Effect of nonsteroidal anti-inflammatory drugs with varied cyclooxygenase-2 selectivity on cyclooxygenase protein and prostanoid concentrations in pyloric and duodenal mucosa of dogs

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Objective—To assess in vivo effects of short-term administration of NSAIDs with varied cyclooxygenase (COX)-2 selectivity on pyloric and duodenal mucosa.

Animals—8 healthy dogs.

 Procedures—Each dog received deracoxib (2 mg/kg, PO, q 24 h for 3 days), firocoxib (5 mg/kg, PO, q 24 h for 3 days), meloxicam (0.2 mg/kg, PO, q 24 h for 1 day followed by 0.1 mg/kg, PO, q 24 h for 2 days), or placebo orally for 3 days; there was a 4-week interval between successive treatments. Prior to and on day 3 of drug administration, pyloric and duodenal mucosae were assessed endoscopically and biopsy specimens obtained for histologic examination. Cyclooxygenase-1 and -2 protein expressions were assessed (western blotting) and prostanoid concentrations measured (ELISAs). Data were analyzed by use of an ANOVA.

 Results—Drug administration did not significantly affect endoscopic mucosal scores, histologic scores, or COX-1 or -2 protein expression. The COX-1 protein expression was significantly higher in the pylorus than in the duodenum. Total prostaglandin and thromboxane B₂ (TXB₂) concentrations were significantly greater in pyloric than in duodenal mucosa. Drug administration had no effect on prostaglandin or TXB₂ concentrations.

 Conclusions and Clinical Relevance—Prostanoid concentrations in gastric and duodenal tissues, and gross and histologic appearances, were not significantly affected by drugs with varied COX-2 selectivity. These findings suggested that, for these experimental conditions, there were no differences among the preferential and selective COX-2 inhibitors with regard to adverse effects on the gastric and duodenal portions of the gastrointestinal tract of dogs. (Am J Vet Res 2009;70:1243–1249)

A dministration of NSAIDs to dogs, although efficacious, has been associated with adverse events, most notably gastrointestinal ulcers. The NSAID-associated gastrointestinal ulcers are most commonly found in the pylorus and proximal portion of the duodenum of dogs.1,2 Many factors likely contribute to ulcer formation in dogs. However, the incidence of ulcers in dogs is unknown because of the nonspecific or mild clinical signs associated with erosions and minor gastric ulcers.3 Risk factors considered to play a role in the development of NSAID-associated ulcers in humans and dogs include a history of bleeding from the gastrointestinal tract or gastric ulcers, concurrent administration of multiple NSAIDs, concomitant use of corticosteroids or anticoagulants, hepatic dysfunction, renal dysfunction, and administration of NSAIDs at a dosage higher than that approved on the label.4,5 A number of these factors were identified in a report1 on gastric perforation in dogs.

Although the mechanisms are not clearly understood, gastric damage related to NSAID administration is the result of a 2-fold mechanism: a topical effect on gastric mucosa and a systemic effect, most likely attributable to decreased production of gastroprotective PGs.6 Injury to the mucosa is initiated topically by weakly acidic NSAIDs, such as aspirin.4 For most of the NSAIDs, including the COX-2–selective NSAIDs,
Because COX-1–derived PGs are believed to play a dominant role in gastric mucosal defense and cytoprotection,7,8 drugs that selectively inhibit COX-2 are considered to have a safer gastrointestinal profile than those that also block COX-1.9,10 However, investigators in several studies9,7,11 detected constitutive COX-2 protein expression in the canine CNS and kidneys. Of greater pertinence, our laboratory group recently published a report11 in which constitutive expression of COX-2 protein was detected in canine gastroduodenal mucosa. In addition, COX-2 was found to be upregulated in inflamed gastrointestinal tissues, which suggested a role in defense and cytoprotection of the gastrointestinal mucosa.13,14 Other studies15,16 have revealed upregulation of COX-2 expression in the margins of healing gastric ulcers. Furthermore, selective inhibition of COX-1 does not predictably cause ulcers, which indicates that blockade of both isoforms may be necessary to induce lesions.15 Mucosal damage induced by ischemia–reperfusion can be markedly aggravated by the inhibition of COX-2.15,16 Results of these aforementioned studies as well as several others have established that COX-2 expression appears to be involved in providing a second line of defense for gastric mucosa as well as serving as a crucial mediator in mucosal repair. Thus, there appear to be 2 important scenarios for which the effects of selective COX-2 inhibitors should be evaluated: normal gastrointestinal mucosa and abnormal or reparative mucosa.

