Gastric and pyloric sphincter muscle function and the developmental-dependent regulation of gastric content emptying in the rat

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Running head: Developmental changes in gastropyloric motor function
Abstract

Background: Feeding intolerance is a common issue in the care of preterm neonates. The condition manifests as delayed gastric content emptying and represents a therapeutic challenge, since the factors accounting for its manifestations are unknown. The main goal of this study was to comparatively investigate the age-related rat gastric and pyloric smooth muscle function and their putative regulators. We hypothesized that a reduced gastric muscle contraction potential early in life, contributes to the delayed gastric emptying of the newborn. Methodology: Newborn and adult rat gastric (fundal) and pyloric sphincter tissues were comparatively studied in vitro. The tissue-specific dissociated smooth muscle cells (SMC) shortening properties were evaluated and the key regulatory proteins Rho-associated kinase 2 (ROCK-2) and myosin light chain kinase (MLCK) expression determined. Results: Adult gastric and pyloric SMC shortening was significantly greater, when compared with the respective newborn counterpart. MLCK and ROCK-2 expression were developmentally regulated and increased with age. The newborn pyloric sphincter muscle expresses a higher neuronal nitric oxide synthase and phosphorylated vasodilator-stimulated phosphoprotein content, when compared with adult tissue. Conclusions: As compared with later in life, the newborn rat gastropyloric muscle has a Ca\(^{2+}\)-related reduced potential for contraction and the pyloric sphincter relaxation-dependent modulators are overexpressed. To the extent that these rodent data can be extrapolated to humans, the neonatal delayed gastric emptying reflects the reduced stomach muscles contraction potential, as opposed to increased pyloric sphincter tone.
Introduction

Feeding intolerance, defined as the presence of a gastric residual volume greater than 50% (34), is one of the most common clinical issues in preterm neonates (19). The factors accounting for delayed gastric emptying in neonates are presently unknown. Their identification, however, is of importance since parenteral nutrition is associated with intravenous catheter complications, such as septicemia, and a longer hospital stay (42).

There is reason to believe that immaturity-related motor function changes account for the preterm infants’ delayed gastric content emptying. Amniography studies of the gastrointestinal motility conducted 50 years ago suggested that the human fetal bowel is inactive prior to 30 weeks’ gestation (33). More recent data demonstrate that fetal gastric emptying activity is absent before 24 weeks’ gestation and progressively increases with advancement of fetal maturity prior to term (41).

Duodenal-jejunal intraluminal pressure recordings obtained in preterm neonates confirmed that below 31 weeks’ gestation the intestinal motor activity was characterized by random and poorly organized contractions of low amplitude (8). Only at term gestation a well-defined intestinal motor activity with features similar to the ones present later in life was documented in neonates (8). This age-dependent pattern of a more organized function was also noted in the gastric antrum, where intragastric pressures of those below 32 weeks amounted to 50% of the values documented in term infants (8).

Gastric content emptying is dependent on a coordinated gastropyloric motor function involving fundic and antrum muscle contraction and pyloric sphincter relaxation (10). In adults, abnormalities in either of these two processes characterizes gastroparesis (43).

Over 20 years ago, age-dependent changes in agonist-induced force were reported in rabbits, cats and guinea-pig gastric fundus and antrum muscle strips (22, 23, 35, 48). These changes are believed to be...
related to a reduced intracellular Ca\(^{2+}\) mobilization following agonist stimulation in the newborn, as compared with the adult gastric muscle (23). In response to an increase in intracellular Ca\(^{2+}\) content, the smooth muscle myosin light chain kinase (MLCK) is activated and this pathway has a key role in the regulation of gastrointestinal motility (21). We previously reported developmental changes in vascular MLCK expression (2). Following acetylcholine-induced stimulation, Ca\(^{2+}\)-regulated myosin light chain phosphorylation was reported not to be age-dependent (26), but the MLCK expression developmental pattern was not evaluated. Lastly, Ca\(^{2+}\) sensitization, via rho-associated kinase 2 (ROCK-2), is equally important in the regulation of gastric muscle contraction (46), but its developmental regulation is unknown.

