Effect of intraluminal distension or ischemic strangulation obstruction of the equine jejunum on jejunal motilin receptors and binding of erythromycin lactobionate

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Objective—To determine whether inflammation of the jejunum of horses decreases the number of motilin receptors and amounts of motilin receptor mRNA and alters erythromycin lactobionate binding affinity to the motilin receptor in jejunal tissues.

Sample Population—Jejunal segments in 6 adult horses.

Procedure—Each horse was anesthetized, and a ventral median celiotomy was performed; 2 segments of jejunum underwent a sham operation, 2 segments underwent ischemic strangulation obstruction (ISO), and 2 segments underwent intraluminal distension (ILD). Treatments were maintained for 120 minutes. From each segment, full-thickness biopsy samples were collected and smooth-muscle homogenates were prepared. Affinity and distribution of motilin binding to these preparations were determined by use of iodine 125 (125I)-labeled synthetic porcine motilin. Via displacement experiments, competition between 125I-labeled motilin and erythromycin lactobionate for binding to motilin receptors in the different segments was investigated. A quantitative real-time PCR technique was used to assay motilin receptor mRNA content in the muscle preparations.

Results—Compared with the ISO or ILD segments, the number of motilin receptors was significantly higher in the sham-operated segments; ILD segments contained the lowest number of motilin receptors. The expression of motilin receptor mRNA was significantly decreased in ILD segments but not in ISO segments. Erythromycin lactobionate displacement of 125I-labeled motilin from motilin receptors did not differ significantly among the jejunal segments.

Conclusions and Clinical Relevance—Results suggest that downregulation and decreased production of motilin receptors in inflamed jejunal tissue contribute to the altered prokinetic response to erythromycin in horses with gastrointestinal disease. (Am J Vet Res 2006;67:815–820)

Gastrointestinal ileus has been defined as the functional inhibition of propulsive intestinal activity, irrespective of its pathophysiologic basis. Terms used to describe the various clinical manifestations of motility disorders in horses are confusing; ileus, postoperative ileus, endotoxemic ileus, idiopathic ileus, and adynamic ileus are used inconsistently. When duration of clinical signs is used to classify ileus, it can be characterized as adynamic (resulting from short-term alterations of motility) or paralytic (resulting from loss of gastrointestinal motility for >72 hours). Ileus can result from diseases of the digestive system or develop secondary to diseases of other systems. Reflex postoperative ileus has been reported in humans and horses. It has been postulated that this develops as a result of an imbalance between sympathetic and parasympathetic nervous input to the intestines. Sympathetic (adrenergic) hyperactivity results in splanchic vasconstriction, reduction of intestinal propulsive motility, and an increase in tone of the gastrointestinal sphincter. Parasympathetic (cholinergic) hypoactivity results in decreases in gastrointestinal motility and secretion. However, it is likely that abnormalities involving the enteric nervous system, which uses several neuromodulators and nitric oxide as neurotransmitters, and damage to the interstitial cells of Cajal that normally generate slow waves also contribute to the development of ileus. Shock, intestinal ischemia, endotoxemia, and prolonged distension and inflammation of the intestinal tract have all been implicated as factors that contribute to the pathogenesis of ileus in horses.

Various pharmacologic agents have been used in horses to promote motility of the gastrointestinal tract. Erythromycin lactobionate, a macrolide antimicrobial, has been characterized in vivo and in vitro as a prokinetic agent in humans, dogs, cats, rabbits, and horses. In clinically normal horses, erythromycin lactobionate accelerates gastric emptying.
whereas in healthy ponies, erythromycin lactobionate can increase cecal emptying as well as ileocecal myoelectric activity. In other species, the action of erythromycin seems to be secondary to binding to smooth-muscle motilin receptors as well as neural motilin receptors. In a recent study, we determined that motilin receptors are present in both the small and large intestine of healthy horses and that one of the prokinetic actions of erythromycin is mediated through binding to motilin receptors.

