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

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Welsh C, Kasirer MY, Pan J, Shifrin Y, Belik J. Pantoprazole decreases gastroesophageal muscle tone in newborn rats via rho-kinase inhibition. Am J Physiol Gastrointest Liver Physiol 307: G390–G396, 2014. First published April 3, 2014; doi:10.1152/ajpgi.00005.2014.—Proton pump inhibitors reduce gastric acid secretion and are commonly utilized in the management of gastroesophageal reflux disease 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 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. To ascertain the signal transduction pathway responsible for the reduction in muscle contraction, we evaluated the tissue ROCK-2 and CPI-17 activity. Pantoprazole reduced myosin light chain phosphatase (PPT-1) and, 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 esophageal sphincter muscle tone and thus paradoxically exacerbate esophageal reflex. Further studies addressing the effect of proton pump inhibitors on gastroesophageal muscle contraction are warranted to justify its therapeutic use in gastroesophageal reflux disease.

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+–ATPase-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 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 tone 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 euthanized with an overdose of pentobarbital sodium (60 mg/kg ip). Immediately after death, the gastric fundus and 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 in the in vitro effect of pantoprazole (PTP) on ROCK-2 activity, as described under Western blot analysis 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 via a modified protocol based on previous reports by others (8, 10). The tissue mucosa and submucosa layers 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 (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 until ready to be mounted.

The muscle strips were secured at either end with 6-0 braided silk. Changes in force were recorded and stored digitally below.
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 N\textsuperscript{G}nitro-L-arginine methyl ester (L-NAME; \(10^{-4}\) M), propranolol (\(10^{-5}\) M), and phentolamine (\(10^{-5}\) M) by using a commercially available stimulator (Cibertec, Madrid, Spain) as follows: 80 V stimulation, 0.5-ms 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 precontracted with carbachol at the concentration required to induce a 75% increase in maximal-induced force (\(E_{75}\)). 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.

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 precontracted muscle strips in a cumulative manner.

**Western blot analysis.** Agonist-induced ROCK-2 activity was assessed by measuring the phosphorylation of the threonine 853 (rat equivalent = threonine 855) residue of myosin light chain phosphatase MYPT-1 subunit, as reported by others (8). For that, the tissue was homogenized and lysate proteins in Laemmli buffer were fractionated on SDS-PAGE, transferred to polyvinylidene difluoride (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 (1:1,000 dilution; BD Biosciences, Mississauga, ON, Canada), CPI-17 (1:1,000 dilution; Santa Cruz) and pCPI-17 (Thr 38; 1:1,000 dilution; Santa Cruz) antibodies. Appropriate IgGs conjugated with horseradish peroxidase were used as secondary antibodies. The enhanced chemiluminescence (Perkin Elmer, Shelton, CT) reagent was used for detection and the band intensities were determined via the Bradford method (9). Equivalent amounts of lysate proteins in Laemmli buffer were fractionated on SDS-PAGE, transferred to 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 (1:1,000 dilution; BD Biosciences, Mississauga, ON, Canada), CPI-17 (1:1,000 dilution; Santa Cruz) and pCPI-17 (Thr 38; 1:1,000 dilution; Santa Cruz) antibodies. Appropri-ate IgGs conjugated with horseradish peroxidase were used as secondary antibodies. The enhanced chemiluminescence (Perkin Elmer, Shelton, CT) reagent was used for detection and the band intensities were quantified by ImageJ software (National Institutes of Health). 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- to 3-wk-old pups. We utilized a previously described method (16), in which the pups are separated from their mother at a precise time and their stomach content was weighed 2 h

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**Fig. 1. Fundic and lower esophageal sphincter (LES) muscle carbachol-induced force dose-response (\(10^{-9}\) to \(10^{-6}\) 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). **\(P < 0.01\) with control samples by 2-way ANOVA with repeated measures. No statistical significant difference for the interaction of the 2 main effects (control vs. PTP and carbachol concentrations) was found. Representative tracings are shown in the insets.
The pups were kept in a 37°C environment to prevent hypothermia and euthanized 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, UT). Data are presented as means ± SE.

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.

PTP effect on gastroesophageal muscle contraction in vitro. The carbachol-induced force dose-response in the absence and presence of PTP is shown in Fig. 1. PTP significantly de-

![Fig. 1](image-url)

### Fig. 1

A: carbachol precontracted fundic and LES (N = 3 for each) muscle relaxation dose-response to PTP (10$^{-9}$ to 10$^{-4}$ M). A representative tracing is shown in the inset. *$P < 0.05$, **$P < 0.01$ compared with control by unpaired Student $t$-test. Max, maximum.

