In vitro investigation of the effects of cyclooxygenase-2 inhibitors on contractile activity of the equine dorsal and ventral colon

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Objective—To evaluate the effect of 2 cyclooxygenase (COX)-2 inhibitors on contractile activity of the circular smooth muscle layer of the equine dorsal and ventral colon.

Sample Population—Samples of the dorsal and ventral colon obtained from 10 healthy horses.

Procedure—Full-thickness tissue samples were collected from the dorsal colon in the area of the diaphragmatic flexure and the ventral colon in the area of the sternal flexure. Samples were cut into strips oriented along the fibers of the circular muscle layer and mounted in a tissue bath system for determination of contractile strength. Incremental amounts of etodolac, nabumetone, and indomethacin were added, and contractile activity was recorded.

Results—Response of the dorsal and ventral colon to nonsteroidal anti-inflammatory drugs (NSAIDs) was variable. Indomethacin induced the greatest reduction in contractile activity, followed by nabumetone. For etodolac, the difference from baseline values was only significantly reduced at the highest concentration used (1×10^{-5}M) for the ventral colon.

Conclusions and Clinical Relevance—The NSAIDs that are designed to target the COX-2 isoform appeared to have variable effects on the contractile activity of the equine dorsal and ventral colon. Etodolac appeared to have the least effect on contractile activity, compared with the effects attributable to nabumetone, and would potentially have the fewest adverse effects relative to motility of the dorsal and ventral colon. (*Am J Vet Res* 2002;63:1496–1500)

Although the use of **nonsteroidal anti-inflammatory drugs (NSAIDs)** is widespread in the management of horses with musculoskeletal pain and inflammation, chronic use of these agents may predispose horses to additional complications including intestinal ulcers and renal dysfunction.[1-3] Such complications are believed to be induced by a reduction in local prostaglandin production via inhibition of **cyclooxygenase (COX)** enzymes.[4] The molecular structure of COX is quite complex and exists in 2 isoforms, specifically COX-1 and COX-2. Products of COX-1 activity are involved in maintaining normal function of the gastric and intestinal mucosa, renal function, microvascular blood flow, and platelet aggregation.[5,6] In contrast, COX-2 is induced within 4 to 24 hours following exposure to inflammatory mediators, such as interleukins and lipopolysaccharide, and is the primary source of prostaglandins that mediate pain, amplification of inflammation, and fever.[6-12] This induction of COX-2 activity also occurs in the intestinal tract during periods of inflammation.[13-16]

Prostaglandins exert a cytoprotective effect on the intestinal tract via stimulation of gastric mucus production, increased mucosal blood flow, and decreased acid secretion via COX-1 activity.[12] Local prostaglandin release is also believed to play an important role in repair of the epithelial barrier following injury.[16] With regard to motility, the specific role of prostaglandins is less clearly defined. Prostaglandin release has been correlated with increased propulsion of feed material via increases in secretion and peristalsis.[17] Although it has been established[15] that inhibition of COX induces mucosal injury, the specific effects of COX-1 and -2 in the regulation of intestinal motility remain to be identified. Variability in the effects of NSAIDs on various regions of the intestinal tract certainly plays a role in the lack of information. For example, in the colon of dogs and humans, indomethacin impairs peristalsis, whereas indomethacin induces phasic contractions in the small intestine of guinea pigs.[18] Studies[19,20] conducted by our laboratory group also revealed that NSAIDs inhibit contractile activity of the colon of horses. Specifically, we found that NSAIDs such as phenylbutazone, which are nonspecific COX-1 and -2 inhibitors, induce a concentration-dependent decrease in contractile activity relative to baseline values in the dorsal colon, ventral colon, and pelvic flexure. Clinical implications of the results of that study were that chronic exposure to NSAIDs may predispose horses to motility disturbances of the colon and subsequently lead to feed impactions.

Selective COX-2 inhibitors are a relatively new class of NSAIDs that may offer additional anti-inflammatory and analgesic efficacy and fewer adverse effects, compared with the traditional NSAIDs.[5] Although studies[5,6,11] have been conducted to evaluate the effect of COX-2 inhibitors on mucosal ulcers and pain, studies on intestinal motility are lacking. Therefore, the objective of the study reported here was to evaluate the effects of 2 COX-2 inhibitors on contractile activity of the circular smooth muscle layer of the dorsal and ventral large colon.