In another study, the effect of erythromycin lactobionate was evaluated in horses after a ventral midline celiotomy; during the first 24 hours, myoelectric activity increased in the ileum and pelvic flexure but not in the cecum. When these horses were administered the same dose of erythromycin lactobionate 8 days after surgery, the cecum was responsive. The authors concluded that the prokinetic effects of erythromycin in healthy horses were 3 to 9 years old (mean age, 5.5 years) and covered with plastic sheets to prevent dehydration.

The experimental protocol was approved by the Animal Care Committee of the University of Guelph.

The purpose of the study reported here was to determine whether inflammation of the jejunum of horses decreases the number of motilin receptors and amounts of motilin receptor mRNA and alters erythromycin lactobionate binding affinity to the motilin receptor in jejunal tissues.

**Materials and Methods**

**Horses**—Six mature horses were used in this study; the horses were 3 to 9 years old (mean age, 5.5 years) and weighed 370 to 570 kg (mean weight, 434 kg). None of the horses had gastrointestinal tract disorders or evidence of systemic disease and were not treated with erythromycin or similar compounds prior to the study. Effects of ILD of the jejunum at a pressure of 25 cm H₂O for 120 minutes, as well as ischemia of the jejunum for 60 minutes followed by reperfusion for 60 minutes, on motilin receptors and effects of the binding of erythromycin on these receptors were evaluated. The experimental protocol was approved by the Animal Care Committee of the University of Guelph.

**Anesthetic protocol**—Horses were sedated with xylazine hydrochloride (0.2 to 0.5 mg/kg, IV), and anesthesia was induced with guaifenesin (100 mg/kg, IV) and ketamine hydrochloride (2.2 mg/kg, IV). Horses were positioned in dorsal recumbency, and anesthesia was maintained by use of halothane in oxygen and intermittent positive-pressure ventilation. Mean blood pressure (measured by direct arterial catheterization) was maintained at ≥70 mm Hg. Lactated Ringer’s solution was administered IV at a rate of 5 to 10 mL/kg/h to maintain circulating volume and blood pressure. To maintain systolic arterial blood pressure at 100 to 120 mm Hg, dobutamine was administered IV as needed throughout the surgery.

**Surgical procedure**—For each horse, the ventral area of the abdomen was clipped and aseptically prepared. In the ventral portion of the abdomen, an incision extending 25 cm cranially from the umbilicus was made. Beginning approximately 100 cm distal to the duodenoileal ligament, two 25-cm-long jejunal segments, each supplied by a jejunal arcade artery and vein, were identified. Each of these experimental segments was separated by a 25-cm-long jejunal segment; each intervening segment was also supplied by a jejunal arcade artery and vein. Two of the experimental segments were randomly designated by use of a Latin square design as ISO segments, 2 were designated as ILD segments, and 2 were designated as sham-operated (control) segments. Throughout the experiment, the segments were moistened with sterile saline (0.9% NaCl) solution and covered with plastic sheets to prevent dehydration.

**Ischemic strangulation obstruction** was created as previously described by applying rubber-shod hemostats across major mesenteric vessels associated with the experimental segment of jejunum. Mural collateral vessels were occluded by application of rubber-shod Doyen clamps across both ends of the experimental segment. Ischemic strangulation obstruction was maintained for 60 minutes and was followed by 60 minutes of reperfusion.

**Intraluminal distension** was created as previously described by occluding the lumen at each end of the segment with circumferential Penrose drains. The latex drains penetrated the mesentery close to the jejunum without occluding extramural vessels. An 18-gauge polyethylene catheter was inserted through the intestinal wall into the lumen, and a 2.0 monofilament glycomer was used to suture the catheter onto the serosa. Sterile lactated Ringer’s solution was infused into the lumen of the intestine until an intramural pressure of 25 cm H₂O was achieved. A pressure transducer attached to another intraluminal catheter was used to monitor pressures in each segment, and pressures were maintained by infusion of fluid as required for 120 minutes. Nonsorbable sutures were used to secure each end of the catheters to mark the beginning and end of the sham-operated segment.

