Pantoprazole decreases gastroesophageal muscle tone in newborn rats via rho-kinase inhibition

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Abstract

Proton pump inhibitors reduce gastric acid secretion and are commonly utilized in the management of gastro-esophageal reflux disease (GERD) across all ages. Yet a decrease in lower esophageal sphincter tone has been reported in vitro in rats through an unknown mechanism, however their effect on the gastroesophageal muscle tone early in life was never studied. Hypothesizing that proton pump inhibitors also reduce gastroesophageal muscle contraction in newborn and juvenile rats, we evaluated the in vitro effect of pantoprazole on gastric and lower esophageal sphincter muscle tissue. Electrical field stimulation (EFS) and carbachol-induced force were significantly (P<0.01) reduced in the presence of pantoprazole, whereas the drug had no effect on the neuromuscular-dependent relaxation. When administered in vivo, pantoprazole (9 mg/kg) significantly (P<0.01) reduced gastric emptying time at both ages. In order to ascertain the signal transduction pathway responsible for the reduction in muscle contraction, we evaluated the tissue rho-associated kinase 2 (ROCK-2) and CPI-17 activity. Pantoprazole reduced myosin light chain phosphatase MYPT-1, but not CPI-17 phosphorylation of gastric and lower esophageal sphincter tissue strongly suggesting that it is a ROCK-2 inhibitor. To the extent that these findings can be extrapolated to human neonates, the use of pantoprazole may impair gastric and lower sphincter muscle tone and thus paradoxically exacerbate esophageal reflux. Further studies addressing the effect of proton pump inhibitors on gastroesophageal muscle contraction are warranted to justify its therapeutic use in GERD.

Key word(s): Lower esophageal sphincter, gastric emptying time, ROCK-2.
Proton pump inhibitors (PPIs) are clinically used to decrease acid secretion and gastroesophageal reflux disease (GERD)-associated symptoms. Their effect is modulated via inhibition of the H⁺-K⁺-ATPases-dependent final step in the transfer of protons to the gastric lumen.

Gastroesophageal reflux is commonly observed in neonates and particularly concerning in preterm infants since its occurrence has been associated with lung aspiration of gastric content and apnea (6). Studies conducted in adult animals have shown that PPIs reduce the lower esophageal sphincter (LES) muscle tone (10, 34). The mechanism accounting for the in vitro PPIs relaxant effect on gastroesophageal muscle contraction is presently unknown, but unrelated to a number of key pathways involved in smooth muscle relaxation (1, 12, 21, 23, 33).

Whether PPIs also have a relaxant effect on the newborn gastroesophageal muscle has not been previously reported and its evaluation was the main goal of the present study. The gastrointestinal sphincter tone is greatly dependent on the smooth muscle expression and/or activity of rho-associated protein kinase 2 (ROCK-2) (7, 8). As such, we hypothesized that PPIs reduce newborn and juvenile rat gastroesophageal muscle contraction via a mechanism involving ROCK-2 inhibition. Studies were conducted in rat gastric fundus and LES muscle.
Methodology

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 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, ON, Canada) bred in house were utilized. All animals were fed regular rodent pellets and housed under standard lighting and temperature conditions. Newborn (3-7 days of age) and juvenile (13-21 days) were studied. All animals were sacrificed with an overdose of pentobarbital sodium (60 mg/kg ip). Immediately after death, the gastric fundus and lower esophageal sphincter (LES) were dissected free and immediately mounted on an isometric myograph. Tissue samples to be utilized for Western blot analysis were either immediately frozen, or incubated fresh on Krebs-Henseleit solution for testing the in vitro effect of PTP on ROCK-2 activity, as described below.