Materials and Methods

Animals—Tissue samples were collected from 10 horses euthanized for reasons unrelated to the gastrointestinal tract. Horses ranged from 3 to 12 years of age (mean, 9.6

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years) and weighed between 400 and 560 kg (mean, 485 kg). This study was approved by the university animal care and use committee.

Collection and processing of tissues—Immediately after horses were euthanized by administration of an overdose of pentobarbital, a 10 × 10-cm full-thickness sample was collected from the dorsal colon in the area of the diaphragmatic flexure and the ventral colon in the area of the sternal flexure of each horse. Tissue sections were then processed as described elsewhere.[20] Briefly, tissues were immersed in a modified Krebs buffer solution (110mM NaCl, 4.6mM KCl, 2.5mM $CaCl_2$, 24.8mM $NaHCO_3$, 1.2mM KH_2PO_4, 1.2mM $MgSO_4$, and 5.6mM dextrose; pH 7.3 to 7.4 when equilibrated with 95% O_2-5% CO_2), pinned flat, and cut into strips 2 mm in width by 10 mm in length and parallel to the fibers of the circular smooth muscle. Strips were mounted on glass hooks in a tissue bath system (20-mL capacity) that contained oxygenated Krebs buffer solution warmed to 37.5 ± 0.5°C as described elsewhere.[20] The other end of the muscle strip was connected to a polygraph chart recorder[a] that measured tension by the use of force transducers.[b] After allowing the tissue to equilibrate in the baths for 45 minutes, strips were stretched to produce a basal tension of 2 g and allowed to equilibrate for an additional 60 minutes. During the equilibration period, Krebs buffer was changed at 20-minute intervals. Although spontaneous activity was generated by some of the muscle strips during this time, substance P[c] (40 µL of a 10^{-4}M solution) was added to generate a consistent contractile pattern.[20] Eight circular smooth muscle strips were evaluated at a time, and there was a minimum of 4 analyses performed for each drug evaluated. Therefore, each drug was tested on a minimum of 32 muscle strips with only 1 drug tested per muscle strip during each experiment. In 2 horses, 16 strips were collected from each horse to minimize the number of animals used. Contractile activity was recorded to establish baseline values prior to the addition of any drugs.

To reduce confounding influences, atropine[d] and guanethidine[e] (1M solutions of each) were added to each bath to inhibit muscarinic receptors and adrenoceptors, respectively; atropine and guanethidine were added to each bath prior to the addition of any NSAIDs. Concentrations of NSAIDs added to the muscle baths were 1×10^{-9}, 3×10^{-9}, 1×10^{-8}, 3×10^{-8}, 1×10^{-7}, 3×10^{-7}, 1×10^{-6}, 3×10^{-6}, and 1×10^{-5}M. Indomethacin,[f] nabumetone,[g] and etodolac[h] were prepared as 10^{-2}M solutions by dissolving them in a 10-mL volume of Krebs buffer solution and 0.0114 g of Na_2CO_3.[i] All reagents were prepared fresh the day of the experiment.

Following addition of each NSAID to the muscle baths, contractile activity of muscle strips was recorded for 180 seconds with a 5-minute interval between additions of subsequent drugs. Inhibition or excitation generated was determined by comparing the strength of contraction generated during the 180-second interval with the baseline contractile force. In another study,[20] we determined that contractility did not change as a function of time or tissue fatigue. Mean in vitro effective concentration of each NSAID (**mean concentration that is 50% of the NSAID concentration that induces a maximal effect [EC_{50}]**) on the circular smooth muscle layers of the dorsal and ventral equine large colon was also determined. This value was calculated by use of nonlinear regression analysis.[j]

Statistical analysis—Statistical analysis was performed by use of an ANOVA to determine whether there was a significant difference in values for treated strips, compared with baseline contractile activity. For each drug, the difference in contractile activity from baseline was compared for the dorsal and ventral colon. The ANOVA was also used to determine whether a significant difference could be detected between the dorsal and ventral colon for each NSAID and whether there was a significant difference in activity among NSAIDs. Post hoc tests (Fisher least-significant difference) were used when a significant difference was detected ($P < 0.05$).