Developmental-dependent data regarding the pyloric sphincter muscle are also lacking. That increased pyloric sphincter tone can manifest early in life and interfere with gastric emptying is best exemplify by a pathological condition named infantile hypertrophic pyloric stenosis (36). The increased sphincter tone in infants with this condition is likely related to reduced pyloric tissue nNOS expression (1) and the resulting lower NO generation (25, 36, 44). In the rodent model of this disease, we have previously shown that increased pyloric sphincter tone is transiently present (47).

Therefore, the goal of the present study was to comparatively evaluate the developmentally-dependent gastric and pyloric sphincter motor function in rats. We hypothesized that developmental-dependent changes in ROCK-2 and MLCK expression in the gastric muscle and NO generation by the pyloric sphincter contribute to the reduced gastric emptying potential early in life.

**Material 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 and Canadian Council on Animal Care regulations, and the study protocol was approved by the Hospital for Sick Children’s Animal Care Committee. Sprague Dawley Rats (Charles River, Montreal, Canada) were utilized in this study.

The adult animals were fed regular rodent pellets and housed under standard lighting and temperature conditions. One-week old, two-weeks of age and adult (> 21 days) rats of both sexes were studied. The animals were killed through cervical dislocation (newborn) or pentobarbital sodium (60 mg/kg ip- adult) and the gastric as well as pyloric tissue was quickly excised. Tissue samples obtained for Western blots were snap-frozen in liquid nitrogen and stored for later processing.

Organ bath studies

The gastric fundus 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).

The fundus strips (average length and width were 8.9 mm and 4.5 mm) were dissected free, at either end with 6x10mm flat surface tissue clips and submerged into a 10 mL tissue bath (Radnoti LLC, Monrovia, California) filled with Krebs-Henseleit solution at 37°C, pH 7.4 and bubbled with 95% O2/5% CO2. One end of the muscle strip was fixed to the bottom of the tissue bath and the top clamp tied to an isometric force transducer myograph (Harvard Apparatus, Saint-Laurent, QC, Canada) using 7-0 braided silk. Isometric changes were digitized and recorded (LabChart Pro, ADInstruments Inc., Colorado Springs CO, USA). 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 128 mM KCl or electrical field stimulation (EFS). EFS was employed as previously reported (25), using a commercially available stimulator (Cibertec, Madrid, Spain) as follows: 80 V [voltage] stimulation, 0.5 msec pulses with 20-second trains of at a frequency of 5 Hz. Three stimulations obtained 10 min apart were employed and averaged to determine the EFS-induced force increase. Force was normalized to the tissue cross-sectional area (length*diameter*2), as previously reported for vascular tissue (17, 18).

Freshly dispersed smooth muscle cells (SMC)

As compared with the sustained agonist-induced tonic force obtained in gastric fundal muscle, the antrum and pyloric sphincter counterpart exhibit irregular phasic activity (Figure 1 A,B,C) that makes age-dependent comparisons problematic. For this reason, to comparatively assess age- and tissue-specific muscle contraction, we elected to measure SMC shortening in response to acetylcholine stimulation, as used by others (32).

Rat gastropyloric tissues were digested in 1ml/mg collagenase and 0.01% Soybean Trypsin Inhibitor (ThermoFisher, Burlington, Ontario, Canada) for 1 h at 37°C, and the smooth muscle cells were dispersed in DMEM cell culture media (Wisent, St. Bruno, Quebec, Canada). One hour after dispersion the SMCs were exposed to acetylcholine at a final media concentration of 10^-4 for 15 min at 37°C and immediately fixed with 1% acrolein. Cells maintained for a similar duration in Calcium-free PBS media served as control, to prevent spontaneous contraction of the smooth muscle cells. Light microscopy images at X20 magnification were obtained using Leica DM IRE2 microscope (Wetzlar, Germany), and the cells length was measured using ImageJ (NIH, Bethesda, MA, USA). Acetylcholine-induced cells length changes (shortening) were expressed as percentage of average control SMC length. Figure 1 D,E
illustrate a control and acetylcholine-stimulated SMC, respectively. A minimum of 50 agonist-stimulated and control cells from each tissue and age group were evaluated. The cells were obtained from 12 newborns and 10 adult animals and used immediately after tissue retrieval.

