

Evaluation of the relationship between lesions in the gastroduodenal region and cyclooxygenase expression in clinically normal dogs

Jenna G. Wooten, PhD; B. Duncan X. Lascelles, BVSc, PhD; Vanessa L. Cook, VetMB, PhD;
J. Mac Law, DVM, PhD; Anthony T. Blikslager, DVM, PhD

Objective—To determine whether clinically normal dogs have lesions in the pylorus and duodenum and to examine the expression of cyclooxygenase (COX) isoforms in the pylorus and duodenum of these dogs.

Animals—27 clinically normal dogs.

Procedures—Physical examination was performed on clinically normal dogs from animal shelters and research projects; the dogs were then euthanized. After the dogs were euthanized, the pylorus and duodenum were photographed and scored for gross appearance of lesions. Samples were obtained for histologic evaluation and determination of COX expression via western blot analyses. Tissues from the pylorus and duodenum were categorized as normal, inflamed, or eroded on the basis of histologic analysis. Each histologic category of tissue was then evaluated to determine the correlation with gross appearance and COX expression.

Results—Of the 27 dogs, 5 had unremarkable histologic findings in the pylorus and duodenum. Inflammation was found in the pylorus of 10 dogs and in the duodenum of 5 dogs. Epithelial erosion was detected in the pylorus of 1 dog and in the duodenum of 3 dogs. Gross appearance was not significantly correlated with histologic appearance. Expression of COX-1 was not upregulated by inflammation, whereas COX-2 expression was increased by inflammation or erosion.

Conclusions and Clinical Relevance—Dogs that appear to be clinically normal may have underlying gastroduodenal lesions associated with upregulation of COX-2. Because of the inability to determine this during routine physical examination, practitioners should be aware of this potential situation when prescribing COX inhibitors. (*Am J Vet Res* 2010;71:630–635)

The NSAIDs are used to alleviate both acute and chronic pain, and they are frequently used to control postoperative pain and inflammation. However, use of NSAIDs has been associated with adverse effects, most notably ulcers of the gastrointestinal tract.^{1,2} Investigators have indicated that NSAID administration is a risk factor for the development of gastrointestinal tract ulcers and perforation in dogs.^{1,3} It has also been reported^{2,3} that NSAID-associated ulcers and perforations are most commonly detected in the pylorus and

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From the Department of Clinical Sciences (Wooten, Lascelles, Blikslager), Gastrointestinal Physiology (Wooten, Cook, Blikslager) and Comparative Pain (Lascelles) Research Laboratories, and Department of Population Health and Pathobiology (Law), College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27606. Dr Cook's present address is Department of Large Animal Clinical Sciences, College of Veterinary Medicine, Michigan State University, East Lansing, MI 48824. Dr. Wooten's present address is the Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC 27599.

Address correspondence to Dr. Blikslager (Anthony_Blikslager@ncsu.edu).

	ABBREVIATION
COX	Cyclooxygenase

proximal portion of the duodenum. When there are pre-existing conditions such as inflammation and erosion of the gastric mucosa, the adverse effects from NSAID administration could be intensified, which could lead to grave results.³ However, there is a lack of information with regard to the pathophysiologic processes for such preexisting conditions.

The site of action of NSAIDs is COX, which results in blockade of prostaglandin synthesis. Prostaglandins have important protective effects on the gastrointestinal mucosa.^{4–6} Thus, inhibition of prostaglandin synthesis, which is thought to be mediated predominately via the action of COX-1, is believed to be the principal reason for NSAID-induced mucosal injury.⁷ Largely on the basis of the finding that COX-2 is inducible and associated with inflammation, a drug class known as the coxibs that selectively inhibits COX-2 (reducing inflammation) while preserving COX-1 function and potentially preventing NSAID-induced gastrointestinal

injury was developed. However, there may be a more complicated role for the COX isoforms, in particular COX-2, because investigators in several studies⁸⁻¹⁰ have determined that some aspects of mucosal protection and repair may depend on COX-2 expression.

