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

Christopher Welsh¹, Yulia Shifrin¹, Jingyi Pan¹ and Jaques Belik¹, ²

¹Physiology & Experimental Medicine Program, Hospital for Sick Children Research Institute; ²Department of Paediatrics and Physiology, University of Toronto, Toronto, Ontario, CANADA M5G 1X8

Authors Contributions: CW and YS contributed equally.

Study concept and manuscript writing: Christopher Welsh, Yulia Shifrin, Jaques Belik

Development of methodology and data acquisition: Christopher Welsh, Yulia Shifrin, Jingyi Pan

Corresponding Author

Jaques Belik MD
The Hospital for Sick Children Toronto
555 University Avenue
Toronto, Ontario M5G 1X8
Canada

Phone: 416-813-2165
Fax: 416-813-5771
E-mail: jaques.belik@sickkids.ca

Running Title: Rho-kinase and infantile pyloric stenosis
Abstract

Infantile hypertrophic pyloric stenosis (IHPS) is a common disease of unknown etiology. The tetrahydrobiopterin (BH4) - deficient *hph-1* newborn mouse has a similar phenotype to the human condition. *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, as well as, ROCK-2 expression and activity were evaluated. Primary pyloric smooth muscle cells from wild-type newborn animals were utilized to evaluate the effect of tetrahydrobiopterin (BH4) deficiency. One-week old *hph-1* mice exhibited a 4-fold 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, while the rate of nNOS uncoupling increased (p<0.01) in one-week 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 *hph-1* mouse’s gastric emptying rate 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, 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.

Key Words: superoxide, rho-associated protein kinase, smooth muscle
**Introduction**

Sphincter tone is determined by the balance between the neuronal nitric oxide (NO) synthase (nNOS)-dependent relaxant and the rho-associated protein kinase 2 (ROCK-2)-dependent contraction effects. nNOS is the most abundant NOS isoform in gastrointestinal smooth muscle (42) and its NO generation potential depends on the enzyme’s coupled dimeric state. 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 1000 live births (34) and manifesting clinically at 3-6 weeks 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 *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 tetrahydrobiopterin (BH4), an essential nNOS cofactor (15, 17, 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 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 cavernosum tissue superoxide...
content is associated with up-regulation 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 NOS (eNOS) expression where upregulation of the former downregulate 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 hyperphenylalaninemia-1 (*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, since 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 MgSO₄·7H₂O, 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). Isometric changes were digitized and recorded (Myodaq; Danish Myo Technology, Aarhus, Denmark). 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 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). Due to 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, USA).

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 Inc., Rockford, IL), and centrifuged at 14,000 g for 30 min. Protein concentrations were determined via the Bradford assay (Bio-Rad, Mississauga, ON, CA). Equivalent amounts of lysate proteins in Laemmli buffer were fractionated on SDS-PAGE, transferred to polyvinylidene di-fluoride membranes and blotted. Membranes were treated with 5% skim milk and exposed at 4°C overnight for the anti-nNOS (1:1000; Invitrogen, Camarillo, CA), anti-MYPT-1 (Thr 852, the equivalent of the human Thr 853; 1:500; Santa Cruz, Santa Cruz, CA) and MYPT-1 (1:1000; BD Biosciences, Mississauga, ON), anti-ROCK-2 (1:1000; Santa Cruz, CA) and α-tubulin (1:10000; Cambridge, MA) antibodies. Appropriate IgGs conjugated with HRP were used as secondary antibodies. The enhanced chemiluminescence (ECL, Perkin Elmer, Shelton, Connecticut, USA) reagent was used for detection and the band intensities were quantified by ImageJ software (National Institutes of Health, Bethesda, USA).

nNOS dimer/monomer ratio

In order to ascertain for 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 technology, Carlsbad, CA, USA). The
dimer over monomer expression ratio was determined by measuring the Western blot’s respective band
densities using ImageJ software.

cGMP tissue content
Cyclic guanine monophosphate (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, Montreal Quebec, Canada) was used for this purpose, following
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 13,000g for 30 min. The lysate H₂O₂ content was determined by the Amplex® Red
hydrogen peroxide/peroxidase assay kit (Molecular Probes, Invitrogen, Carlsbad, CA, USA) according to
the manufacturer’s protocol. Absorbance was measured at ~560 nm using POLARstar Omega microplate
reader (BMG LABTECH GmbH, Ortenberg, Germany). Tissue total protein was determined by Bradford
method, using Bio-Rad protein assay (Bio-Rad, Mississauga, ON, Canada) and the values used for H₂O₂
content data normalization.