**Western blot analysis**

Freshly dissected rat pylorus, fundus and antrum tissues were immediately frozen in liquid nitrogen. Alternatively, tissues were subjected to collagenase digestion as described above, and dissociated smooth muscle cells were pelleted by centrifugation at 200 G for 10 min at room temperature. Tissues and cell pellets were then homogenized in 10 mmol/l Tris–HCl pH 7.4 lysis buffer-containing 1% Triton X-100 and protease/phosphatase inhibitors (Roche Diagnostics Canada, Laval, Quebec, Canada). Protein content was determined by Bradford method using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, US). Equal amounts of lysate proteins in Laemmli buffer were separated by SDS-PAGE, transferred onto PVDF membrane and immunoblotted using the following antibodies: mouse nNOS (1:1000, 1:1000; Invitrogen, Camarillo, CA, US), rabbit phosphorylated vasodilator-stimulated phosphoprotein (pVASP) (1:3000; Cell Signaling Technology, Danvers, MA, US), rabbit VASP (1:3000, Cell Signaling Technology, Danvers, MA, US), mouse tubulin (1:5000, 1:1000; Invitrogen, Camarillo, CA, US), rabbit pMYPT1 (Thr 853; 1:500; Santa Cruz, Santa Cruz, CA, US), mouse MYPT-1 (1:1000; BD Biosciences, Mississauga, ON, Canada), goat ROCK-2 (1:1000; Santa Cruz, CA, US), mouse MLCK (1:1000; Sigma-Aldrich, Oakville, ON, Canada), anti-mouse IgG HRP-conjugated (1:10000, Sigma-Aldrich, Oakville, ON, Canada), anti-rabbit IgG HRP-conjugated (1:5000, Cell Signaling Technology, Danvers, MA, US), and anti-goat IgG HRP-conjugated (1:5000; Santa Cruz, Santa Cruz, CA, US). Detection was performed with the enhanced chemiluminescence reagent (Perkin Elmer, Shelton, CT, US). Band intensities were quantified using ImageJ (National Institutes of Health-NIH, Bethesda, MD, US) and expressed relative to tubulin.

**Statistical methods**
Data were first evaluated to determine Gaussian distribution by Skewness, Kurtosis and Omnibus testing. Normally distributed data were analyzed by parametric data. Age differences were statistically evaluated by unpaired Student's t-test, or one-way analysis of variance (ANOVA) with multiple comparisons obtained by the Tukey-Kramer test, when more than two groups were assessed. 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

Smooth muscle contraction potential

Newborn and adult fundal muscle strips were comparatively evaluated to determine their force-generating potential. Smooth muscle contraction induced either with KCl and carbachol, or via EFS-mediated nerve stimulation resulted in a significantly higher force in adult tissue, when compared with newborn strips (Figure 2).

The smooth muscle agonist-induced shortening potential was comparatively assessed in one week, two-week old and adult fundal and pyloric SMCs. The magnitude of the acetylcholine-induced fundic and pyloric sphincter SMC shortening was age-dependent and significantly greater in adult, when compared with cells derived from one- and two-week old animals (Figure 3A and 3B). To ascertain whether there are differences in contraction potential between antrum- and fundus-derived SMCs, we comparatively evaluated these cells in one-week and adult rats. No significant tissue difference in the magnitude of agonist-induced SMC shortening was seen at either age (Figure 3C and 3D).