We hypothesized that erosions and ulcers in the gastrointestinal tract would be found in clinically normal dogs and that these lesions would be associated with increased COX-2 expression. Our objectives were to collect material from clinically normal dogs that had been euthanized and determine whether lesions were evident in the gastrointestinal tract. If so, we would evaluate whether COX-1 and COX-2 expression was altered in tissues with histologic lesions, compared with results for histologically normal tissue.

Materials and Methods

Dogs—Twenty-seven adult dogs (18 females and 9 males) that weighed between 7 and 45 kg were used in the study. Dogs were obtained from the College of Veterinary Medicine Laboratory Animal Resources (research dogs) or from a county animal shelter. All dogs were considered unfit for adoption. Research dogs were euthanized as part of another study that did not involve the gastrointestinal tract. No dogs were euthanized solely for the collection of material for this study. The study was approved by the Institutional Animal Care and Use Committee at North Carolina State University.

Experimental protocol—A brief physical examination was performed on each dog by a veterinarian associated with the study to ensure that all dogs were clinically normal. The physical examination included assessment of physical appearance, mucous membrane color, capillary refill time, heart rate, and respiratory rate. Dogs were euthanized in accordance with AVMA-approved protocols by a veterinarian at each location. Immediately after the dogs were euthanized, the stomach and duodenum were removed and opened along the antimesenteric border (greater curvature for the stomach). Photographs were taken of the pylorus and duodenum. Gross appearance of the pylorus and proximal portion of the duodenum was graded by use of a standardized scoring system (0 = apparently normal appearance, 1 = mild bile staining or extremely mild changes, 2 = mild hyperemia, 3 = moderate hyperemia, and 4 = erosions or evidence of ulcers).

Further analysis of the pylorus and proximal portion of the duodenum was performed. Tissue samples were obtained from the pylorus and proximal portion of the duodenum. Each sample was divided into 2 equal halves along the proximal-to-distal axis of the gastrointestinal tract. One set of samples for the pylorus and duodenum was snap-frozen in liquid nitrogen within 8 seconds after collection, stored at -80°C , and subsequently used for western blot analysis of COX-1 and COX-2 expression. The central component of the other set of samples was immediately placed in neutral-buffered 10% formalin for histologic evaluation. Selection of the half that was to be used for western blot analysis and the half that was to be used for histologic analysis was randomized by use of a statistical software package^a for the group of dogs.

Western blot analysis—Samples from the pylorus and duodenum were immersed in 1 mL of modified radioimmunoprecipitation buffer (0.14M NaCl, 50mM sodium Tris [pH, 7.2], 0.5% deoxycholic acid, 1% of a nonionic surfactant,^b 0.1% SDS, and 1% of a commercially available detergent^c) that included the protease inhibitors aprotinin, phenylmethylsulfonyl fluoride, and sodium orthovanadate. Samples were homogenized on ice, and the supernatants were extracted by centrifugation. Protein analysis of extracted samples was performed by use of a commercial protein assay,^d and equal amounts of protein from each sample (35 μg) were mixed and boiled with a commercial proprietary sample buffer.^e Lysates were then loaded into wells of precast gels,^f and protein electrophoresis was performed in accordance with standard protocols. Proteins were transferred to polyvinylidene fluoride membranes, and membranes were then placed in a blocking buffer containing 5% milk, 10mM Tris HCl, 150mM NaCl, and 0.5% Tween-20 for 2 hours at 4°C . Membranes were incubated overnight in a solution of goat polyclonal COX-1 antibody^g (dilution, 1:300; 200 $\mu\text{g}/\text{mL}$) or goat polyclonal COX-2 primary antibody^f (dilution, 1:300; 200 $\mu\text{g}/\text{mL}$). Membranes then were incubated in horseradish peroxidase-conjugated donkey anti-goat secondary antibody^g (dilution, 1:2,000; 0.5 $\mu\text{g}/\text{mL}$) and developed by the addition of enhanced chemiluminescence reagents. This approach is similar to that used in another study¹¹ with canine gastric tissues in which a species-specific antibody was not available.

