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

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Sobchak C, Fajardo AF, Shifrin Y, Pan J, Belik J. Gastric and pyloric sphincter muscle function and the developmental-dependent regulation of gastric content emptying in the rat. Am J Physiol Gastrointest Liver Physiol 310: G1169–G1175, 2016. First published April 28, 2016; doi:10.1152/ajpgi.00046.2016.—Feeding intolerance is a common issue in the care of preterm neonates. The condition manifests as delayed emptying of gastric contents 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 function of rat gastric and pyloric smooth muscle 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. Newborn and adult rat gastric (fundus) and pyloric sphincter tissues were comparatively studied in vitro. Shortening of the tissue-specific dissociated smooth muscle cell was evaluated, and expression of the key regulatory proteins Rho-associated kinase 2 and myosin light chain kinase was determined. Gastric and pyloric smooth muscle cell shortening was significantly greater in the adult than the respective newborn counterpart. Expression of myosin light chain kinase and Rho-associated kinase 2 was developmentally regulated and increased with age. Pyloric sphincter muscle expresses a higher neuronal nitric oxide synthase and phosphorylated vasodilator-stimulated phosphoprotein content in newborn than adult tissue. Compared with later in life, the newborn rat gastropyloric muscle has a Ca2+-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 delayed gastric emptying in the newborn reflects reduced stomach muscle contraction potential, as opposed to increased pyloric sphincter tone.

stomach; smooth muscle; newborn; feeding intolerance

FEEDING INTOLERANCE, defined as a gastric residual volume $>50\%$ (34), is one of the most common clinical issues in preterm neonates (19). The factors accounting for delayed gastric emptying in neonates are unknown. Their identification, however, is of importance, since parenteral nutrition is associated with intravascular catheter complications such as septicemia and a longer hospital stay (42).

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

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

Emptying of gastric contents is dependent on a coordinated gastropyloric motor function involving fundus and antrum muscle contraction and pyloric sphincter relaxation (10). In adults, abnormalities in either of these processes characterize gastroparesis (43).

Over 20 years ago, age-dependent changes in agonist-induced force were reported in gastric fundus and antrum muscle strips from rabbits, cats, and guinea pigs (22, 23, 35, 48). These changes are believed to be related to a reduced intracellular Ca2+ mobilization following agonist stimulation in newborn compared with adult gastric muscle (23). In response to an increase in intracellular Ca2+ content, smooth muscle myosin light chain (MLC) kinase (MLCK) is activated, and this pathway has a key role in the regulation of GI motility (21). We previously reported developmental changes in vascular MLCK expression (2). After acetylcholine-induced stimulation, Ca2+-regulated MLC phosphorylation was reported not to be age-dependent (26), but the developmental pattern of MLCK expression was not evaluated. Finally, Ca2+ 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. The ability of increased pyloric sphincter tone to manifest early in life and interfere with gastric emptying is best exemplified by the pathological condition infantile hypertrophic pyloric stenosis (36). The increased sphincter tone in infants with this condition is likely related to reduced pyloric tissue neuronal nitric oxide (NO) synthase (nNOS) expression (1) and the resulting lower NO generation (25, 36, 44). In the rodent model of this disease, we previously showed that increased pyloric sphincter tone is transiently present (47).
Therefore, the goal of the present study was to comparatively evaluate the developmental-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.

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 accordance with the Canadian Animals for Research Act and Canadian Council on Animal Care regulations, and the study protocol was approved by the Animal Care Committee of the Hospital for Sick Children.

Sprague-Dawley rats (Charles River, Montreal, QC, Canada) of both sexes were studied at 1, 2, and >3 wk (adult) of age. They were fed regular rodent pellets and housed under standard lighting and temperature conditions. The animals were killed by cervical dislocation (newborn) or pentobarbital sodium injection [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 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) 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 was 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, Colorado Springs, CO).

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 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 stimulation, 0.5-ms pulses with 20-s trains at a frequency of 5 Hz. Three stimulations, applied 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. Compared with the sustained agonist-induced tonic force obtained in gastric fundus muscle, the antrum and pyloric sphincter counterparts exhibit irregular phasic activity (Fig. 1, A–C), which makes age-dependent comparisons problematic. For this reason, to comparatively assess age- and tissue-specific muscle contraction, we elected to measure smooth muscle cell (SMC) shortening in response to acetylcholine stimulation, as used by others (32).