ROCK-2 and MLCK
In order to evaluate the developmental changes in the agonist-induced Ca\textsuperscript{2+} sensitization, we proceeded to evaluate the tissue-specific SMC ROCK-2 and MLCK expression. When compared with newborn samples, both fundus and pyloric sphincter SMC ROCK-2 expression and activity, as well as MLCK expression were significantly higher in the adult extracts (Figure 4).

*Pyloric sphincter muscle nNOS and pVASP expression*

Pyloric sphincter muscle relaxation is modulated by nNOS mediated nitric oxide generation and the downstream cGMP-dependent pVASP, the latter found to be present in gastropyloric tissue of rodents (20). For this reason, the developmental-dependent expression of these enzymes was examined. When compared with adult cells, the newborn pyloric sphincter muscle showed a higher nNOS and pVASP expression (Figure 5) suggestive of a greater potential for relaxation.

**Discussion**

Feeding intolerance is a common clinical occurrence in preterm infants (19). The factors accounting for this disorder are poorly understood, but developmental regulation of the gastropyloric motor function likely plays a role. Previous studies documented a reduced agonist-stimulated gastric muscle contraction in newborn animals, when compared with adult counterparts (22, 23, 26, 35, 48). Age-dependent changes in Ca\textsuperscript{2+} mobilization was identified in these reports as accounting for the decreased fundic and antrum muscle contraction early in life.

In the present study, we obtained developmental gastropyloric data in rats and confirmed that the potential for force generation and smooth muscle shortening, is significantly lower in newborn gastric and pyloric tissues, when compared with their adult counterparts. In addition, we documented that the expression of nNOS and pVASP, two important regulators of sphincter muscle relaxation, was higher in one-week old pyloric SMC, when compared with adult cells. Based on the present data and the above
referenced reports, Figure 6 outlines the factors accounting for the developmental-dependent regulation of gastropyloric function.

The gastropyloric and intestinal motor activity reflects the integrated maturation of the wall muscle, gastroenteric and extrinsic nervous system, as well as, humoral environment (40). In the present study, we mostly evaluated freshly dispersed SMC, thus allowing us to comparatively determine their motor activity independently of the extrinsic innervation and humoral factors.

Fundal and antral muscles contribute to the gastric motor activity. Previous studies have shown that in humans, the normal gastric slow waves that characterize antrum muscle activity are not present at birth, first recordable at the age of 2–4 months, and only reach the adult pattern by the age of 4–11 years (11). In adults, gastric content emptying is dependent on both gastric anatomical regions. In contrast, only the fundic muscle appears to play a role in preterm infants’ milk clearance from the stomach (11). For this reason, in the present study, we mainly focused on the age-dependent mechanical properties of the fundal muscle. Yet, a similar magnitude of shortening was documented for the fundus- and antrum-derived SMC at both ages (Figure 3 C,D).

Gastropyloric smooth muscle contraction involves two Ca\(^{2+}\)-dependent mechanisms. The first is manifested by a rise in intracellular Ca\(^{2+}\) as a result of either a Ca\(^{2+}\) channel regulated mobilization from the extracellular compartment, or 1,4,5-triphosphate stimulation-induced Ca\(^{2+}\) release from the sarcoplasmic reticulum. Age-dependent changes in muscle Ca\(^{2+}\) mobilization have been reported in feline (15, 22, 23), guinea-pig (29) and rabbit antrum(48), as well as guinea-pig fundus(35).

The rise in intracellular Ca\(^{2+}\) stimulates myosin light chain phosphorylation via MLCK resulting in muscle contraction (5). In diabetic rats, decreased gastrointestinal tissues MLCK expression is associated with reduced gastric content emptying (21). Age-dependent differences in gastric and pyloric muscle MLCK expression were demonstrated in the present study.
The second process is called Ca\textsuperscript{2+}-sensitization and involves muscle contraction via MLCP inhibition (30). Amongst other factors, activation of the RhoA/Rho-kinase pathway induces Ca\textsuperscript{2+}-sensitization. In mammalian cells two Rho-kinases are present with the type 2 (ROCK-2) being functional in the GI tract (3, 16, 24, 38). ROCK-2 inhibits MLCP activity via phosphorylation of the regulatory subunit MYPT1 (6).