B: stomach content/body weight ratio following saline (Control), or PTP (9 mg/kg ip injection) in newborn (N = 4) and juvenile (N = 3) animals. *$P < 0.05$, **$P < 0.01$ compared with age-matched control by unpaired Student $t$-test.

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Fig. 2. Fundic and LES muscle contraction (A) in the absence (Control; N = 3 and 4, respectively) and presence (N = 3 and 5) of PTP following electrical field stimulation (EFS). The experiments were conducted in the presence of $N^\omega$-nitro-$L$-arginine methyl ester (10$^{-4}$ M), propranolol (10$^{-5}$ M), and phentolamine (10$^{-5}$ M). Fundic and LES (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 insets. *$P < 0.05$, **$P < 0.01$ compared with same age control samples by unpaired Student $t$-test. Max, maximum.

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Fig. 3. A: carbachol precontracted fundic and LES (N = 3 for each) muscle relaxation dose-response to PTP (10$^{-9}$ to 10$^{-4}$ M). A representative tracing is shown in the inset. B: stomach content/body weight ratio following saline (Control), or PTP (9 mg/kg ip injection) in newborn (N = 4) and juvenile (N = 3) animals. *$P < 0.05$, **$P < 0.01$ compared with age-matched control by unpaired Student $t$-test.
creased \((P < 0.01)\) the force dose-response in the fundus (Fig. 1, A and C) and LES (Fig. 1, B and D) muscle strips, compared with untreated control samples. PTP also significantly decreased the EFS-induced fundic \((P < 0.05)\) and LES \((P < 0.01)\) muscle contraction (Fig. 2A), but had no effect on the EFS-induced fundic and LES muscle relaxation (Fig. 2, B and C, respectively). Incubation with the cyclooxygenase blocker indomethacin \((10^{-4} \text{ M})\) did not alter the PTP-induced force reduction following EFS or carbachol stimulation (data not shown).

To assess the PTP-induced relaxation, we evaluated fundic muscle precontracted with carbachol. As shown in Fig. 3A, PTP-induced fundic and LES muscle response was concentration dependent, resulting in complete relaxation at \(10^{-3} \text{ M}\).

\textit{PTP delays newborn gastric emptying.} We proceeded to evaluate whether PTP has an in vivo effect on gastric emptying. 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 (Fig. 3B).

\textit{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 (Fig. 4, A and C). PTP alone had no effect on either fundus or LES basal MYPT-1 phosphorylation levels (Fig. 4, B and D).

We further evaluated the PTP effect on CPI-17 phosphorylation (T38) in LES tissue (Fig. 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 phosphorylation (Fig. 4A).

**Fig. 4.** Fundic (A) and LES (C) tissue pMYPT-1(T853 residue) content normalized to total MYPT-1 expression in the absence of treatment (control; \(N = 3\)), with carbachol alone \((N = 3)\), or with carbachol in combination with PTP \((10^{-4} \text{ M}; N = 3\)). 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 \((N = 4)\). Insets show representative Western blots. *\(P < 0.05\) and **\(P < 0.01\) by 1-way ANOVA with Tukey-Kramér multiple comparison testing.
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(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 (Fig. 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 precontracted 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, its clinical intravenous usage in neonates (15), and the fact that the PPI relaxation effect of PTP on adult rat gastroesophageal smooth muscle is greater compared with other PPIs (34).

In adult rodents PPIs induce a decrease in LES muscle tone in vitro (10, 34). The PPI-induced reduction in LES tone is not unique to gastroesophageal muscle, 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), and gallbladder (1) smooth muscle and corpus cavernosum (23).

Yet the mechanism accounting for the PPI effect on smooth muscle contraction is presently unknown and likely unrelated to nitric oxide (23, 33), K+ channel (1, 12), or cyclooxygenase (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+-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 (5), and vascular smooth muscle cells (17, 18). Thus it is appealing to link the PPI effect on smooth muscle tone to its potential action on the H+-K+-ATPases. Yet all PPIs are prodrugs that need acidic environment to become active (15); 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 PTP 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. 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.

PTP is 98% bound to protein in serum (13, 20) and such a low free drug availability has been incriminated in its lack of hemodynamic and cardiac in vivo effects in adult rats (32). The data from the present study showing delayed gastric emptying

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. Insets show representative Western blots. **P < 0.01 and *P < 0.05 by 1-way ANOVA with Tukey-Kramer multiple comparison testing.
following intraperitoneal PTP 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 PPI 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.

REFERENCES


