Expression of cyclooxygenase isoforms in ulcerated tissues of the nonglandular portion of the stomach in horses

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Objective—To characterize the expression of the cyclooxygenase (COX)-1 and COX-2 isoforms in naturally occurring ulcers of the nonglandular portion of the stomach in horses.

Specimen Population—38 specimens from ulcerated stomachs and 10 specimens from healthy stomachs.

Procedures—Specimens were collected at an abattoir; for each specimen of squamous gastric mucosa, 1 portion was fixed in neutral-buffered 10% formalin for immunohistochemical analysis and another was frozen at −70°C for immunoblotting analysis. Immunoreactivity to 2 antibodies, MF241 (selective for COX-1) and MF243 (selective for COX-2), was evaluated by a veterinary pathologist using a scoring system. Expression of COX-1 and COX-2 was confirmed by use of immunoblotting analyses.

Results—All specimens from healthy stomachs strongly expressed COX-1, whereas only 2 of 10 expressed COX-2. The expression of both isoforms varied greatly in the ulcerated mucosal specimens. Expression of COX-1 was significantly lower and expression of COX-2 was significantly higher in ulcerated versus healthy specimens.

Conclusions and Clinical Relevance—Increased expression of COX-2 in gastric ulcers of the squamous portion of the stomach in horses suggested a role for this enzyme in gastric ulcer healing. (Am J Vet Res 2010;71:592–596)

The nonglandular (or squamous) form of EGUS is a common disease complex of horses. The high prevalence reported by different authors ranges from 40% in western performance horses to 93.6% in Standardbreds in active race training. In adult horses, ulcers are typically formed in the squamous mucosa alongside the margo plicatus, but they can also be found in the glandular and pyloric areas. Although many theories related to the mechanical consequences of exercise and other potential risk factors have been proposed to explain the location and pathogenesis of gastric ulcers in the nonglandular portion of the equine stomach, the molecular pathogeneses of ulcer formation and healing have yet to be elucidated. The role of COX-1 and COX-2 on gastric mucosal defense has been studied in several species, yet information is lacking concerning horses. In horses, ulceration of the glandular portion of the stomach resembles the situation in other species. However, the pathophysiologic features of nonglandular equine gastric ulcers are thought to be diverse because of different physiologic and defense mechanisms in equine mucosa. In the glandular gastric mucosa, a complex protection system exists to strengthen its resistance against injury. Prostaglandins play a crucial role in this system. In the gastrointestinal tract, maintenance of mucosal integrity was initially attributed exclusively to the COX-1 isoform with no contribution of COX-2. Induction of COX-2 was associated with pathophysiologic reactions such as inflammation. Studies reveal that COX-2 may also have regulatory functions under physiologic conditions. These observations indicate that COX-1 and COX-2 either alone or in concert contribute to maintaining gastric mucosal integrity in species in which glandular ulcers predominate. Only 1 report to date has described COX activity in equine nonglandular and glandular gastric mucosa. The authors observed that, in both mucosa types, COX-1 was the most important enzyme involved in PGE production under basal circumstances and COX-2 was the primary enzyme in bradykinin-stimulated tissue in vitro.

ABBREVIATIONS

<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
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<td>DAB</td>
<td>Diaminobenzidine</td>
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<td>EGUS</td>
<td>Equine gastric ulcer syndrome</td>
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Cyclooxygenase-1 and COX-2 also play a role in ulcer healing in several species in which the stomach is composed primarily of glandular mucosa. For example, in rats with chronic ulcers, the immunoreactivity of COX-2 is low in healthy gastric wall tissue and greater in tissue located at the ulcer base. Further evidence that COX-2 plays an important role in the acceleration of ulcer healing has also been revealed in a previous study in which administration of selective COX-2 inhibitors prevented ulcer healing. In fact, NSAIDs that inhibit COX-1 and COX-2 impair the ulcer healing process in species with predominantly glandular mucosa. Because of the development of COX-2 selective agents for use in horses, it has become imperative to comprehend the role of this enzyme on the molecular pathogenesis of EGUS. The objective of the study reported here was therefore to characterize the expression of COX-1 and COX-2 isoforms in naturally occurring nonglandular gastric ulcers in horses to establish whether the use of selective NSAIDs might be dangerous to horses.