The purpose of the study reported here was to assess the in vivo effects of short-term administration of NSAIDs with varying COX-2 selectivity on normal pyloric and duodenal mucosa of dogs. We hypothesized that NSAIDs with greater COX-2 selectivity would reduce the adverse effects on the mucosa associated with increased prostanooid production.

Materials and Methods

Animals—Eight adult purpose-bred crossbred dogs (4 females and 4 males) weighing 8 to 13 kg were used in the study. Physical examination was performed on each dog prior to the beginning of the study to ensure that all dogs were healthy. In addition, a CBC, serum biochemical analysis, and urinalysis were performed immediately prior to commencement of the study. Gastroduodenoscopy was performed on each dog prior to the start of the study to rule out preexisting gastroduodenal disease. The study was approved by the Animal Care and Use Committee at North Carolina State University.

Experimental protocol—The study was conducted as a randomized block design with placebo control and a crossover. Each dog was assigned by use of a randomization procedure to receive deracoxib (2 mg/kg, PO, q 24 h for 3 days), firocoxib (3 mg/kg, PO, q 24 h for 3 days), meloxicam (0.2 mg/kg, PO, q 24 h for 1 day followed by 0.1 mg/kg, PO, q 24 h for 2 days), and a placebo (PO, q 24 h for 3 days); the first day of each treatment was designated as day 1. Each dog received each treatment, and there was a 4-week washout period between subsequent treatments. Commercially available products were used, and dogs were dosed within ± 10% of the recommended dose according to package inserts. Dogs were treated for 3 days to allow each drug to reach a theoretical steady-state concentration in the plasma and, presumably, the gastrointestinal tract.

Four weeks before initiation of the study (baseline) and on day 3 of each treatment, the pyloric and duodenal mucosae were scored, and mucosal biopsy specimens were obtained endoscopically. On day 3, endoscopy was performed 2 hours after administration of the treatments. Food typically was not withheld from the dogs prior to treatment administration, but food was withheld for 24 hours prior to endoscopy.

For the endoscopic procedures, anesthesia was induced in the dogs by administration of propofol (10 to 15 mg/kg, IV, to effect). Dogs were orotracheally intubated, and anesthesia was maintained by administration of isoflurane vaporized in 100% oxygen. Gastroduodenoscopy and biopsy were performed by 1 investigator (SLM), who used a flexible videoscope.4 Each procedure was recorded onto a DVD. All endoscopic procedures were performed the same time each morning (10 AM) to avoid diurnal and feeding-associated changes in gastric physiologic processes. Biopsy specimens were collected, with a distance of at least 2 cm between adjacent biopsy locations. Mucosal biopsy specimens were collected from the pylorus and duodenum, snap-frozen in liquid nitrogen within 6 to 8 seconds, and stored at −80°C until subsequently used for western blot analysis of COX-1 and COX-2 expression and measurement of total PG and TXB2 concentrations. Care was taken to ensure that each biopsy specimen was handled identically. In addition, separate mucosal biopsy specimens were immediately placed in neutral-buffered 10% formalin for histologic evaluation.

Western blot analysis—Each biopsy specimen (1 from the pylorus and 1 from the duodenum of each dog) was added to a vial containing 200 μL of modified radioimmunoprecipitation bufler, including the protease inhibitors aprotinin, phenylmethylsulfonyl fluoride, and sodium orthovanadate. Specimens were homogenized on ice, and supernatants were extracted via centrifugation. Protein analysis of extracted samples was performed, and equal concentrations of protein from each specimen were mixed and boiled with sample buffer. Lysates were then loaded into wells containing gels,1 and protein electrophoresis was performed in accordance with standard protocols. Proteins were transferred to a polyvinylidene fluoride transfer membrane and blocked in 5% milk with 0.05% Tween-20. Membranes were washed with PBS solution and incubated overnight in a solution (1:300) of polyclonal COX-1 or COX-2 primary antibody. Membranes were then incubated in a horseradish peroxidase–conjugated secondary antibody and developed by addition of enhanced chemiluminescence reagents. Expression of β-actin1 was used to verify that the same amount of protein was loaded into each well. Recombinant COX protein1 was used as a positive control sample, and a
molecular-weight indicator (protein standard) was used to ensure canine COX protein bands corresponded to the appropriate number of kilodaltons. Negative control samples were used as a quality-control procedure for some gels. This approach was similar to that used in another study for species in which a specific antibody was not available.