After 120 minutes, a 10 × 5-cm full-thickness biopsy specimen was collected from each of the 6 segments. The biopsy specimen was obtained from the center of each segment at the antimesenteric side, and the horse was euthanized via IV administration of an overdose of pentobarbital sodium. The segments were gently lavaged with physiologic saline solution to remove ingesta. Mucosa and submucosa were dissected free from the smooth-muscle tissue by use of scissors or a scalpel blade. The individual smooth-muscle samples were rinsed again with physiologic saline solution, wrapped in tinfoil, snap-frozen in liquid nitrogen, and stored at −70°C until further processing.

**Crude membrane preparation**—The technique for crude membrane preparation from tissues of horses has been described previously. Each section of smooth-muscle tissue was weighed (mean sample weight, 2.65 g; range, 2.2 to 3.5 g), finely minced, and homogenized in 10 volumes of buffer (containing 250 mM sucrose and 50 mM tris-HCl; pH, 7.4) by use of a homogenizer at 1,500 rounds/min for 2 minutes. The homogenates were centrifuged at 1,000 × g for 15 minutes and washed 4 times in 150 mM NaCl solution. The homogenates were resuspended in 5 volumes of 50 mM tris-HCl buffer (containing 1.5% bovine serum albumin and 10 mM MgCl₂; pH, 8.0) and divided into 1-mL aliquots for use in binding and displacement experiments.

**Assessment of the number of motilin receptors**—Binding experiments were performed as described else
Briefly, total binding was determined at room temperature (approx 21°C) by incubating 1.6, 3.2, 6.4, 12.8, 25.6, and 51.2 nM iodine 125 (125I)-labeled synthetic porcine motilin with the crude membrane preparations from ISO, ILD, and control segments (6 horses [3 repetitions each]) in 50 mM tris-HCl buffer (containing 1.5% bovine serum albumin and 10 mM MgCl₂; pH 8.0). Incubation of identical samples in the presence of a 100-fold excess of unlabeled motilin enabled assessment of nonspecific binding. After a 90-minute incubation, bound and unbound motilin were separated by centrifugation for 15 minutes at 1,000 X g. The supernatant (containing unbound motilin) was discarded. Radioactivity of the pellets (representative of bound motilin) was measured by use of a gamma counter. Mean total protein amount per pellet was determined by use of a protein assay. Binding values were expressed per milligram of protein. Specific binding was determined by subtracting the amount of nonspecific binding from the total amount of binding. Values of Kᵦ and Bₘₐₓ of the various membrane preparations were determined via nonlinear least squares analysis.

Displacement experiments—In the competition experiments, a displacement curve was obtained by incubating crude membrane preparations from ISO, ILD, and control segments with 3 nM 125I-labeled synthetic porcine motilin and increasing concentrations (10⁻¹, 10⁻², 10⁻³, and 10⁻⁴M) of erythromycin lactobionate (n = 6 horses [3 repetitions each] at each concentration) in 50 mM tris buffer (containing 1.5% bovine serum albumin and 10 mM MgCl₂; pH 8.0). Nonspecific binding was assessed by incubating identical preparations in the presence of 50 nM unlabeled motilin. After a 90-minute incubation at room temperature, bound and unbound motilin were separated by centrifugation for 15 minutes at 1,000 X g. The radioactivity of the pellet (representative of bound motilin) was measured by use of a gamma counter. Specific binding was determined as outlined previously. The negative logarithm of the pIC₅₀ value was obtained from the displacement curve.