Western blots were analyzed by use of a commercial densitometric package.^h For each blot, a box was manually drawn around the largest band, and the density of that band was recorded. A box of the same size was then placed around each of the other bands, and the density of each of those bands was recorded. Densitometric analysis was performed for all dogs.

Histologic analysis—Slides of gastric and duodenal biopsy specimens were stained with H&E and evaluated for inflammation and ulcers (erosions) by a board-certified veterinary pathologist (JML) who used a standardized scoring system (0 = no remarkable changes, 1 = mild inflammation [or erosion], 2 = moderate inflammation [or erosion], and 3 = marked evidence of inflammation [or erosion]). Values did not sum to 27 because some gastroduodenal tissues had ≥ 1 histologic lesion in the pylorus, proximal portion of the duodenum, or both. Samples were assigned to 1 of 3 broad categories: histologically normal appearance of the pyloric and duodenal mucosa (ie, control tissue); evidence of inflammation in the pylorus, duodenum, or both; or evidence of epithelial erosion in the pylorus, duodenum, or both.

Statistical analysis—The correlation between grade of lesion assigned on the basis of the gross appearance and grade of lesion assigned on the basis of histologic examination was tested by use of a rank Spearman order correlation. For western blot densitometric analyses, samples for 3 dogs with inflammation and 3 dogs with erosion were placed on the same blot to assure spurious results were not obtained from mul-

multiple blots. The densitometric data were expressed as a percentage of the value for a corresponding control tissue. These data were assessed for normality and for equal variance. Following these tests, a 3-way ANOVA for the effect of COX isoform (COX-1 vs COX-2),

gastrointestinal region (pylorus vs duodenum), and lesion (inflammation vs erosion) was performed. Values of $P < 0.05$ were considered significant for all analyses, which were performed by use of a commercial statistical package.^a