Specimens from the pylorus and duodenum for all the treatments of a specific dog were assayed on a single gel. This made it possible to compare COX expression after each treatment within each dog. By use of the densitometry values, COX protein concentrations after each treatment were expressed as a percentage of the baseline value for each dog in each region (pylorus and duodenum). To compare overall COX protein expression in the duodenum and pylorus, densitometric values within each dog were expressed as a percentage of the baseline value.

Prostanoid analysis—Each biopsy specimen was placed in a vial containing 200 µL of Tris buffer (50mM Tris-HCl, 150mM NaCl, 1mM EDTA [pH 7.4]), including aprotinin, phenylmethylsulfonyl fluoride, and orthovanadate. Specimens were homogenized on ice, and supernatants were extracted via centrifugation. Protein analysis of extracted samples was performed. Concentrations of PG and TXB were measured with commercially available ELISA kits. Results were expressed as the number of picograms of prostanoid per microgram of protein in the tissue.

Mucosal scores and histologic analysis—After completion of the study, all endoscopy videos were reviewed by an investigator (SLM) who was not aware of the treatment for each dog for each endoscopic procedure. The mucosa was scored for lesions by use of a subjective scoring system. Slides with sections of gastric and duodenal biopsy specimens were stained with H&E and evaluated for inflammation and ulceration by a board-certified veterinary pathologist (JML). The pathologist was not aware of the treatment groups.

Data analysis—A 2-way repeated-measures ANOVA was used to compare densitometric data for COX-1 and COX-2 protein, PG, and TXB concentrations. A Tukey test was used to identify specific differences among treatments. Significance was set at values of \( P < 0.05 \). An ANOVA on ranks was used when the data were not normally distributed.
Results

All dogs had anticipated results for physical examination, and laboratory values were within the expected reference ranges. No clinically relevant adverse effects were detected for any treatment administered.

Drug administration had no effect on COX-1 or COX-2 protein expression in the pylorus or duodenum (Figures 1 and 2). Expression of COX-1 was significantly higher in the pylorus, compared with expression in the duodenum.

Overall, total PG concentrations were significantly higher in the pyloric mucosa than in the duodenal mucosa (mean ± SEM baseline concentrations, 1,043 ± 222 pg/µg of protein and 283 ± 49 pg/µg of protein, respectively; Figure 3). Treatment had no effect on PG concentrations in pyloric and duodenal mucosa. Concentrations of TXB₂ were significantly higher in the pyloric mucosa than in the duodenal mucosa (mean ± SEM baseline concentrations, 1,649 ± 125 pg/µg of protein and 187 ± 50 pg/µg of protein, respectively; Figure 4). Treatment had no effect on TXB₂ concentrations in the pylorus or duodenum.

Histologic evaluation revealed no evidence of ulceration or notable inflammation in any biopsy specimen. Mild inflammation was detected in 16 of 80 (20.0%) biopsy specimens. There was no significant effect of treatment on histologic score in the pylorus (P = 0.518) or duodenum (P = 0.918). Helicobacter spp were detected in 30 of 80 (37.5%) biopsy specimens. During endoscopic evaluation, bleeding was not detected in the pylorus or duodenum.

Endoscopic mucosal scores were significantly higher for the pylorus, compared with mucosal scores for the duodenum. Overall, there was no significant effect of treatment on mucosal score.

Discussion

To our knowledge, there have been no in vivo studies conducted to evaluate the short-term administration of NSAIDs with varying COX-2 selectivity while concurrently assessing COX protein and prostanoid concentrations in dogs. Meloxicam is considered a preferential COX-2 inhibitor in vitro because it can inhibit COX-2 more readily than COX-1. In vitro studies have revealed that meloxicam inhibits COX-2 activity 10 to 12 times as effectively as it inhibits COX-1 activity. Deracoxib and firocoxib are called coxibs and described as selective COX-2 inhibitors.

Selectivity ratios have been evaluated in several in vitro studies. Results vary depending on which in vitro assay was used, and it is not known how the results will
translate to in vivo circumstances. Each of the aforementioned drugs is believed to spare the COX-1 enzyme, thereby potentially increasing the safety profile of NSAIDs while retaining efficacy; however, this postulated increase in safety has not been proven in canine medicine. There is still potential for inhibition of COX-1, depending on the tissue concentration of the drug. The association between NSAIDs and gastroduodenal perforation has been assessed in 2 retrospective clinical studies.\textsuperscript{1,2,3,4} In one of those studies,\textsuperscript{1} almost all dogs with ulcers had received an inappropriately high dose of an NSAID, concurrent administration of other NSAIDs or corticosteroids, or rapid switching from one NSAID to another. In the other study,\textsuperscript{5,6} it was suggested that the selectivity for COX-2 may decrease at higher doses of meloxicam, thereby increasing COX-1 inhibition. The authors of that study\textsuperscript{4} suggested that this increase in COX-1 inhibition may explain adverse gastrointestinal tract events.