Results

We did not detect a significant difference in contractile activity between the dorsal and ventral colon at the same concentration of each NSAID. Whereas indomethacin and nabumetone produced a concentration-dependent decrease in contractile activity for the dorsal and ventral colon (**Fig 1 and 2**), the magnitude of the inhibition was greater for indomethacin. For example, at a concentration of 3×10^{-8}M in the dorsal colon, mean decrease from baseline value was 3.82 N for indomethacin, compared with 1.18 N for nabumetone. Activity of the dorsal and ventral colon was not significantly inhibited from baseline values following administration of etodolac at concentrations of $< 3 \times 10^{-7}$ and 1×10^{-6}M, respectively (**Fig 3**). The only significant ($P = 0.02$) difference from baseline value for the ventral colon was at a concentration of 1×10^{-5}M etodolac. For indomethacin, contractile effects of concentrations $> 3 \times 10^{-9}$M were inhibited significantly ($P = 0.01$).

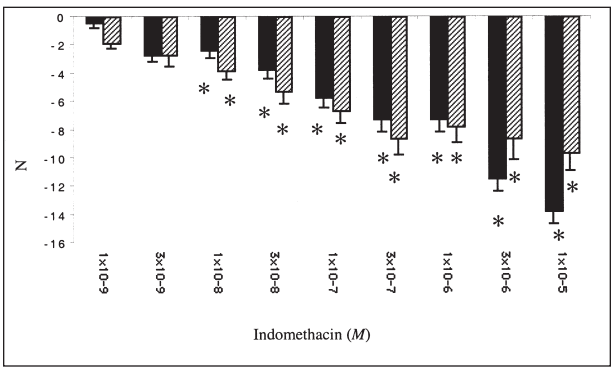

Figure 1—Mean ± SD inhibition of contractile response following the addition of various concentrations of indomethacin to tissue baths containing samples obtained from the ventral (black bar) and dorsal (diagonal striped bar) colon of 10 horses. *Within each tissue location, values differ significantly ($P < 0.05$) from baseline values.

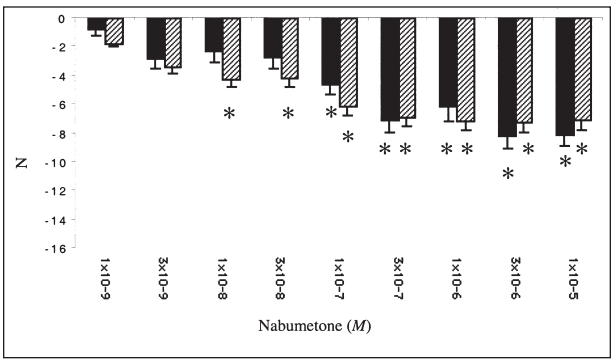

Figure 2—Mean ± SD inhibition of contractile response following the addition of various concentrations of nabumetone to tissue baths containing samples obtained from the ventral (black bar) and dorsal (diagonal striped bar) colon of 10 horses. *See* Figure 1 for key.

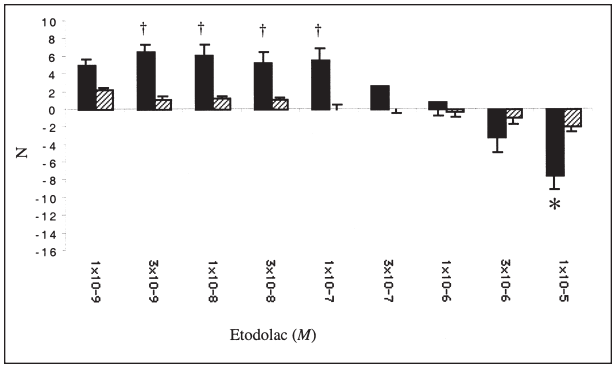

Figure 3—Mean ± SD inhibition of contractile response following the addition of various concentrations of etodolac to tissue baths containing samples obtained from the ventral (black bar) and dorsal (diagonal striped bar) colon of 10 horses. †Within a concentration, values differ significantly ($P < 0.05$) between the dorsal and ventral colon. *See* Figure 1 for remainder of key.