Gastric fundus and LES smooth muscle mechanical response

Gastric longitudinally oriented fundic, or circular LES muscle strips (average 2 mm long and 5 mm wide) were studied using a modified protocol based on previous
reports by others (8, 10). The tissue mucosa and submucosa lawyers were carefully removed by sharp dissection and particular care taken with the newborn and juvenile strips to avoid damage to the underlying muscle layer. The tissue was maintained in ice-cold Krebs-Henseleit solution (NaCl, 115 mM; NaHCO₃, 25 mM; NaHPO₄, 1.38 mM; KCl, 2.51 mM; MgSO₄ 7 H₂O, 2.46 mM; CaCl₂, 1.91 mM; and dextrose, 5.56 mM) bubbled with 95% O₂/5% CO₂ until ready to be mounted. The muscle strips were secured at either end with 6x10mm flat surface tissue clips, suspended between two flat lead electrodes, and submerged into a 25 mL tissue bath (Radnoti LLC, Monrovia, California, USA) filled with Krebs-Henseleit solution at 37°C 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 using 7-0 braided silk. Changes in force were recorded and stored digitally for processing (LabChart 7, AD instruments, Colorado Springs, CO, USA). The muscle strips were equilibrated in the bath for the first 45 minutes while replacing the Krebs solution every 15 minutes and stretched to their previously determined optimal length of either 3 mN (newborn), or 5 mN (juvenile). All subsequent agonist-induced force measurements were obtained at the optimal resting tension and normalized to either the tissue cross sectional area, or the KCl (128 mM) initial response. The cross-sectional area was calculated from the weight and length of the tissue and assumes that its density is equal to 1.0 (4).

The muscle contraction potential was evaluated in response to either carbachol, or electrical field stimulation (EFS). EFS-induced force measurements were obtained in the presence of L-NAME (10⁻⁴ M), propranolol (10⁻⁵ M) and phentolamine (10⁻⁵ M) using a commercially available stimulator (Cibertec, Madrid,
Spain) as follows: 80 V stimulation, 0.5 msec pulses with 20-s trains of at a frequency of 5 Hz, as previously reported (31). Three stimulations obtained 10 min apart were employed and averaged to determine the EFS-induced force increase.

Muscle relaxation was induced by EFS as previously described (31). Briefly, the muscle strips were pre-contracted with carbachol at the concentration required to induce a 75% increase in maximal-induced force (E₇₅). The EFS-induced relaxation protocol utilized was as follows: 40 V stimulation, 0.2 ms (duration) with 30-s trains at 5-min intervals at 2, 4, 8, and 12 Hz (pulse frequency). EFS-induced relaxation was expressed as a percentage of maximum carbachol-induced contraction.

In order to evaluate the PTP effect on the muscle contraction, the tissue was preincubated with this compound for 20 min at a bath concentration of 10⁻⁴ M. To evaluate its dose-response effect, PTP was added to pre-contracted muscle strips in a cumulative manner.

Western blot analysis

Agonist-induced ROCK-2 activity was assessed by measuring the phosphorylation of the threonine 853 residue of myosin light chain phosphatase MYPT-1 subunit, as reported by others (8). For that, the tissue was pre-incubated for one hour at 37°C with L-NAME (10⁻⁴ M) in the presence or absence of PTP (10⁻⁴ M), and stimulated with carbachol (10⁻⁶ M) for 1 minute. Carbachol-naïve samples served as controls. Immediately after carbachol exposure, the tissue was immersed in ice-cold acetone, 10% trichloroacetic acid and 10mM dithiothreitol for 2 min then transferred into micro-centrifuge tubes and snap frozen in liquid nitrogen. In order to ascertain the EFS effect (100 V, 0.5 msec pulses with 20-s trains of 5 Hz) on ROCK-
2 and CPI-17 activity, immediately following stimulation the tissue was submerged in chilled acetone (10mM), DTT (10%) and trichloroacetic acid (TCA) for 2 min, snap frozen in liquid N₂ and stored at -80°C for future western blotting analysis.

The frozen tissue samples were subsequently lysed in 10 mM Tris-HCl pH 7.4 buffer containing 1% Triton X-100 and a protease/phosphatase inhibitor cocktail (Thermo Fisher Scientific Inc., Rockford, IL), and centrifuged at 14,000 g for 30 min. Protein concentrations were determined via the Bradford method (9). Equivalent amounts of lysate proteins in Laemmli buffer were fractionated on SDS-PAGE, transferred to polyvinylidene di-fluoride (PVDF) membranes and blotted. Membranes were treated with 5% skim milk and exposed overnight at 4°C to anti-MYPT-1 (Thr 853; 1:500 dilution; Santa Cruz, Santa Cruz, CA), MYPT-1 (1:1000 dilution; BD Biosciences, Mississauga, ON), CPI-17 (1:1000 dilution; Santa Cruz, Santa Cruz, CA) and pCPI-17 (Thr 38; 1:1000 dilution; Santa Cruz, Santa Cruz, CA) antibodies. Appropriate IgGs conjugated with horseradish peroxidase were used as secondary antibodies. The enhanced chemiluminescence (Perkin Elmer, Shelton, Connecticut, USA) reagent was used for detection and the band intensities were quantified by ImageJ software (National Institutes of Health, US). Whenever comparisons were made, all samples were present on the same PVDF membrane.