The study reported here was designed to assess the in vivo effects of NSAIDs on pyloric and duodenal mucosa, which can be at risk for development of ulcers.\textsuperscript{7} None of the dogs had clinical signs of gastrointestinal tract disease in this study, but the duration of treatment was only 3 days. Ideally, such a study would be conducted to evaluate multiple time points over an extended period. However, such an approach has its limitations, including potential trauma to the stomach as a result of multiple endoscopies. With that in mind, this study was designed to initially assess an early time period and evaluate the effect of initial administration of NSAIDs. The 3-day time period was chosen on the basis of results of another study\textsuperscript{22} conducted to evaluate the 3-day time point; in that study,\textsuperscript{22} all the drugs evaluated significantly suppressed gastric PGE\textsubscript{2} concentrations. In another study\textsuperscript{22} conducted by our laboratory group, we had found significant differences in effects between aspirin, deracoxib, and carprofen at the 3-day time point. Thus, we wanted to conduct a follow-up study to compare NSAIDs with varying COX-2 selectivities.

In the study reported here, there was no significant effect of treatment on histologic score of the mucosa. Only a few gastric biopsy specimens had mild inflammation, but these findings were not related to a specific treatment or dog. Histologic findings similar to those reported in this study were reported in another endoscopic study.\textsuperscript{21} In that study,\textsuperscript{21} Helicobacter spp were detected histologically in all of the dogs. In another study\textsuperscript{12} conducted by our laboratory group, we also found Helicobacter spp during histologic analyses. In the present study, Helicobacter spp were found in 30 of 80 biopsy specimens. We did not determine the species of Helicobacter organisms, and no dogs were excluded on the basis of these findings. The clinical relevance of Helicobacter spp is unclear in canine gastroenterology. However, Helicobacter organisms are commonly found in clinically normal dogs,\textsuperscript{22} and the number of positive results in the study reported here was low, compared with the number reported in another report\textsuperscript{26} in which 100% of healthy laboratory dogs without any evidence of gastrointestinal tract disease had positive results when tested for Helicobacter spp. In the present study, mucosal scores were significantly higher in the pylorus, compared with scores in the duodenum, which indicated greater mucosal irritation in the pylorus. This may have been attributable to irritating effects of ingesta and gastric acid on the pyloric mucosa, with bile acids having a protective effect in the duodenum.

Treatment did not affect expression of COX-1 or COX-2 protein in the pylorus and duodenum during the period of this study. Specific canine antibodies are not available for COX-1 or -2. There is the possibility of nonspecific binding, but the use of negative control samples, a molecular-weight ladder, and respective recombinant protein as a marker and the high degree of homology across mammalian species with regard to COX proteins all indicate that we detected COX-1 and -2 specifically. Expression of COX-1 was significantly higher in the pylorus, compared with expression in the duodenum. The clinical relevance of this is unclear, but it may suggest higher COX-1 concentrations aid in protection of this region of the stomach. Constitutive expression of COX-2 protein was detected in gastric tissues in the present study, and this result is consistent with results of another study\textsuperscript{12} conducted by our laboratory group. The findings from this present study and that other study\textsuperscript{12} contrast with results of a study\textsuperscript{22} in which investigators used tissues from a single dog but found no COX-2 protein expression in gastrointestinal tract tissues. In the gastrointestinal tract of healthy humans and a number of other animals, COX-2 is undetectable or expressed in extremely low amounts.\textsuperscript{7} Consistent with its role as an inducible enzyme during conditions of inflammation, COX-2 can be upregulated in inflamed gastrointestinal tract tissues in humans and rodents.\textsuperscript{13,14} Thus, COX-2 is generally reported to be expressed in inflammatory conditions. However, analysis of results of the study reported here suggested a more complicated role of the COX-2 isoform because it was constitutively expressed in normal conditions. Cyclooxygenase-2 may play a role in mucosal protection and resolution of inflammation in the gastrointestinal tract. Indeed, evidence from several studies\textsuperscript{14,26,31} suggests that COX-2 plays a role in mucosal protection. This protective role was further emphasized in those same studies when administration of a selective COX-1 inhibitor did not affect gastric healing, which was in contrast to the findings for administration of a selective COX-2 inhibitor.\textsuperscript{30,31} Additionally, experiments performed on COX-1–deficient mice suggested that COX-2–derived PGs contribute to mucosal protection\textsuperscript{31} and that inhibition of those PGs by NSAIDs can cause mucosal injury. Another study\textsuperscript{32} revealed that COX-2 expression can be upregulated rapidly within 1 hour after oral administration of aspirin or indomethacin, which suggests that upregulation was the result of a protective mechanism. Studies evaluating carrageenan-induced paw edema and carrageenan-induced pleurisy\textsuperscript{32} in wild-type and COX-2–deficient mice have also emphasized the role of COX-2 in resolution of and regulation of inflammation. The latter study\textsuperscript{32} revealed that there were 2 phases of COX-2 expression. An early peak (at approx 2 hours) was associated with onset of inflammation, leukocyte infiltration, PGE\textsubscript{2} production, and COX activity. Nonselective and COX-2–selective NSAIDs can inhibit this early phase of the inflamma-
tory response.\textsuperscript{39} A second and much greater peak in COX-2 expression was detected 48 hours after irritant injection. At this peak, the number of leukocytes in the pleural cavity decreased to within the reference range, which resulted in resolution of the inflammation. No PGE\textsubscript{1} was detected; however, another PGH\textsubscript{2} metabolite, PGD\textsubscript{2}, accompanied the increase in COX-2 expression. Administration of COX-2-selective inhibitors from 24 to 48 hours after injection of an irritant (ie, during the resolution phase) abolished PGD\textsubscript{2} production and prolonged the inflammatory response by preventing a decrease in leukocytes. A typical course of resolution was evident with coadministration of PGD\textsubscript{2} and cyclopentenone PGs, which indicated products of PGD\textsubscript{2} may be responsible for initiating resolution of inflammation.

