoA-regulated mechanism increases ROCK expression and activity of pulmonary artery vascular and cavernosum smooth muscle (23, 27). Interestingly, in the present study, nNOS inhibition with the nonspecific blocker l-NAME also increased ROCK-2 activation in primary pyloric smooth muscle cells, suggesting that both ROS and reduced NOS-dependent cGMP generation are key regulators of pyloric tissue ROCK-2 activation. nNOS knockout mice exhibit pyloric hypertrophy and gastric stasis that does not resolve with age (1, 14). However, the pyloric sphincter of these animals has normal basal muscle motility and myogenic activity compared with wild-type controls (40).

To ascertain the mechanism accounting for the differential effects of BH4 deficiency in fundal and pyloric sphincter smooth muscle, we evaluated these tissues in newborn wild-type mice. A short-term exposure to hypoxanthine + xanthine oxidase induces oxidative stress via superoxide generation (19), and this oxidative stress pathway is functional in gastrointestinal tissue (3). With the use of this approach, a significant difference in the ROCK-2 activation response was noted when...
comparing wild-type neonatal fundal and pyloric tissue. The oxidative stress-induced ROCK-2 activation in pyloric, but not fundal, tissue mirrored the enhanced contractile potential of the hph-1 newborn sphincter muscle, when compared with age-matched, wild-type animals. This suggests that the differential effects of BH4 deficiency in fundal and pyloric sphincter tissue are related to the oxidative stress-dependent ROCK-2 activation response.

The interaction between ROCK-2 and nNOS is complex, and there is reason to speculate that ROCK-2 activation downregulates pyloric tissue nNOS expression. In human endothelial cells, RhoA (activator of ROCK) negatively regulates eNOS expression through RhoA geranylation inhibition, which is essential for RhoA membrane binding (26). Others have also shown that, in human umbilical vein endothelial cells, RhoA/ROCK, not only downregulates eNOS gene expression, but also inhibits eNOS phosphorylation, which is essential for the enzyme activation (30).

Limited data are available on the pyloric muscle function of subjects with IHPS. Pyloric sphincter manometry measurements obtained in IHPS subjects (average 42 days old) showed abnormal motor activity characterized by high-amplitude spasitic contractions of the pylorus (18, 22). The present study data suggest that enhanced contraction, as opposed to abnormal relaxation, accounts for the newborn hph-1 mice gastric stasis. This is based on the fact that, in response to agonist stimulation, the hph-1 pyloric muscle frequency of contraction, time to reach maximum force, and the strength of the contraction are all increased, when compared with wild-type control animals. The pyloric muscle rate of relaxation after agonist-induced stimulation was not different among genotypes.

Whether BH4 deficiency plays a role in the IHPS pathogenesis remains unclear. Breast milk, as opposed to infant formulas, has a high BH4 content (46), and IHPS rarely occurs in breastfed infants that are exclusively breastfed (24). However, in a small study involving only six infants, BH4 supplementation failed to reverse the IHPS clinical manifestations (8). To the extent of our knowledge, no other clinical trials have been conducted where the therapeutic effect of BH4 was tested in IHPS.

In summary, we confirmed that the pyloric tissue nNOS expression is downregulated at 1 wk of age in hph-1 mice, when compared with wild-type controls. We propose that this finding is a downstream effect, as opposed to the main factor responsible for gastric stasis in the hph-1 mice. To the extent that these data can be extrapolated to the human condition, ROS generation may account for the pathobiology of IHPS. Further investigation of ROS-mediated ROCK-2 signaling in neonatal pyloric tissue and the potential beneficial effect of exogenous BH4 supplementation is warranted.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: C.W., Y.S., and J.B. conception and design of research; C.W., Y.S., and J.P. performed experiments; C.W., Y.S., and J.B. analyzed data; C.W., Y.S., J.P., and J.B. interpreted results of experiments; C.W. and Y.S. prepared figures; C.W. and Y.S. drafted manuscript; C.W., Y.S., and J.B. approved final version of manuscript.

REFERENCES