Quantitative real-time PCR procedure—Snap-frozen tissues (100 mg) from ISO, ILD, and control segments were homogenized in isolation reagent by use of a homogenizer. Total RNA was isolated according to the manufacturer’s instructions. Samples were treated to remove contaminated DNA from the RNA preparations. Purity and concentrations of the RNA samples were determined via spectrophotometry. Three micrograms of RNA from each sample was then reverse transcribed by use of a synthesis system for the reverse transcriptase-PCR procedure.

To the authors’ knowledge, the CDNA sequence for the equine motilin receptor has not been previously reported. Therefore, we designed the following PCR primers on the basis of the rabbit motilin receptor sequence, focusing on the region of highest homology between rabbit and human cDNAs: forward, 5' TTTGCTGGTTGCCCTTCT 3'; reverse, 5' AGGGTGTCGTTGCGCTTCCT 3'. The partial equine motilin receptor sequence was used to design equine-specific motilin receptor primers: forward, 5' TTGTCGGTGCGCCCCTCT 3'; reverse, 5' AGGGTGTCGTTGCGCTTCCT 3'. These primers were used to perform quantitative reverse transcriptase-PCR procedures. The equine motilin receptor primers were used via 50 cycles at 95°C for 15 seconds, 55°C for 5 seconds, and 72°C for 10 seconds; glucose-6-phosphate dehydrogenase was used as the housekeeping gene under the same conditions as those applied to the gene of interest. Analysis was performed by use of computer software.

Statistical analysis—Statistical analysis was performed with a randomized complete block design to determine whether there was a significant difference between the different segments of jejunum with regard to Kᵦ, Bₘₐₓ, and pIC₅₀ values and mRNA ratios. A Shapiro-Wilk test and plots of the residuals were used to determine whether the residuals were normally distributed and the data fit the assumptions of normality. Tukey multiple means comparisons were used if a significant difference was detected. A value of P < 0.05 was considered significant.

Results

All 6 horses required periodic infusion of sterile lactated Ringer’s solution into the lumen of ILD segments of the jejunum to maintain an intramural pressure of 25 cm H₂O. Fluid was instilled into the lumen when the intramural pressure was less than 20 cm H₂O. In an ISO segment in 1 horse, a rubber-shod hemostat fell off from a mesenteric vessel and had to be replaced during the procedure.

Number of motilin receptors—The mean ± SEM Bₘₐₓ for control, ISO, and ILD segments was 357.01 ± 9.34 fmol/mg of protein, 324.39 ± 9.18 fmol/mg of protein, and 193.47 ± 5.5 fmol/mg of protein, respectively. Values were calculated from 5 measurements of each crude membrane preparation for each segment of the jejunum obtained from 6 horses. Mean Bₘₐₓ differed significantly among control, ISO, and ILD segments. Control segments had a significantly higher Bₘₐₓ, compared with the value for ISO or ILD segments. The Bₘₐₓ for ISO segments was significantly higher, compared with the value for ILD segments. Therefore, ILD or ISO of equine jejunum resulted in a significant decrease in the number of motilin receptors, compared with the number of receptors in the control segments.

Affinity of motilin for the receptor—Mean Kᵦ ± SEM for the equine motilin receptor in control, ISO, and ILD segments was 6.36 ± 0.53 nM, 6.35 ± 1.58 nM, and 6.36 ± 0.53 nM, respectively.

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Figure 1—Sequence of the equine motilin receptor CDNA fragment from which equine-specific motilin receptor primers were designed. Sequence of the equine motilin receptor cDNA fragment via PCR procedures. Underlined typeface indicates the primers derived from the published human sequence that were used in the quantitative real-time PCR analysis. Highlighted typeface indicates primers derived from the published human sequence that were used in the quantitative real-time PCR analysis.
affected by ISO or ILD.

The expression of motilin receptor mRNA was significantly decreased in ILD segments but not in ISO segments. The expression of motilin receptor mRNA in jejunal segments was significantly different between control, ISO, and ILD segments. Thus, the affinity of 125I-labeled synthetic porcine motilin for the equine motilin receptor did not change with ISD or ILD.