On the basis of the aforementioned information, it appears that COX-2 may play an important role in mucosal defense and the repair process. In the study reported here, we did not detect adverse effects of inhibition of COX-2 in normal canine gastroduodenal mucosa for the conditions of the study; however, the role of COX-2 and the consequences of COX-2 inhibition require further study.

In complex tissues such as the gastrointestinal mucosa, it is difficult to conclude the COX enzyme isoform that generates each prostanoid. In the study reported here, total PG production was measured to investigate the synthesis of all PGs from both COX enzymes. Tissue concentrations of prostanoids were measured at the time of biopsy. Other investigators have collected biopsy specimens and measured the total amount of prostanoids that can be produced when the specimens are subsequently stimulated ex vivo.\textsuperscript{22} However, measurement of tissue prostanoid concentrations reflects the effect of a treatment on the actual tissue concentrations of prostanoids in vivo. In the present study as well as in other studies, COX-1 expression was higher in the pylorus\textsuperscript{12} and stomach’ than in the duodenum. This may explain the reason that PG concentrations were significantly higher in the pylorus than in the duodenum. Investigators in one of those studies\textsuperscript{3} found higher PGE\textsubscript{2} production from human duodenal tissue, compared with production in gastric tissue, but the reverse was true for duodenal tissues obtained from rats and rhesus monkeys; PG concentrations were not examined in canine tissues in that study. In the present study, higher PG concentrations correlated with higher concentrations of COX-1 protein, which is similar to results of another study.\textsuperscript{7} Investigators have used the assay method involving stimulation of PG production to determine that oral administration of deramaxx for 3 days decreases gastric PGE\textsubscript{2} concentrations but not PGE\textsubscript{1} concentrations.\textsuperscript{30} In the study reported here, none of the treatments appeared to inhibit PGs. Because we used drugs with reported preferential selectivity for COX-2, it was possible that the PG concentrations measured in this study were more reflective of COX-1 activity, despite the fact that COX-2 protein was also detected in the mucosa.

Thromboxane is a likely indicator of COX-1 activity in the gastrointestinal tract of pigs.\textsuperscript{37} Results of another study\textsuperscript{32} conducted by our laboratory group suggested that TXB\textsubscript{2} can be linked to COX-1 activity in the canine gastrointestinal tract. In that other study\textsuperscript{12} and the study reported here, TXB\textsubscript{2} concentrations were significantly higher in the pylorus than in the duodenum. This phenomenon appeared to reflect higher COX-1 protein expression in the pylorus. Drug administration had no effect on TXB\textsubscript{2} concentrations in the pylorus or duodenum, which suggested no clinically relevant inhibition of COX-1 for the dosages chosen for the 3-day period.

The study reported here revealed that various selective and preferential COX-2 inhibitors had no significant effects on prostanoid production in gastroduodenal mucosa. Furthermore, there was not a significant relationship between the degree of selectivity and gastrointestinal injury or histologic appearance for the 3-day period of drug administration.

\textbf{References}