Tissue superoxide dismutase (SOD)-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, Life technologies, Carlsbad, CA, USA) 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. In order to further confirm the specificity of the measurement for superoxide determination, the second group of tissue homogenates were pre-incubated with PEG-superoxide dismutase (SOD; Sigma) (250 U/ml) for 30 minutes at 37°C in the dark prior to adding 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, USA). 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 tissue from wild-type newborn mice were dissected and the mucosa and submucosa layers carefully removed by sharp dissection. Tissues were pre-incubated 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, St. Louis, CA, USA) 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 mouse 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 1mg/ml collagenase in DMEM supplemented with 10% FBS and 10% antibiotics/antimycotics medium, serum and antibiotic/antimycotic solutions (Wisent, St-Bruno, QB, CA). Cell suspension was pelleted by centrifuging at 400g for 5 minutes and the pellet was re-suspended in DMEM, containing 10% FBS, 10% antibiotics/antimycotics. Cells were cultured on 6-well tissue culture plate until confluency, serum-starved for 1h 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 48h 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 analysis of variance (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, Utah, USA). Data are presented as means±SEM.

Results

Pyloric muscle contraction potential is increased in the one-week old hph-1 mice
We first comparatively evaluated the pyloric muscle mechanical properties of one-week old mice of both genotypes. The pyloric muscle contraction response to KCl and bethanechol stimulation was higher (P<0.01; Figure 1A and B respectively) and the slope of the agonist-stimulated force development increased (P<0.01; Figure 1C,D) in hph-1 mice, as compared with wild-type animals. No genotype difference in the pyloric muscle relaxation rate was observed (Figure 1C,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; Figure 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 Figure 2, the one-week old hph-1 mice pyloric tissue nNOS expression, the dimer/monomer ratio and the cGMP content were all significantly reduced (P<0.01; Figure 2A-C respectively), when compared with similar age 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 (Figure 3 A,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 (Figure 4A) and activity (Figure 4B) were significantly increased (P<0.05) in one-week old hph-1 mice, when compared with age-matched controls.

In order 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 (HX/XO). ROS exposure markedly increased pyloric tissue ROCK-2 activity whereas no changes were observed in similar age fundal tissue (Figure 5A,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 (Figure 5C) and increased ROCK-2 activity (Figure 5D), when compared with the untreated group. NO deficiency induced via L-NAME-induced nNOS inhibition had no effect on the cells H₂O₂ generation, but equally increased ROCK-2 activity (Figure 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. Yet, 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 6-7 days old pups, when compared with same age, wild-type control animals (Figure 6). The higher stomach content/body weight ratio of 6-7 days of age hph-1 pups, when compared with same age 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 1-3 days old and adult mice of both genotypes. No significant genotype differences for both parameters were documented at either age (Figure 7A,B and C,D respectively). In order to ascertain as to 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 Figure 7E,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). Yet, 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 Figure 8.

The hph-1 newborn mouse is the most suitable animal model for the study of the IHPS pathobiology, since 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. Yet, rodents lack the vomiting reflex and this precludes them from getting as malnourished 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 d 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 1-3 day old mice. We propose that the lack of changes in these parameters account for the absence of gastric stasis immediately after birth, in hph-1 mice. Yet, in spite of 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. have shown that the porcine lung eNOS activity increases with age during the first week of life (4) 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 since 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 CPI-17 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.
Based on 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 Rho-A 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 nNOS-dependent cGMP generation are key regulators of pyloric tissue ROCK-2 activation. nNOS knock-out mice exhibit pyloric hypertrophy and gastric stasis that does not resolve with age (1, 14). Yet, the pyloric sphincter of these animals has normal basal muscle motility and myogenic activity when compared to wild-type controls (40).

In order 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). Utilizing 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 same age 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 spastic contractions of the pylorus (18, 22). The present study data suggest that enhanced contraction, as opposed to abnormal relaxation, account 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 post agonist-induced stimulation was not different amongst 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 infants that are exclusively breastfed (24). Yet, in a small study involving only six infants, BH4 supplementation failed to reverse the IHPS clinical manifestations (8). To the best 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 one week 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 this 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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**Figures**

**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). The slope of phasic contraction/relaxation in response to bethanechol (C; N=4 per genotype) and U46619 (D; N=4 per genotype), as well as, the frequency of contraction for both agonists (E; N=4 per genotype) is shown. All data were obtained from for the wild-type and hph-1 newborn mice (5-8 d) pyloric muscle. *Insert:* 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 when compared to age-matched wild-type samples by unpaired Student’s t-test.**

**Fig. 2.** One-week old wild-type and hph-1 (N=3-4 per genotype and age) pyloric tissue neuronal nitric oxide synthase (nNOS) protein expression (A), cyclic guanosine monophosphate (cGMP) content (normalized to tissue weight) (B) and nNOS dimer / monomer ratio (C). Representative Western blots are shown. *P<0.05, **P<0.01, when compared to age-matched wild-type animal’s samples by unpaired Student’s t-test.