**Displacement experiments with erythromycin**—Mean pIC₅₀ ± SEM in control segments was 2.76 ± 0.31 nM, which was not significantly different from the value in ISO segments (mean pIC₅₀, 2.68 ± 0.28 nM) or ILD segments (mean pIC₅₀, 2.72 ± 0.30; Figure 2). The concentration of erythromycin lactobionate required to displace porcine motilin from the equine motilin receptor did not change with ISD or ILD of the jejunum.

**Expression of motilin receptor mRNA**—The normalized ratio for motilin receptor mRNA in control, ILD, and ISO segments was 0.97 ± 0.14, 0.63 ± 0.12, and 0.95 ± 0.11, respectively. There was a significantly lower ratio for motilin receptor mRNA in ILD segments than in control or ISO segments. There was no significant difference in the mRNA ratio between control and ISO segments. The expression of motilin receptor mRNA was significantly decreased in ILD segments but not in ISO segments.

**Discussion**

In some horses with colic, intestinal ischemia or distension causes inflammation, which is partly responsible for development of ileus postoperatively. An ischemic intestine model has been developed in horses and was used successfully to study effects of various drugs. The ischemic episode used in that model resulted in decreased motility and thickening of the intestine and was found to simulate the type of injury that develops in some equine abdominal crises. Histologically, after 1 hour of ischemia, hemorrhage or edema developed in the small intestinal interstitial layer and mild separation of epithelium from the basement membrane at the villus tip was detected. Sixty minutes of ischemia followed by 60 minutes of reperfusion exacerbated the ultrastructural damage in the jejunum, especially in the mucosa in which the damage increased substantially, compared with that resulting from ischemia only. In another study, control, ISD, and ILD segments were established in the same horse at the same time and ultrastructural damage in the intestinal wall was detected. Therefore, we decided to use the latter model to induce inflammation in the jejunum of horses and thereby evaluate changes in motilin receptor density and mRNA expression as well as the binding ability of erythromycin to the motilin receptor in inflamed equine intestine. We decided not to perform histologic evaluation on experimental segments because of multiple reports describing histologic changes after ISO or ILD.

In a previous investigation by our group, binding studies with 125I-labeled synthetic porcine motilin were performed to assess the distribution of motilin receptors in the gastrointestinal tract of horses. In that investigation, the highest number of motilin receptors was detected in the duodenum. In the study reported here, the jejunum was used to create experimental segments because there is no significant difference in the number of motilin receptors between the duodenum and jejunum; the jejunum is much more accessible via a ventral midline exploratory laparotomy, compared with the duodenum; and the jejunum is a common site for small intestinal lesions. Establishing 2 control segments, 2 ISO segments, and 2 ILD segments in a randomized sequence in each of 6 horses further minimized the chance that a decrease in motilin receptors was attributable to anatomic location because it is known that the number of motilin receptors decreases abnormally in many species.

In the present study, 60 minutes of ISO followed by 60 minutes of reperfusion resulted in a significant decrease of the number of motilin receptors, compared with findings in control segments. However, the number of motilin receptors correspondingly decreased only by approximately 10%, and the biological importance of this decrease is unknown. The decrease in motilin receptor numbers was more pronounced after 2 hours of ILD than after ischemia and reperfusion. This may be attributable to the fact that ISO results in major damage to the mucosa (and to the smooth muscle of the jejunum to a lesser degree), whereas distension had a more severe effect on the seromuscular layer than on the mucosa. In guinea pigs and rabbits, most of the intestinal motilin receptors are located in the smooth-muscle layer. Because distension causes more damage to the seromuscular layer than ischemia, it might be expected that the motilin receptors would be more affected by distension than by ischemia. This is also supported by our finding that, compared with control segments, the expression of motilin receptor mRNA in jejunal segments was significantly decreased by ILD but not by ISO.