**In vivo measurement of gastric emptying time**

Gastric emptying time was evaluated in 1-3 week old pups. We utilized a previously described method (16), where the pups are separated from their mother at a precise time and their stomach content weighed 2 hours later. The pups were kept in a 37°C environment to prevent hypothermia and sacrificed with a barbiturate
overdose. The stomach content was normalized to the pup’s body weight. Normal saline (vehicle used to dissolve PTP), or PTP (9 mg/kg) was administered intraperitoneally to the animals immediately after maternal separation. The dose of PTP chosen for the newborn and juvenile animals was based on previously published reports utilizing this regimen in adult rats (2, 32).

**Data Analysis**

Data were evaluated either by one- or two-way analysis of variance (ANOVA) with multiple comparisons obtained by the Tukey-Kramer test, or unpaired Student’s t-test. 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:**

Since we did not observe any age-dependent differences in the measured parameters, the newborn and juvenile data in the figures and text were combined.

**Pantoprazole effect on gastroesophageal muscle contraction in vitro**

The carbachol-induced force dose-response in the absence and presence of PTP is shown in Figure 1. PTP significantly decreased (P<0.01) the force dose-response in the fundus (Figure 1A and 1C) and LES (Figure 1B and 1D) muscle strips, when compared with untreated control samples. PTP also significantly decreased the EFS-induced fundic (P<0.05) and LES (P<0.01) muscle contraction (Figure 2A), but
had no effect on the EFS-induced fundic and LES muscle relaxation (Figure 2B and C respectively). Incubation with the cyclooxygenase blocker indomethacin (10^{-4} M) did not alter the PTP-induced force reduction following EFS, or carbachol stimulation (data not shown).

In order to assess the PTP-induced relaxation, we evaluated fundic muscle pre-contracted with carbachol. As shown in Figure 3A, PTP-induced fundic and LES muscle response was concentration-dependent resulting in complete relaxation at 10^{-3} M.

**Pantoprazole delays newborn gastric emptying**

We proceeded to evaluate whether PTP has an *in vivo* effect on gastric emptying. As compared with saline injected animals, PTP significantly increased gastric emptying time (greater stomach content weight) in both newborn (P<0.05) and juvenile (P<0.01) rats (Figure 3B).

**PTP is a gastroesophageal muscle ROCK inhibitor**

To evaluate whether PTP-induced smooth muscle relaxation involves inhibition of ROCK-2 activity, we measured its effect on the carbachol-induced pMYPT-1(T853) phosphorylation, since carbachol has been previously shown to activate ROCK-2 (7, 8). In both fundus and LES tissue, PTP abolished (P<0.01) the carbachol-induced ROCK-2 activity (Figure 4A and C). PTP alone had no effect on either fundus or LES basal MYPT-1 phosphorylation levels (Figure 4B and D).
We further evaluated the PTP effect on CPI-17 phosphorylation (T38) in LES tissue (Figure 4E). No significant changes in CPI-17 phosphorylation were noted in response to carbachol or PTP.

Lastly the EFS stimulation effect on ROCK-2 activity was evaluated in the fundic muscle. EFS stimulation resulted in a significantly increased fundic muscle MYPT-1 (P<0.01), but not CPI-17, phosphorylation and the EFS-induced ROCK-2 activation was reduced (P<0.05) in the presence of PTP (Figure 5).

**Discussion**

In the present study, we documented that in the presence of PTP, the gastric fundus and LES muscle contraction is significantly reduced and this drug has a direct muscle relaxant effect in pre-contracted gastric muscle. PTP does not alter the EFS-dependent relaxation potential of fundic and LES muscle indicating that the drug effect is solely dependent on reduced contraction potential. PTP abolished the *in vitro* carbachol-induced ROCK-2 activation of fundic and LES tissue indicating that its muscle relaxant effect is modulated via ROCK-2 inhibition. Lastly, PTP administration to newborn and juvenile rats resulted in increased gastric emptying time confirming the *in vitro* effect on the fundic muscle.

To the best of our knowledge, this is the first study addressing the PTP effect on gastric and LES smooth muscle contractility early in life. We chose to study PTP given its water solubility, clinical intravenous usage in neonates (15) and that the PPI relaxation effect of PTP on adult rat gastroesophageal smooth muscle is greater when compared with other PPIs (34).
In adult rodents PPIs induce a decrease in LES muscle tone \textit{in vitro} (10, 34).