Rat gastropyloric tissues were digested in 1 ml/mg collagenase and 0.01% soybean trypsin inhibitor (ThermoFisher, Burlington, ON, Canada) for 1 h at 37°C, and the SMCs were dispersed in DMEM cell culture medium (Wisent, St. Bruno, QC, Canada). At 1 h after dispersion, the SMCs were exposed to acetylcholine at a final medium concentration of 10⁻⁴ M for 15 min at 37°C and immediately fixed with 1% acrolein. Cells maintained for a similar duration in Ca²⁺-free PBS medium served as control to prevent spontaneous contraction of the SMCs. Light microscopy images at ×20 magnification were obtained using an inverted fluorescence microscope (DM IRE2, Leica,

Fig. 1. A and B: representative gastric fundus and antrum muscle strips were stimulated with increasing concentrations of carbachol. A sustained and progressively higher force is observed for the fundus muscle strip following each agonist stimulation, whereas the antrum muscle strip develops irregular phasic motor activity. C: pyloric sphincter muscle phasic activity in response to carbachol stimulation. D and E: pyloric smooth muscle cells under basal conditions and following acetylcholine (10⁻⁴ M) stimulation to induce contraction (length change). Both images were obtained at ×20 magnification following acrolein fixation.
Wetzelr, Germany), and cell length was measured using ImageJ (National Institutes of Health, Bethesda, MD). Acetylcholine-induced cell length changes (shortening) are expressed as percentage of average control SMC length. A control SMC and an acetylcholine-stimulated SMC are illustrated in Fig. 1, D and E, respectively. A minimum of 30 agonist-stimulated and control cells from each tissue and age group were evaluated. The cells were obtained from 12 newborn 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 SMCs were pelleted by centrifugation at 200 g for 10 min at room temperature. Tissue and cell pellets were 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, QC, Canada). Protein content was determined by the Bradford method using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of lysate proteins in Laemmli buffer were separated by SDS-PAGE, transferred onto a PVDF membrane, and immunoblotted using the following antibodies: mouse nNOS (1:1,000 dilution; Invitrogen, Camarillo, CA), rabbit phosphorylated vasodilator-stimulated phosphoprotein (pVASP, 1:3,000 dilution; Cell Signaling Technology), mouse tubulin (1:5,000 or 1:1,000 dilution; Sigma-Aldrich), goat ROCK-2 (1:1,000 dilution; Santa Cruz Biotechnology), mouse MLCK (1:1,000 dilution; Sigma-Aldrich), anti-mouse horseradish peroxidase (HRP)-conjugated IgG (1:10,000 dilution; Cell Signal Technology), and anti-goat HRP-conjugated IgG (1:5,000 dilution; Santa Cruz Biotechnology). Detection was performed with the enhanced chemiluminescence reagent (Perkin Elmer, Shelton, CT). Band intensities were quantified using ImageJ 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, 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, UT).

RESULTS

Smooth muscle contraction potential. Newborn and adult fundus muscle strips were comparatively evaluated to determine their force-generating potential. Smooth muscle contraction induced with KCl and carbachol or via EFS-mediated
nerve stimulation resulted in a significantly higher force in adult than newborn muscle strips (Fig. 2).

The smooth muscle agonist-induced shortening potential was comparatively assessed in 1-wk-old, 2-wk-old, and adult fundus and pyloric SMCs. The magnitude of the acetylcholine-induced fundus and pyloric sphincter SMC shortening was age-dependent and significantly greater in adult than 1- and 2-wk-old animals (Fig. 3, A and B). To ascertain whether there are differences in contraction potential between antrum- and fundus-derived SMCs, we comparatively evaluated these cells in 1-wk-old and adult rats. No significant tissue difference in the magnitude of agonist-induced SMC shortening was seen at either age (Fig. 3, C and D).

**ROCK-2 and MLCK.** To evaluate the developmental changes in the agonist-induced Ca\(^{2+}\)/H\(_{11001}\) sensitization, we proceeded to evaluate the tissue-specific SMC ROCK-2 and MLCK expression. Both fundus and pyloric sphincter SMC ROCK-2 expression and activity, as well as MLCK expression, were significantly higher in adult than newborn extracts (Fig. 4).