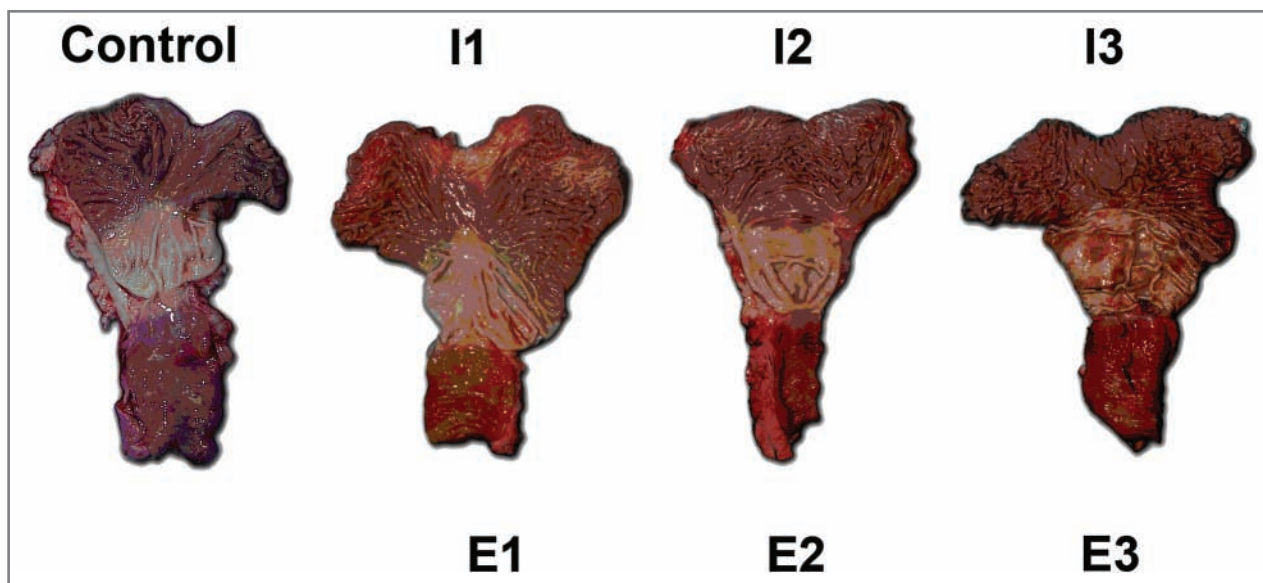


Figure 1—Photographs of the gross appearance of the pylorus (top) and proximal portion of the duodenum (bottom) of representative clinically normal dogs. Gross appearance of the pylorus and proximal portion of the duodenum was graded by use of a standardized scoring system (0 = apparently normal appearance, 1 = mild bile staining or extremely mild changes, 2 = mild hyperemia, 3 = moderate hyperemia, and 4 = erosions or evidence of ulcers). Samples were categorized on the basis of results of histologic examination by use of the following system: 0 = no remarkable changes (histologically normal [control]), 1 = mild inflammation (or erosion), 2 = moderate inflammation (or erosion), and 3 = marked evidence of inflammation (or erosion), and samples were then assigned into 1 of 3 broad histologic categories: histologically normal appearance of the pyloric and duodenal mucosa (control tissue); evidence of inflammation in the pylorus, duodenum, or both; or evidence of epithelial erosion in the pylorus, duodenum, or both. For the pylorus, tissues were categorized as histologically normal (control) or with histologic evidence of increasing severity of inflammation (I1, I2, and I3, respectively). For the proximal portion of the duodenum, tissues were categorized as histologically normal (control) or with histologic evidence of increasing severity of epithelial erosion (E1, E2, and E3, respectively). Gross appearance of the mucosa appeared similar regardless of the histologic category; however, there was grossly visible evidence of bile staining for the control and E1 tissues and a hyperemic appearance in the proximal portion of the duodenum for tissues E1, E2, and E3 but no grossly visible erosion.