**Fig. 3.** One-week 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). Both assays data were normalized to tissue protein content. *P<0.05, **P<0.01, when compared to age-matched wild-type animal’s samples, by unpaired Student’s t-test.
**Fig. 4.** One-week old wild-type and *hph-1* (N=3 per genotype and age) pyloric rho-associated protein kinase 2 (ROCK-2) protein expression (A) and ROCK-2 activity (B) assessed through pMYPT-1 content normalized to total MYPT-1 expression (N=3 per genotype and age) in wild-type and *hph-1* mice. Figure inserts show representative Western blots. *P<0.05, **P<0.01, when compared to age-matched wild-type control animals, by unpaired Student’s t-test.

**Fig. 5.** One-week 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 H$_2$O$_2$ 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 DAHP or L-NAME. Representative Western blot are shown. *P<0.05, **P<0.01 when compared to untreated control cells by one-way ANOVA and Tukey-Kramer multiple comparison testing.

**Fig. 6.** A: Stomach content/body weight ratio in 1-3 d (N=7 for both genotypes), 6-7 d (N=11 and 15) and adults (N=8 and 4) wild-type and *hph-1* mice respectively. Adult data here shown was previously reported by our research group (47). **P<0.01 when compared to age-matched wild-type controls by Mann-Whitney U test.

**Fig. 7.** A: Newborn 1-3 d 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 H$_2$O$_2$ content (E and F) (N=3 per genotype; normalized to tissue protein content). Representative Western blots are shown.
Fig. 8. Outline of the proposed mechanism by which tetrahydrobiopterin (BH4)-deficiency promotes increased pyloric sphincter tone and neuronal nitric oxide synthase (nNOS) expression downregulation in the newborn hph-1 mice. cGMP: intracellular increase in cyclic guanosine monophosphate; ROCK-2: rho-associated protein kinase.


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**Figure 1**

A) Force (mN/mm²) response to Bethanechol and U46619 in Wild-type and *hph-1* mutant cells.

B) AUC (mN*s) response to Bethanechol and U46619 in Wild-type and *hph-1* mutant cells.

C) Slope of phasic peaks (mN/s) response to Bethanechol in Wild-type and *hph-1* mutant cells.

D) Slope of phasic peaks (mN/s) response to U46619 in Wild-type and *hph-1* mutant cells.

E) Frequency of phasic contractions (s⁻¹) response to Bethanechol and U46619 in Wild-type and *hph-1* mutant cells.
Figure 2

A

nNOS / Tubulin

Wild-type  hph-1

B

cGMP (pmol/mg tissue)

Wild-type  hph-1

C

dimer/monomer ratio

Wild-type  hph-1
Figure 3

**A**

H$_2$O$_2$ Content (µmol/µg protein)

- Wild-type
- hph-1

**B**

Chemiluminescence/mg protein

- Wild-type
- hph-1
Figure 4

A

B
Figure 5

(A) Pylorus Smooth Muscle Cells

(B) Fundus Smooth Muscle Cells

(C) 

H₂O₂ Content (μmol/μg protein)

Control  DAHP  L-NAME

(D) 

pMYPT-1/MYPT-1

Control  DAHP  L-NAME
Figure 6

Stomach content: Body weight

Wild-type

$\text{hph-1}$

1-3 d  6-7 d  Adult

**
Figure 7

1-3 d Newborn

A

nNOS/Tubulin

Wild-type  hph-1

B

nNOS/Tubulin

Wild-type  hph-1

C

pMYPT-1/MYPT-1

Wild-type  hph-1

D

pMYPT-1/MYPT-1

Wild-type  hph-1

E

H₂O₂ Content (µmol/µg protein)

Wild-type  hph-1

F

H₂O₂ Content (µmol/µg protein)

Wild-type  hph-1

Adult
Figure 8

nNOS $\rightarrow$ BH4 Deficiency $\rightarrow$ nNOS uncoupling $\rightarrow$

- $\uparrow$ Superoxide
- $\downarrow$ cGMP

- $\uparrow$ ROCK-2

- $\uparrow$ Pyloric sphincter tone

$\downarrow$ nNOS expression