**Pyloric sphincter muscle nNOS and pVASP expression.** Pyloric sphincter muscle relaxation is modulated by nNOS-mediated NO generation and the downstream cGMP-dependent pVASP, the latter of which was found to be present in gastropyloric tissue of rodents (20). For this reason, the developmental-dependent expression of these enzymes was examined. Levels of nNOS and pVASP expression were higher in newborn than adult pyloric sphincter muscle (Fig. 5), which is 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 compared with adult counterparts (22, 23, 26, 35, 48). Age-dependent changes in Ca\(^{2+}\) mobilization were identified in these reports as accounting for the decreased fundus 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 than adult gastric and pyloric tissues. In addition, we documented that the expression of nNOS and pVASP, two important regulators of sphincter muscle relaxation, was higher...
in 1-wk-old than adult pyloric SMCs. On the basis of the present data and the above-referenced reports, Fig. 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, and humoral environment (40). In the present study we mostly evaluated freshly dispersed SMCs, thus allowing us to comparatively determine their motor activity independently of the extrinsic innervation and humoral factors.

Fundus and antrum 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, are first recordable at 2–4 mo of age, and only reach the adult pattern by 4–11 yr of age (11). In adults, gastric content emptying is dependent on both gastric activity and pyloric muscle contractions. The gastropyloric and intestinal motor activity reflects the integrated maturation of the wall muscle, gastroenteric and extrinsic nervous system, and humoral environment (40). In the present study we focused on the age-dependent mechanical properties of the fundus muscle. Yet a similar magnitude of shortening was documented for the fundus- and antrum-derived SMCs at both ages (Fig. 3, C and D).

Gastropyloric smooth muscle contraction involves two Ca\(^{2+}\)-dependent mechanisms. The first is manifested by a rise of intracellular Ca\(^{2+}\) as a result of 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 (48) antrum, as well as guinea pig fundus (35).

The rise of intracellular Ca\(^{2+}\) stimulates MLC phosphorylation via MLCK, resulting in muscle contraction (5). In diabetic rats, decreased GI tissue MLCK expression is associated with reduced gastric content emptying (21). Age-dependent differences in gastric and pyloric muscle MLCK expression are demonstrated in the present study.

The second process is called Ca\(^{2+}\) sensitization and involves muscle contraction via MLC phosphate (MLCP) inhibition (30). Among other factors, activation of the RhoA/ROCK pathway induces Ca\(^{2+}\) sensitization. In mammalian cells, two types of ROCK are present, with ROCK-2 being functional in the GI tract (3, 16, 24, 38). ROCK-2 inhibits MLCP activity via phosphorylation of the regulatory subunit MYPT1 (5). 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 a mechanism is operative in rodent gastric tissue and in the fundus and has been shown to be more important than intracellular Ca\(^{2+}\) changes (5).

In the present study we showed higher ROCK-2 expression and activity in adult than 1-wk-old gastric and pyloric SMCs. This suggests that, aside from the reported reduced Ca\(^{2+}\) mobilization early in life, age-dependent changes in Ca\(^{2+}\) sensitization also contribute to less gastric muscle shortening potential in the newborn than adult rat.

nNOS-derived NO is the most important GI muscle relaxant agonist (12) and induces MLCP dephosphorylation (7). NO promotes cGMP generation, which in turn activates protein kinase G, resulting in phosphorylation of the VASP. VASP is present in rodent GI tissue smooth muscle, and its expression is highest in the newborn and decreases later in life (20). We previously showed that vascular SMCs contain pVASP and that exposure to a NO donor upregulates its expression (17). In the present study we documented that pyloric sphincter SMC nNOS and pVASP expression are significantly higher in newborns. pVASP, phosphorylated vasodilator-stimulated phosphoprotein; nNOS, neuronal nitric oxide synthase; MLCK, myosin light chain kinase; ROCK, Rho-associated kinase 2.
born than adult tissue samples. Such findings strongly suggest that the pyloric sphincter muscle has a greater potential for relaxation early than later in life.

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

Newborn rodents are a suitable animal model to address the maturational-dependent regulation of gastropyloric motor function in humans. In the rat at birth, GI function is developmentally comparable to that of 32-wk-gestation infants and reaches full-term equivalent by 1–2 wk of age (14). We previously demonstrated delayed gastric emptying in newborn compared with adult rats (27). By utilizing high-fidelity ultrasound, we also recently demonstrated a doubling of the rate of gastric content emptying in mice during the 1st wk 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 development-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 recently showed 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, data from the present study suggest that gastropyloric motor activity in newborn rats is best characterized by a lower pyloric sphincter tone and reduced gastric muscle contraction compared with adult animals. Such findings imply that decreased gastric motor activity, and not increased pyloric sphincter tone, accounts for the reduced gastric content emptying early in life. The data from the present animal study warrant clinical validation to further inform therapeutic strategies to address delayed gastric emptying in preterm infants.

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

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