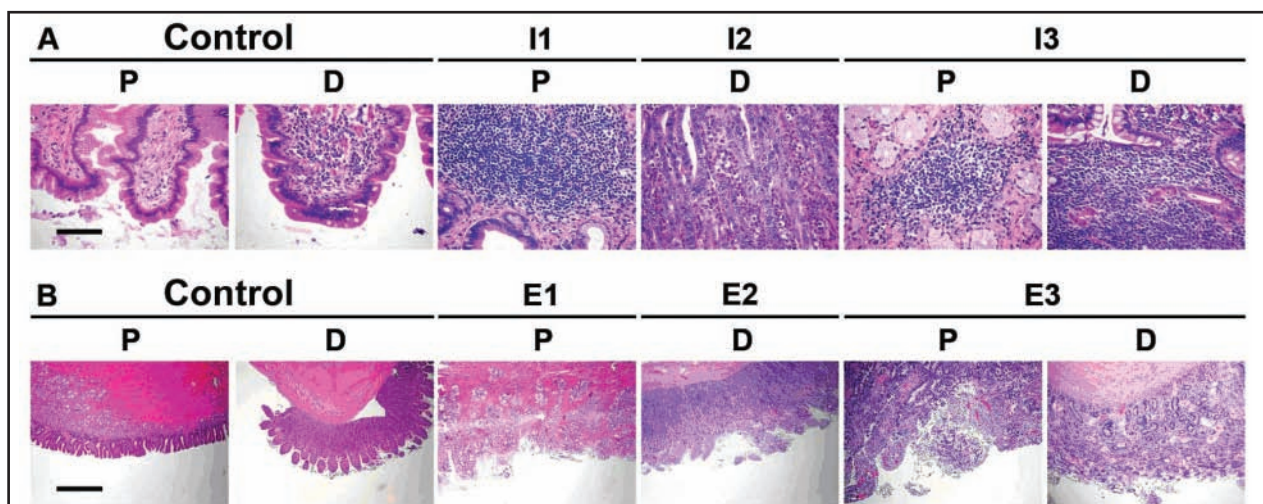


Figure 2—Histologic appearance of the mucosa of the pylorus (P) and proximal portion of the duodenum (D) obtained from representative clinically normal dogs. In panel A, tissues in the pylorus and duodenum were histologically normal (control) or had histologic evidence of increasing severity of inflammation (I1, I2, and I3, respectively). Multiple lymphoid follicles were identified in the pylorus of I1. Moderate eosinophilic enteritis was identified in the duodenum of I2, whereas there was evidence of inflammation (consisting of small de novo lymphoid follicles in the pylorus and reactive lymphoid follicles in the duodenum) in both regions of I3. In panel B, tissues in the pylorus and duodenum were histologically normal (control) or had histologic evidence of increasing severity of epithelial erosion (E1, E2, and E3, respectively). There was a focal erosion of the mucosal surface in the pyloric region of E1 and evidence of mild lymphoplasmacytic enteritis with central erosion in the duodenum of E2. Moderate eosinophilic enteritis along with a small central erosion in the pylorus and a mild central erosion in the duodenum was evident in E3. H&E stain; bar = 300 μ m. See Figure 1 for remainder of key.

Results

All 27 dogs were considered clinically normal on the basis of results of physical examination.

Gross examination revealed that the pylorus and proximal portion of the duodenum were apparently normal, and no erosions were visible on any of the pyloric or duodenal mucosa (Figure 1). The gross appearance of the mucosa appeared similar in all dogs, regardless of the histologic category. However, the mu-

Table 1—Comparison of gross appearance and histologic category for mucosa of the pylorus and proximal portion of the duodenum obtained from 27 clinically normal dogs.

Variable	Tissue	Pylorus		Duodenum	
		Gross	Histologic	Gross	Histologic
Inflammation	Control	0	0	0	0
	1	0	2	1	0
	2	0	0	2	2
	3	2	2	2	2
Erosion	Control	0	0	0	0
	1	1	3	0	3
	2	1	1	2	3
	3	1	3	3	3

Gross appearance of the pylorus and proximal portion of the duodenum was graded by use of a standardized scoring system (0 = apparently normal appearance, 1 = mild bile staining or extremely mild changes, 2 = mild hyperemia, 3 = moderate hyperemia, and 4 = erosions or evidence of ulcers). Tissues were categorized on the basis of results of histologic examination by use of the following system: 0 = no remarkable changes (histologically normal [control]), 1 = mild inflammation (or erosion), 2 = moderate inflammation (or erosion), and 3 = marked evidence of inflammation (or erosion). Values do not sum to 27 because some gastroduodenal tissues had ≥ 1 histologic lesion in the pylorus, proximal portion of the duodenum, or both.

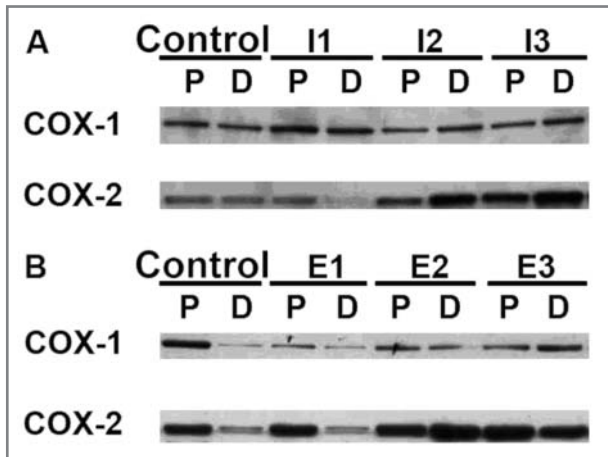


Figure 3—Photographs of results of western blot analysis of COX expression in canine gastroduodenal mucosa. A—Expression of COX-1 and COX-2 was determined in tissues from the pylorus (P) or proximal portion of the duodenum (D) that were histologically normal (control) or had histologic evidence of increasing severity of inflammation (I1, I2, and I3, respectively). B—Expression of COX-1 and COX-2 was determined in tissues from the pylorus or duodenum that were histologically normal (control) or had histologic evidence of increasing severity of erosion (E1, E2, and E3, respectively). Results of a 3-way ANOVA for the entire blot in which the value for the 3 tissues with inflammation and erosion, respectively, were compared with the value for the control tissue revealed that COX-2 expression was significantly ($P < 0.05$) increased, compared with COX-1 expression. See Figure 1 for remainder of key.

cosa was considered hyperemic in all areas in which erosions were detected histologically.