Materials and Methods

Specimen collection and anti-COX antibodies—Ulcerated and healthy stomachs from horses slaughtered at an abattoir in St-Aimé, QC, Canada, by use of the free-bullet method were used in this study. Care was taken to include ulcerated and peripheral mucosa for each specimen representing the ulcerated stomachs. All specimens were immersed in saline (0.9% NaCl) solution to remove gastric contents before being fixed in neutral-buffered 10% formalin or being transported on ice until they could be placed in a freezer at −70°C for immunohistochemical analysis.

Healthy and ulcerated specimens were evaluated by examination of H&E-saffron–stained sections by a veterinary pathologist. Stomachs with mucosal erosions or healthy stomachs with evidence of inflammation were excluded. Ten healthy and 38 ulcerated specimens were included in this study. Two anti-COX antibodies (MF241 and MF243) were used. The MF241 antibody was raised in rabbits against ovine placental COX-1, and its selectivity for COX-1 has been determined. The MF243 antibody was raised in rabbits against ovine placental COX-2, and its selectivity for equine COX-2 has been characterized.

Immunohistochemical analysis—Immunohistochemical staining was performed by use of a described method. Briefly, formalin-fixed tissues were paraffin embedded, and 3-µm-thick sections were prepared, deparaffinized in toluene, and hydrated through a graded alcohol series. Endogenous peroxidase was quenched by incubating the slides in 0.3% hydrogen peroxide in methanol for 30 minutes. After rinsing in PBS solution for 15 minutes, sections were incubated with normal goat serum (1:7.4 dilution) for 20 minutes at 22°C. Primary antibodies diluted in PBS solution were applied (MF241 at 1:4,000 dilution and MF243 at 1:10,000 dilution), and sections were incubated overnight at 4°C. Control sections were incubated with nonimmunoreactive rabbit serum. After rinsing in PBS solution for 10 minutes, a biotinylated goat anti-rabbit antibody (1:222 dilution) was applied and sections were incubated for 45 minutes at 22°C. Sections were washed in PBS solution for 10 minutes and incubated with the avidin DH–biotinylated horseradish peroxidase H reagents for 45 minutes at 20°C. After washing in PBS solution for 10 minutes, the reaction was revealed by use of DAB tetrahydrochloride (0.5 mg/mL) in Tris buffer (pH, 7.6) as the chromogen and 0.03% hydrogen peroxide as the substrate. Sections were counterstained with Gill hematoxylin and mounted. Immunoreactivity was evaluated by an independent observer using the scoring system proposed by Lajoie et al (0 = no staining, 1 = 0% to 10% positively stained cells, 2 = 11% to 30% positively stained cells, and 3 = ≥ 31% positively stained cells).

Solubilized cell extracts and immunoblotting analysis—The immunoblotting technique used in this study has been described for porcine stomachs. Proteins were resolved by use of 1-dimensional SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes. Blocking of membranes was accomplished by use of 5% nonfat dry milk in solution (0.1% Tween-20 and 10mM Tris-buffered saline solution [pH, 7.5]) for 1 hour at 20°C, then membranes were washed twice for 2 minutes at 20°C with the same solution. After blocking, membranes were incubated with anti-COX antibodies (MF241 at 1:4,000 dilution and MF243 at 1:7,500 dilution) in 0.05% solution (0.05% Tween-20 and 10mM Tris-buffered saline solution [pH, 7.5]) containing 2% nonfat dry milk for 2 hours at 20°C. Membranes were incubated with a horseradish peroxidase–labeled donkey anti-rabbit secondary antibody (1:15,000 dilution) for 1 hour at 20°C. The membranes were washed, and the bound secondary antibody was detected by use of an enhanced chemiluminescence detection kit. The signal was detected on x-ray film.

Statistical analysis—Statistical analyses were performed with computer software. The Cochran-ManTEL-HAENSEL test assuming unequal distances between scores was used to establish the association of the gastric mucosa status and the COX immunoreactivity expression and the correlation between the expression of COX-1 and COX-2. A value of P ≤ 0.05 was considered significant.

Results

Healthy specimens—All healthy stomachs strongly expressed COX-1, but only 2 of 10 expressed COX-2 (Table 1). The COX-1 staining in healthy stomachs was generally seen in cells located in the lamina propria under the epithelial surface and identified as fibroblasts; in

Table 1—Staining score frequency for COX-1 (proportion) and COX-2 (percentage) in healthy (n = 10) or ulcerated (38) gastric mucosal specimens of horses.