The PPIs-induced reduction in LES tone is not unique to gastroesophageal, since these compounds have been shown to relax all smooth muscle preparations where their effect was evaluated. These include the human myometrium (27, 33), bladder muscle (19) and myocardium (24, 26), as well as animal tissue intestinal (14), vascular (12), airway (21), gallbladder (1) smooth muscle and corpus cavernosum (23).

Yet the mechanism accounting for the PPIs effect on smooth muscle contraction is presently unknown and likely unrelated to nitric oxide (23, 33), K⁺ channels (1, 12), or cyclooxygenases (1, 12) pathways. The PTP effect on myocardium fibers was attributed to impaired sarcoplasmic reticulum Ca²⁺ uptake and reduced Ca²⁺ influx leading to decrease in the muscle's responsiveness to Ca²⁺ (24).

PPIs primary target is the gastric mucosae parietal cells where following acidic activation they form covalent disulfide bonds with cysteines of the H(+)K(+-)adenosine triphosphatase (H(+)K(+-)ATPase) resulting in the inactivation of the pump (22, 29). Yet H⁺K⁺-ATPases are also expressed in other tissues including renal (11), colonic epithelial cells (5), vascular smooth muscle cells (17, 18). Thus it is appealing to link the PPIs effect on smooth muscle tone to its potential action on the H⁺K⁺-ATPases. Yet, all PPI's are pro-drugs that need acidic environment to become active (15) and since the bath pH was neutral in this study, it is therefore unlikely that pH was involved in their muscle relaxant effect. Indeed, Schillinger et al. (24) showed that the negative inotropic effect of pantoprazole on myometrium fibers was not accompanied by changes in the intracellular pH. Further support that the
mechanism of action is not through the proton pump inhibition comes from a study looking at the PTP effect on rabbit prostatic smooth muscle strips. In that study the impaired contraction and relaxation effect did not occur in response to acidification of the medium and the different PPIs muscle effect was the same despite differences in their potency to block the H⁺-K⁺-ATPase (3).

The primary signaling pathway responsible for gastrointestinal smooth muscle contraction involves Ca²⁺ sensitization. ROCK-2 phosphorylates MYPT-1 (primarily at the threonine 853 residue) and CPI-17 (primarily at threonine 38), inhibiting myosin light chain phosphatase activity and thus promoting smooth muscle contraction (7). In the present study we demonstrated that carbachol and EFS induce MYPT-1, but not CPI-17 phosphorylation in newborn and juvenile rat fundic and LES and that ROCK-2 tissue activation is suppressed by PTP.

In a recent report where the EFS-induced stimulation was evaluated in adult mice (8), it was concluded that the resultant gastric fundic muscle contraction was mediated via CPI-17 phosphorylation. The apparent discrepancy between ours and Bhetwal et al’s (8) data may relate to the fact that the present experiments were performed in newborn rats, as opposed to adult mice.

Pantoprazole is 98% bound to protein in serum (13, 20) and such a low free drug availability has been incriminated on its lack of hemodynamic and cardiac in vivo effects in adult rats (32). The data from the present study showing delayed gastric emptying following ip pantoprazole administration, however, strongly indicate that the drug in vitro effects are also evident in newborn and juvenile rats in vivo.
The North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition Foundation guidelines states that “no PPI has been approved for use in infants younger than 1 year of age, and there are special concerns pertaining to prescription of PPIs in infants” (28). Yet, PPIs are commonly prescribed for neonates and deemed to be well tolerated (25, 30). Neonates and infants appeared to have developmentally-related PPIs pharmacokinetics exhibiting a longer drug half-life (15, 20, 29). The in vitro PTP concentration shown in the present study to induce significant gastroesophageal muscle relaxation and ROCK-2 inhibition is within the range of reported maximum serum concentrations of this drug in children (15, 20).

In summary, we have shown that PTP, a commonly utilized proton pump inhibitor medication relaxes the gastric fundic and LES smooth muscle in neonatal and juvenile rats, similar to previously shown effects in adult rodents. Here we make the novel observation that the mechanism accounting for the drug effect involves rho-kinase inhibition. PTP is often utilized in neonates to prevent the esophageal and pulmonary complications associated with GERD by reducing and/or inhibiting gastric acid secretion. The present animal data suggest that PTP may promote gastroesophageal reflux by reducing gastric contraction and lowering LES muscle tone. Further investigation on the potential deleterious effects associated with the therapeutic use of PPIs in neonates is warranted.