Reduced RhoA/ROCK activation is associated with GI dysmotility (38) and plays an important role in GI motility, as well as sphincter tone (4, 6, 13, 28). Such mechanism is operative in rodents’ gastric tissue and in the fundus, shown to more important than intracellular Ca\textsuperscript{2+} changes (6).

In the present study we showed higher ROCK-2 expression and activity in adult gastric and pyloric SMC, when compared with their one-week of age counterpart. This suggest that aside from the reported reduced Ca\textsuperscript{2+} mobilization early in life, age-dependent changes in Ca\textsuperscript{2+} sensitization also contribute to the lesser gastric muscle shortening potential of the newborn rat, when compared with the adult counterpart.

Neuronal nitric oxide (NO) synthase (nNOS)-derived NO is the most important GI muscle relaxant agonist (12) and induces MLCP dephosphorylation (7). NO promotes cGMP generation that in turn activates protein kinase G resulting in phosphorylation of the vasodilator-stimulated phosphoprotein (VASP).

VASP is present in rodent’s gastrointestinal tissue smooth muscle and its expression is highest in the newborn and its decreases later in life (20). We have previously shown that vascular SMC contain pVASP and exposure to a NO donor upregulates its expression (17). In the present study, we documented that the pyloric sphincter SMC nNOS and pVASP expression are significantly higher in newborn tissue, when compared with adult samples. Such findings strongly suggest that during neonatal life, the pyloric sphincter muscle has a greater potential for relaxation, when compared with later in life.

Gastric content emptying is related to gestational and postnatal ages, such that it is reduced in preterm infants, when compared with term neonates (37, 39, 40). It is also widely accepted that, when
compared with adults, neonates show delayed gastric emptying (37, 39). Since gastric content clearance is dependent on a number of factors to include, consistency, volume and osmolarity to name a few, it is difficult to evaluate the developmentally-related rate of emptying. Attempts to create mathematical models that combine the available past published data obtained from preterm infants, children and adults by distinct methods (9) is fraught with false assumptions that preclude any meaningful developmental-related comparisons.

Newborn rodents are a suitable animal model to address the maturational-dependent regulation of gastropyloric motor function in humans. At birth, the rat gastrointestinal function is developmentally comparable to that of 32-week gestation infants and reach term equivalent by 1-2 weeks of age (14). We have previously demonstrated that, when compared to adults, newborns rats have delayed gastric emptying (27). By utilizing high fidelity ultrasound, we also recently demonstrated that the gastric content emptying rate in mice doubles during the first week of life (45). Such data are in keeping with the age-dependent increase in gastric emptying documented in clinical studies (37, 39).

The findings of the present animal study are of significant translational value given the importance of determining the developmentally-dependent gastric and pyloric sphincter motor regulation. This knowledge can inform new strategies to prevent and/or treat feeding intolerance in the preterm population. Several prokinetic therapeutic agents are presently utilized in neonates exhibiting feeding intolerance (31) on the basis of their effectiveness in adults. We have recently shown that, as opposed to its established effectiveness later in life, the prokinetic agent metoclopramide has no effect on newborn rats due to maturational-dependent dysregulation of its GI receptors (27).

Together the present study data suggest that newborn rats’ gastropyloric motor activity is best characterized by a lower pyloric sphincter tone and reduced gastric muscle contraction, when compared with adult animals. Such findings imply that decreased gastric motor activity, and not increased pyloric
sphincter tone, account for the reduced gastric content emptying early in life. The data from the present animal study warrants clinical validation to further inform therapeutic strategies to address the preterm infants’ delayed gastric emptied.