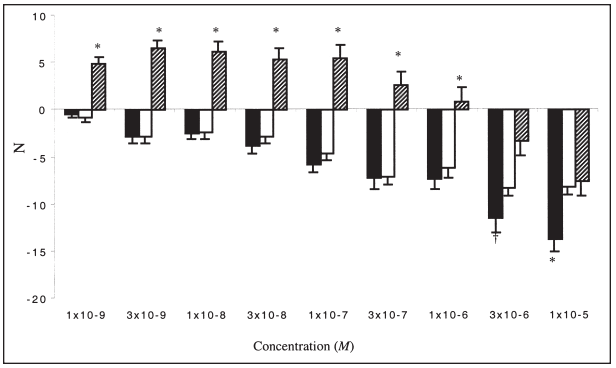

Figure 4—Mean ± SD inhibition of contractile response following the addition of various concentrations of indomethacin (black bar), nabumetone (white bar), or etodolac (diagonal striped bar) to tissue baths containing samples obtained from the ventral colon of 10 horses. *Within a concentration, value differs significantly ($P < 0.05$) from values for the other 2 agents. †Within a concentration, value for indomethacin differs significantly ($P < 0.05$) from the value for etodolac.

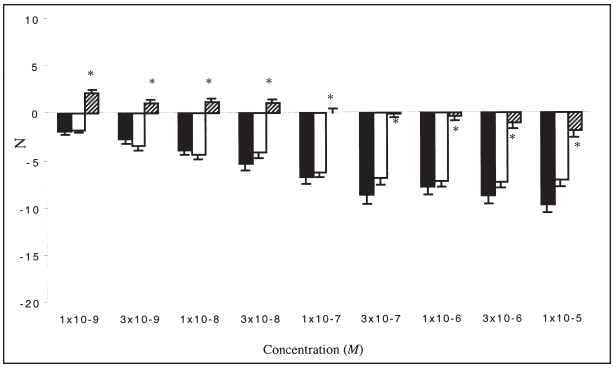

Figure 5—Mean ± SD inhibition of contractile response following the addition of various concentrations of indomethacin (black bar), nabumetone (white bar), or etodolac (diagonal striped bar) to tissue baths containing samples obtained from the dorsal colon of 10 horses. *See* Figure 4 for key.

Addition of nabumetone produced a pattern of contractile activity in the dorsal and ventral colon that was similar to the pattern after addition of indomethacin. Specifically, there was a progressive decrease in contractile activity with increasing concentrations of nabumetone. Generally, the dorsal colon also appeared more sensitive than the ventral colon in that contractile activity was more inhibited in the dorsal than in the ventral colon at lower concentrations. At concentrations of 3×10^{-6} and 1×10^{-5}M nabumetone, contractile activity was more inhibited in the ventral colon (8.21 and 8.18 N versus 7.31 and 7.11 N in the dorsal and ventral colon, respectively). At the same concentration, there was not a significant difference in contractile activity between the dorsal and ventral colon. For the dorsal colon, concentrations $> 3 \times 10^{-9}$M nabumetone were significantly ($P = 0.02$) more inhibited relative to baseline activity (Fig 2). Activity of the ventral colon was significantly ($P = 0.02$) more inhibited relative to baseline activity at concentrations $\geq 1 \times 10^{-7}$M.

Following addition of etodolac, the ventral colon appeared more sensitive to the drug than was the dorsal colon, because there was an increase in contractile activity at lower concentrations (1×10^{-9} to 1×10^{-6}M), compared with baseline activity (Fig 3). There was a significant difference in activity between the dorsal and ventral colon at concentrations of 3×10^{-9}M ($P = 0.04$), 1×10^{-8}M ($P = 0.02$), 3×10^{-8}M ($P = 0.04$), and 1×10^{-5}M ($P = 0.03$). For the dorsal colon, there was not a significant ($P = 0.08$) difference from baseline activity at any concentration of etodolac evaluated. For the ventral colon, 1×10^{-5}M etodolac was the only concentration for which contractile activity differed significantly ($P = 0.03$) from baseline activity.