Grants

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**Figure Legends**

Fig. 1. Fundic and LES muscle carbachol-induced force dose-response ($10^{-9} – 10^{-5}$M) normalized to cross-sectional area (A {N=14} and B {N=4} respectively) and KCl-induced contraction (C and D respectively; N=3 for each group) in the absence and presence of pantoprazole (PTP). **P<0.01 when compared with control samples by two-way ANOVA with repeated measures. No statistical significant difference for the interaction of the two main effects (control versus PTP and carbachol concentrations) was found. Representative tracings are shown in the figure inserts.

Fig. 2. Fundic and LES muscle contraction (Panel A) in the absence (Control; N=3 and 4 respectively) and presence (N=3 and 5) of pantoprazole (PTP) following electrical field stimulation (EFS). The experiments were conducted in the presence of L-NAME ($10^{-4}$M), propranolol ($10^{-5}$M) and phentolamine ($10^{-5}$M). Fundic and LES (Panel B and C respectively) muscle relaxation in the absence (Control; N=18 and 7 respectively) and presence (N=8 and 3) of PTP following EFS stimulation. Representative tracings for fundus and LES are shown in the figure inserts.* P<0.05, **P<0.01 when compared with same age control samples by unpaired Student t-test.

Fig. 3. A: Carbachol pre-contracted fundic and LES (N=3 for each) muscle relaxation dose-response to pantoprazole ($10^{-9} – 10^{-3}$M). Representative tracing is shown in the figure insert. B: Stomach content/body weight ratio following saline (Control), or pantoprazole (PTP; 9mg/kg ip injection) in newborn (N=4) and juvenile (N=3) animals. *P<0.05, **P<0.01 as compared with age-matched control by unpaired Student t-test.
**Fig. 4.** Fundic (A) and LES (C) tissue pMYPT-1(T853 residue) content normalized to total MYPT-1 expression in the absence (Control; N=3), carbachol alone (N=3) or in combination with pantoprazole (10^-4 M; N=3). Fundus (B) and LES tissue (D) pMYPT-1(T853) content normalized to MYPT-1 content in the absence and presence of PTP (N=3 for each group). LES (E) tissue pCPI-17(T38 residue) content normalized to total CPI-17 expression in the absence (Control; N=4) or presence of PTP (N=4) and PTP + carbachol (N=4). Figure inserts show representative Western blots. *P<0.05 and **P<0.01 by one-way ANOVA with Tukey-Kramer multiple comparison testing.

**Fig. 5.** Gastric Fundus tissue. Unstimulated control (N=3), following EFS stimulation (N=3) and the combination of EFS and PTP (10^-4 M N=3) tissue for (A) pMYPT-1(T853 residue) normalized to total MYPT-1 expression and (B) pCPI-17(T38 residue) content normalized to CPI-17 expression. Figure inserts show the representative Western blots. **P<0.01 and *P<0.05 by one-way ANOVA with Tukey-Kramer multiple comparison testing.


Figure 1

Fundus

A

![Graph showing force (mN/mm²) versus log [Carbachol] (M) for Control and PTP groups.]

C

![Graph showing force/KCl versus log [Carbachol] (M) for Control and PTP groups.]

LES

B

![Graph showing force (mN/mm²) versus log [Carbachol] (M) for Control and PTP groups.]

D

![Graph showing force/KCl versus log [Carbachol] (M) for Control and PTP groups.]

Legend:
- Control
- PTP

 Annotations:
- *= 3 mN
- ** Statistical significance indicated by asterisks
Figure 2

A

EFS-Induced Force

Frequency (Hz)

Relaxation (% of Max Contraction)

Fundus

LES

Control

PTP

B

Fundus

Relaxation (% of Max Contraction)

Frequency (Hz)

C

LES

Relaxation (% of Max Contraction)

Frequency (Hz)
Figure 3

A

Relaxation (% of Max Contraction) vs. Log [Pantoprazole] (M)

Inset: 2 mN

B

Stomach Content / Body Weight

Newborn  Juvenile

Control  PTP

*  **
Figure 4

**Fundus**

A

B

**LES**

C

D

**LES**

E

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* p < 0.05
** p < 0.01
Figure 5

A

B

Control EFS PTP + EFS

Control EFS PTP + EFS