The affinity of 125I-labeled synthetic porcine motilin to the equine motilin receptor was approximately 6.3 nM, which is similar to a previously reported affinity of 6.1 nM. The affinity of the motilin receptor to motilin was not affected by inflammation, which
implies that the functionality of the receptor likely is not disturbed by inflammation induced by the procedures used in our study. This is in agreement with findings in rabbits, in which inflammation did not affect the binding affinity of motilin to the colonic receptor.22

The ability of erythromycin lactobionate to bind to the equine motilin receptor was also not affected by ISO or ILD. In an in vitro study25 in horses, it was shown that smooth-muscle strips harvested from a jejunum that underwent distension and decompression had a decreased contractility response to erythromycin, compared with untreated strips of jejunal smooth muscle. The authors of that report25 concluded that the release of inflammatory mediators or nonadrenergic noncholinergic inhibitory neurotransmitters could be involved in the decreased contractile response. Given the fact that, in the present study, both the number of motilin receptors as well as the expression of motilin receptor mRNA decreased with inflammation but the affinity of erythromycin to the motilin receptor was unaffected, it seems plausible that the diminished response to erythromycin in inflamed segments of jejunum was a consequence of downregulation or decreased synthesis of motilin receptors.

In a study25 by another group, the prokinetic effect of erythromycin lactobionate (0.5 mg/kg, IV) on myoelectric activity of the ileum, cecum, and pelvic flexure (large colon) in horses after surgery was evaluated. Erythromycin increased contractility in the ileum and pelvic flexure but had no effect on the cecum in the first 24 hours of the postoperative period. Eight days after surgery, the cecum was responsive to administration of erythromycin.25 It is well documented that the motility-inhibiting effects of anesthetic drugs last for only approximately 9 hours after general anesthesia.50 Furthermore, in healthy horses, erythromycin induced motility in the cecum and right ventral colon and promoted cecal emptying of radiolabeled markers.23 Our group has also determined that motilin receptors are present in the cecum of horses and that erythromycin binds to this receptor.27 In the study reported here, the effect of inflammation on the cecum was not evaluated, but we speculate that the lack of response of the cecum to erythromycin in the postoperative period reported by Roussel et al45 was a result of downregulation of motilin receptors, rather than a functional disturbance of the erythromycin–motilin receptor interaction.

One potential mechanism for the decrease in motilin receptor expression is internalization of motilin receptors.21 It was recently determined that motilin receptors belong to the family of class I G-protein receptors.33 It is now known that after motilin binds to the motilin receptor, the receptor becomes desensitized and rapidly internalized, whereupon it becomes coupled to a G_{13} receptor that leads to Ca-dependent initial contraction of the smooth-muscle cell. The internalized motilin receptor also becomes coupled to a G_{13} receptor, which results in Ca-independent sustained contraction of the smooth-muscle cell.34 Prolonged internalization has been described in other species as a cause of decreased duodenal contractility.25 In the present study, downregulation at the mRNA level was detected in the ILD segments, indicating an additional potentially important control mechanism. The lack of an equivalent decrease at the mRNA level in the ISO segments suggests that additional mechanisms, such as redistribution, may occur under ischemic conditions. When the expression of cell surface receptor mRNA is not affected, inflammation can affect the expression or activation of G-proteins coupled to these receptors. This has been determined for muscarinic M2 and M3 receptors; their mRNA expression was unaffected by inflammation of the ileum, but modulation of expression and activation of G-protein receptors was disturbed.35 The relative importance of each regulatory mechanism, as well as the underlying pathways involved, are important areas for further investigation.

Reportedly, the duration of postoperative ileus in horses can range from 1 to 7 days26 or 1 to 9 days.11 Downregulation, as well as upregulation, of various receptors occurs during inflammation of the intestinal wall in other species31,32 and in the jejunum of horses; it would be important to explore how long these receptors are affected during ileus.

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