A comparison of the effect of the NSAIDs on motility in the ventral and dorsal colon of the large intestine was summarized **(Fig 4 and 5)**. In the dorsal colon, etodolac produced significantly ($P = 0.018$) less inhibition, compared with the amount of inhibition produced by nabumetone and indomethacin, at all concentrations. In the ventral colon, etodolac produced significantly ($P = 0.04$) less inhibition than nabumetone and indomethacin at all concentrations $< 3 \times 10^{-6}$M. At 3×10^{-6} M etodolac, contractile effects were significantly ($P = 0.005$) less inhibited than effects attributable to indomethacin but did not differ significantly from the effects attributable to nabumetone. At the highest concentration (ie, 1×10^{-5}M), effects attributable to nabumetone and etodolac were significantly ($P = 0.04$ and 0.03, respectively) less inhibited than effects attributable to indomethacin.

The EC_{50} values were calculated. The EC_{50} for indomethacin in the ventral colon was 2.35×10^{-6}M, whereas it was 3.84×10^{-6}M in the dorsal colon. The EC_{50} for nabumetone in the ventral colon was 2.44×10^{-6}M, whereas it was 3.64×10^{-6}M in the dorsal colon. The EC_{50} for etodolac in the ventral colon was 3.16×10^{-5}M, whereas it was 1.50×10^{-5}M in the dorsal colon. There was a difference of at least 1 logarithm in the EC_{50} between indomethacin and etodolac, suggesting that indomethacin had more activity against COX-1 receptors than COX-2 receptors, compared with the effects of etodolac.

Discussion

Indomethacin is an example of a NSAID that has activity against COX-1 and -2, although it is less effective against COX-2 than against COX-1.[21,22] In the study reported here, indomethacin was included to

represent the criterion-referenced standard of a nonselective inhibitor of COX-1 and -2. The finding that this NSAID induced a concentration-dependent decrease in contractile activity of the circular smooth muscle layer of the colon was similar to results from another study,[20] which supports the contention that indomethacin has greater inhibition of COX-1. Nabumetone had greater inhibition of COX-2, compared with the effects of indomethacin, but less inhibition of COX-2 than was evident for etodolac. Therefore, etodolac likely had lesser effects because of more selective inhibition of COX-2, whereas nabumetone inhibited contractile activity via some inhibition of COX-1. In a study[16] in which investigators evaluated the effect of NSAIDs that were specific and nonspecific for COX-2 in samples of ischemic intestine, there was continued production of prostaglandins in tissues exposed to etodolac, as opposed to tissues exposed to flunixin meglumine. Therefore, etodolac may be a better choice in animals with intestinal inflammation because of its minimal effect on the reparative process and motility, compared with nonspecific inhibitors of COX.[16]

The inhibitory effect of nabumetone in the study reported here is similar to that detected in the equine colon for another COX-2 inhibitor, carprofen.[20] Carprofen, similar to the other NSAIDs evaluated, consistently decreased contractile strength of smooth muscle in the dorsal colon and pelvic flexure. In the ventral colon, there was not a significant effect on contractile activity relative to baseline until the highest concentration (1×10^{-5}M) was administered. It is possible that NSAIDs have an effect that varies among intestinal locations, such that the dorsal colon appeared more sensitive to all the NSAIDs used in this study, albeit to varying degrees, relative to the ventral colon. This effect would likely be related to variability in the location of the COX enzyme. It is also interesting to speculate that COX activity may be greater in the dorsal colon, which may also explain the greater incidence of colitis in this location, relative to the ventral colon, following long-term administration of NSAIDs.[23]

In the study reported here, the highest concentration of each NSAID caused a universal inhibitory effect on the tissues. This is unlikely to be a toxic pharmacologic effect, because in a preliminary study, activity was restored following the addition of 1×10^{-6}M acetylcholine. Therefore, it is more likely that NSAIDs that were used at amounts greater than physiologic concentrations inhibited intestinal motility through mechanisms other than via CXO-1 activity; conversely, the concentration of each NSAID may have been sufficient to cause effects through inhibition of COX-1 activity. The concentrations of NSAIDs used in our study were determined in preliminary studies to be the lowest quantity that had a minimal effect on tissue activity, which enabled us to detect effects with increasing concentrations. It is difficult to extrapolate in vitro data to in vivo concentrations. Concentrations used in studies[16,24,25] that evaluated the effect of NSAIDs in horses were similar to those used in the study reported here. In 1 of those studies,[16] flunixin meglumine and etodolac were compared. In that study, concentrations of 2.7×10^{-5}M were used on the basis of extrapolation of peak serum concentrations achieved following IV administration of phenylbutazone. Generally, concentrations in excess of 1×10^{-5}M are greater than physiologic concentrations. In the study reported here, we evaluated the effect of NSAIDs on contractile activity of normal intestines. Because intestinal injury can lead to the activation of other inflammatory processes via bradykinin, nitric oxide, and histamine, additional studies are indicated on the basis that because inhibition of a single enzymatic system may not yield the total expected effect.