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Figures

Figure 1: Representative gastric fundus (Panel A) and antral (Panel B) muscle strips stimulated with increasing concentrations of carbachol. A sustained and progressively higher force is observed for the fundal strip following each agonist stimulation, whereas the antral muscle develops irregular phasic motor activity. Panel C: Pyloric sphincter muscle phasic activity in response to carbachol stimulation. Panel D: Pyloric smooth muscle cells under basal conditions (Control) and following acetylcholine (10^{-4} M) stimulation (Stimulated) to induce contraction (length change). Both images were obtained at 20x magnification following acrolein fixation.

Figure 2: One week and adult gastric fundus muscle-derived force generation in response to 128 mM KCl, Carbachol (10^{-6} M) and electrical field stimulation (EFS; see methodology for details). One week old (N= 9-42), adult (N= 11-13). ** P<0.01 when compared with one-week samples, by unpaired t-test.

Figure 3: Gastric fundus and pyloric sphincter smooth muscle cells from one-week (1 wk; N=89-93 cells), two-weeks (2 wks; N=19-88) and adult (N=111-146) animal. Agonist-induced shortening is expressed as a percentage of basal cell length (minimum 50 cells measured from each age and tissue). ** P<0.01 as compared with adult cells by one-way ANOVA.

Figure 4: Rho-associated kinase 2 (ROCK-2) protein expression, activity (pMYPT/MYPT) and myosin light chain kinase (MLCK) expression from gastric fundus and pyloric sphincter smooth muscle cells’ extracts (N=3 per tissue and age) from one-week (1 wk) and adult animals. * P<0.05 when compared with one-week old samples by unpaired student t-test. ROCK-2 and MLCK expression were normalized to tubulin.

Figure 5: Neuronal nitric oxide synthase (nNOS) and phosphorylated vasodilator-stimulated phosphoprotein (pVASP) protein expression from pyloric sphincter smooth muscle cells’ extracts (N=3
per age) from one-week (1 wk) and adult animals. nNOS and pVASP expression was normalized, respectively to tubulin and VASP.

Figure 6: Proposed mechanism accounting for the newborn delayed gastric content emptying. Phosphorylated vasodilator-stimulated phosphoprotein (pVASP); Neuronal nitric oxide synthase (nNOS); myosin light chain kinase (MLCK); Rho-associated kinase 2 (ROCK-2)


Figure 2

KCl

Force [mN/mm²]

One Week  |  Adult
---|---
0  | 2
0  | 1

Carbachol

Force [mN/mm²]

One Week  |  Adult
---|---
0  | 4
0  | 2

EFS

Force [mN/mm²]

One Week  |  Adult
---|---
0  | 0.5
0  | 0.25
Figure 4

Fundus

A

B

C

Pylorus

D

E

F

ROCK2/tubulin

pMYPT/MYPT1

MLCK/tubulin

ROCK2/tubulin

pMYPT/MYPT1

MLCK/tubulin

0.0
1.0
2.0
3.0
4.0
5.0
0.0
1.0
2.0
3.0
4.0
5.0
0.0
1.0
2.0
3.0
4.0
5.0

1 wk
Adult

0.0
1.0
2.0
3.0

Fundus

Pylorus
Figure 5

[Graph showing nNOS and tubulin levels in 1 week and adult stages, with statistical significance denoted by * and **]

[Graph showing pVASP and VASP levels in 1 week and adult stages, with statistical significance denoted by * and **]
Figure 6

**Pylorus**
- ↑ nNOS
- ↓ pVasp
- ↓ Sphincter Muscle Tone
- ↑ Gastric Emptying

**Fundus**
- ↓ Ca²⁺ Sensitization (ROCK-2)
- ↓ MLCK
- ↓ Ca²⁺ Mobilization
- ↓ Muscle Contraction
- ↓ Gastric Emptying

**Pylorus Sphincter** ➔ **Fundus** ➔ **Pylorus**