Histologic examination revealed that 5 dogs had an unremarkable histologic appearance for both the pyloric and duodenal mucosa. Inflammation was detected in the pylorus of 10 dogs, in the duodenum of 5 dogs, and in both the pylorus and duodenum of 2 dogs. Evidence of epithelial erosion was detected in the pylorus of 1 dog, the duodenum of 3 dogs, and both the pylorus and duodenum of 1 dog (Figure 2).

Correlation between gross appearance of the mucosa and histologic category was evaluated. There was not a significant correlation between gross appearance and histologic category for the pylorus ($r = 0.52$) or proximal portion of the duodenum ($r = 0.38$; Table 1).

The 3-way ANOVA performed on densitometric analyses of COX expression revealed that the statistical design was balanced and that the data were normally distributed and had equal variance. In addition, this test revealed that COX-2 expression was significantly ($P = 0.002$) increased, compared with COX-1 expression (Figure 3; Table 2). There was no significant difference between the expression of the COX isoforms in the inflammation lesions, compared with expression in the tissues with erosions. There were no significant differences in results between the pylorus and proximal portion of the duodenum, and there were no significant interactions among the variables to provide further insights into the significant upregulation of COX-2.

Discussion

In the study reported here, categorization of gastrointestinal mucosal integrity was based on histologic evaluation because it is reportedly¹² difficult to use gross appearance of the gastrointestinal mucosal surface alone as an indication of mucosal injury. Histologic evaluation revealed 3 general categories of mucosa: histologically normal appearance of the pyloric and duodenal mucosa; evidence of inflammation in the pylorus, duodenum, or both; and evidence of epithelial erosion in the pylorus, duodenum, or both. Inflammation has often been detected in clinically normal dogs, and histologic findings for the present study are similar to those reported for an endoscopic study.¹³ However, histologic appearance could not be predicted from the gross appearance of the gastroduodenal surface. Nonetheless, the proximal portion of the duodenum had a

Table 2—Results of densitometric analysis for COX expression on the basis of COX isoform, histologic lesion, and anatomic location for western blot analysis of tissues obtained from the pylorus and proximal portion of the duodenum of 27 clinically normal dogs.

Factor	Group	LSM \pm SEM
COX	COX-1	116.7 \pm 3.2
	COX-2	163.7 \pm 3.2*
Lesion	Inflammation	150.2 \pm 3.2
	Erosion	130.3 \pm 3.2
Region	Pylorus	140.6 \pm 3.2
	Duodenum	139.9 \pm 3.2

*Value differs significantly ($P < 0.05$; 3-way ANOVA) from the value for COX-1.
LSM = Least squares mean.

hyperemic appearance in all dogs with histologic evidence of erosions, but gross visual evidence of erosions was not detected in any of the dogs.

In the diagnosis and management of gastrointestinal tract disease, characterization of inflammatory conditions is usually based on the evaluation of mucosal biopsy specimens obtained endoscopically. However, investigators in 1 study¹² found that this characterization could be influenced by unintentional error, including error introduced during the endoscopic biopsy procedure. In the study reported here, a board-certified veterinary pathologist who was not aware of the results of gross evaluation of the pylorus and proximal portion of the duodenum characterized the histologic samples. Lack of a standard for defining morphological and inflammatory conditions made it difficult to compare our results with those from other studies. To develop a set of standards for the diagnosis and treatment of gastrointestinal tract disease in companion animals, the World Small Animal Veterinary Association established a Gastrointestinal Standardization Group.¹⁴ This group developed histologic standards for interpreting the nature and severity of morphological changes and published a monograph¹⁴ of the histopathologic changes in the small intestine that are attributable to inflammatory disease. Evaluation of samples in our study was based on this system, although only 1 board-certified veterinary pathologist examined the tissue specimens.