In the intestinal tract, it has been established that inhibition of COX-1 activity leads to mucosal injury via alterations in local microcirculation, mucus production, and the promotion of adhesion of WBCs to the endothelium.[6] In animals in which intestinal injury has already occurred, use of nonspecific NSAIDs may actually amplify the injury by disrupting normal physiologic processes. Unfortunately in the equine industry, most of the NSAIDs traditionally used are nonselective, and only recently has interest become more focused on a search for alternatives. However, COX isoenzymes are quite complex, and it is likely that researchers could develop a single magic bullet. Currently, the specific mechanism of action for the commonly used NSAIDs remains unknown.[11] Because COX-2 activity increases dramatically during inflammatory insults, it is intuitively obvious that this search has become focused on the effects of selective COX-2 inhibitors.

Although some NSAIDs bind the active site of COX, others irreversibly alter the binding site, and others reversibly inhibit COX-1. Therefore, effects of these inhibitors vary depending on the concentration of the NSAIDs, binding site affinity, and availability of arachidonic acid.[12,26] This may account for the discrepancy in contractile responses seen between nabumetone and etodolac in the study reported here. Etodolac may act more selectively on COX-2 activity, whereas the chemical nature of nabumetone may favor less discriminatory inhibition of COX, which could include some inhibition of COX-1.

It has been determined that there are substantial differences in the selectivity of COX-2 inhibitors for the COX-1 or -2 isoforms. In a study[27] in which researchers evaluated the COX selectivity of various commercial NSAIDs, the ratio for COX-2 to COX-1 for indomethacin, etodolac, and nabumetone was 0.30, 179, and 1.6, respectively, as determined on the basis of the mean 50% inhibitory concentration (ie, IC_{50}) value. Indomethacin had the lowest specificity for COX-2 and highest selectivity for COX-1, whereas nabumetone predominantly had activity against COX-1, relative to COX-2, although it is still considered an inhibitor of COX-2.

It is also likely that anatomic location has an important role in the effects NSAIDs will have. In clinical trials in humans, etodolac and nabumetone have been associated with the lowest incidence of gastric complications despite the fact they differ significantly in COX-2 selectivity.[k] In the study reported here, the inhibitory effect of nabumetone may reflect greater COX-1 activity, relative to COX-2, in these areas of the intestinal tract. In other tissues, it has also been docu-

mented that COX-2 inhibition produces physiologic effects that are indistinguishable from traditional NSAID inhibition of COX-1 and -2.[4] The explanation for this finding is unknown and indicates the need to separately evaluate these NSAIDs.

[a]Model 70 polygraph, Grass Astromed, West Warwick, RI.
[b]FTO3, Grass Astromed, West Warwick, RI.
[c]Substance P, Sigma Chemical Co, St Louis, Mo.
[d]Atropine, Sigma Chemical Co, St Louis, Mo.
[e]Guanethidine, Sigma Chemical Co, St Louis, Mo.
[f]Indomethacin, Sigma Chemical Co, St Louis, Mo.
[g]Nabumetone, Sigma Chemical Co, St Louis, Mo.
[h]Etodolac, Mylan Pharmaceuticals Inc, Morgantown, WV.
[i]Sodium carbonate, Sigma Chemical Co, St Louis, Mo.
[j]Statview 4.51, Abacus Concepts Inc, Berkeley, Calif.
[k]Singh G, Terry R, Ramey D, et al. Comparative GI toxicity of NSAIDS (abstr), in *Proceedings*. XIXth Int Lab Anim Res Cong Rheumatol 1997;159.

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