Endoscopy (gross appearance rather than results of histologic examination) is considered to be a reliable method for evaluating gastric hemorrhage and ulcers.¹⁵⁻¹⁷ However, results of the study reported here indicate the importance of histologic evaluation of biopsy specimens in conjunction with endoscopy to accurately detect underlying lesions. In the present study, we found that assessment of the gross appearance of the gastrointestinal tract of a dog will likely not provide definitive evidence of whether there are histologic lesions. Characterizing whether there are any gastroduodenal lesions is of interest when considering the administration of NSAIDs.

Analysis of our results indicated that COX-2 expression was significantly upregulated in dogs with inflammation or erosion, which suggests that administration of nonselective NSAIDs or COX-2 inhibitors could be problematic if increased expression of COX-2 has a reparative role, as has been reported in another study.¹⁸ Additionally, it is not known whether there is any difference between selective COX-2 inhibitors and nonselective NSAIDs in their propensity to inhibit upregulation of COX-2. Collectively, this places practitioners in a difficult position in terms of maximizing safety for NSAID administration. First, there are dogs that appear clinically normal on the basis of results of physical examination but that have lesions in the gastrointestinal tract. Second, COX-2 may be required for protective and repair processes in gastroduodenal tissues with inflammation or erosions. Ultimately, the most practical advice would be to use NSAIDs at the labeled dose for the shortest period in case a dog has an undetected lesion. In addition, owners should be alerted to the adverse effects of NSAIDs.

In injured gastric mucosa, there is evidence that COX-2 plays an important role in mucosal defense and

the repair process.¹⁸ The role of COX-2 appears to be multifaceted, and depending on the physiologic process involved, inhibition of COX-2 will lead to corresponding risks or benefits. In 1 study,¹⁹ edema in the paw of wild-type and COX-2-deficient mice was evaluated, and COX-2 apparently had a role in the resolution of inflammation. Injection of carrageenan into a paw in each of these mice induced inflammation to a similar extent in both COX-2-deficient and wild-type animals. In wild-type mice, swelling subsided within 24 to 48 hours. In COX-2-deficient mice, NSAIDs reduced swelling of the injected paw, which suggested that COX-1-derived prostaglandins were responsible for the inflammatory response. Swelling of the paw in the COX-2-deficient mice was still apparent up to 1 week after injection. Analysis of these findings indicates that both COX-1 and COX-2 contribute to prostaglandin production at the site of inflammation. In addition, it can be concluded that prostaglandins derived via COX-2 have a role during the resolution phase of inflammation as well as during the early stages of the inflammatory response.

Expression of COX-2 was increased in inflamed gastric tissue in 1 study,²⁰ and it appeared to play an important role in the repair of mucosal damage. Upregulation of COX-2 after gastric injury has been correlated with an increase in epithelial cell proliferation.²¹ In addition, administration of selective COX-2 inhibitors to rodents with gastric ulcers results in delayed healing of ulcers.²² In studies^{23,24} with a similar experimental design, administration of a selective COX-1 inhibitor did not affect gastric healing. Moreover, results of a study⁹ performed on COX-1-deficient mice suggested that COX-2-derived prostaglandins contribute to mucosal protection and that inhibition of those prostaglandins by NSAIDs caused mucosal injury. Other investigators²⁵ have found that COX-2 can be upregulated rapidly within 1 hour after oral administration of aspirin or indomethacin, which suggests that upregulation is a protective mechanism. In the present study, COX-2 upregulation was detected at sites of gastroduodenal injury. This upregulation should be considered a contribution to resolution of the lesions until additional studies can be conducted.

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- a. Systat, Chicago, Ill.
 - b. Triton X-100, Sigma-Aldrich, St Louis, Mo.
 - c. NP-40, Sigma-Aldrich, St Louis, Mo.
 - d. BCA protein assay, Pierce, Rockford, Ill.
 - e. XT Sample Buffer, Bio-Rad Laboratories, Hercules, Calif.
 - f. Criterion XT, Bio-Rad Laboratories, Hercules, Calif.
 - g. Santa Cruz Biotechnology Inc, Santa Cruz, Calif.
 - h. SigmaScan, San Jose, Calif.

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