<table>
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<tr>
<td>Specimen</td>
<td>0</td>
</tr>
<tr>
<td>Healthy</td>
<td>0/10</td>
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<tr>
<td>Ulcerated</td>
<td>2.6</td>
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Table 1—Staining score frequency for COX-1 (proportion) and COX-2 (percentage) in healthy (n = 10) or ulcerated (38) gastric mucosal specimens of horses.
blood vessels located in the mucosal, submucosal, and muscularis layers; and sometimes in smooth muscle cells (Figure 1). The surface epithelium had consistently negative results for COX-1. When present, COX-2 staining was observed in small cells in the muscularis mucosa, which were thought to be in the capillaries. The COX-2 staining yielded negative results in the surface epithelium, mucosa, and submucosa.

Ulcerated specimens—Both COX-1 and COX-2 were expressed in ulcerated specimens, although the intensity of staining varied considerably (Table 1). When present, COX-1 immunoreactivity in ulcerated specimens was principally located in fibroblast-like cells under the ulcerated surface (Figure 2). Compared with COX-1 expression in healthy specimens, significantly ($P < 0.001$) lower COX-1 expression was observed in ulcerated tissues. The COX-2 immunostaining was predominantly located in the cytoplasm of elongated fibroblast-like cells in the granulation tissue proliferating under the ulcerated area. The COX-2 immunoreactivity was also evident in the cytoplasm of mucosal epithelial cells bordering the ulcers (Figure 3). The COX-2 expression was significantly ($P < 0.001$) higher in ulcerated tissues, compared with that in the healthy specimens. There was no significant ($P = 0.41$) correlation between the expressions of COX-1 and COX-2.

Immunoblotting—When a selective anti–COX-1 antibody was used, a 69,000-kDa band was detected in healthy and ulcerated stomach specimens. In several extracts of gastric ulcers, COX-1 protein concentrations were markedly reduced, compared with those in healthy stomachs (Figure 4), confirming the immunohistological observation of lower COX-1 expression in ulcerated tissues. When a selective anti–COX-2 antibody was used, no signal was detected in healthy specimens, but strong COX-2 induction is evident in all ulcerated specimens. Values on left margin indicate base pairs.

Figure 1—Photomicrographs of COX-1 and COX-2 expression in healthy squamous gastric mucosa specimens from horses. A—Section stained with antibody MF241 (selective against COX-1). Notice immunoreactivity mostly in fibroblast-like cells in the submucosa. B—Section stained with antibody MF243 (selective against COX-2). Notice that there are no positively stained cells. Immunohistochemical stain with hematoxylin counterstain; bar = 70 µm.

Figure 2—Photomicrographs of COX-1 expression in ulcerated squamous gastric mucosa specimens from horses. A—Notice that COX-1–positive cells are reduced in number in many ulcers. B—Numerous COX-1–positive cells are still evident in some specimens. Immunohistochemical stain with hematoxylin counterstain; bar = 70 µm.

Figure 3—Photomicrographs of COX-2 expression in ulcerated squamous gastric mucosa specimens from horses. A and B—Two ulcerated mucosal specimens; notice several COX-2–positive fibroblast-like cells. C—Notice that COX-2 expression is also evident in epithelial cells bordering the ulcer. D—Negative control staining with normal rabbit serum (without primary antibody). Immunohistochemical stain with hematoxylin counterstain; bar = 70 µm.

Figure 4—Immunoblot analysis of COX-1 and COX-2 isoforms in healthy (normal) and ulcerated squamous gastric tissues from horses. A—Blots probed with antibody MF241 (selective for COX-1). Notice that COX-1 protein is strongly expressed in healthy specimens but markedly diminished in 3 of 5 ulcerated specimens. B—Blots probed with antibody MF243 (selective for COX-2). No COX-2 protein is detected in healthy specimens, but strong COX-2 induction is evident in all ulcerated specimens. Values on left margin indicate base pairs.
Discussion

To the authors’ knowledge, this is the first study conducted to evaluate the expression of COX-1 and COX-2 in ulcers of the nonglandular portion of the equine stomach. Results indicated that healthy nonglandular equine gastric mucosa expressed COX-1 and that most stomach specimens did not express COX-2. This observation is in agreement with results of previous studies17,20,21 performed on human squamous esophagus mucosa and on nonglandular gastric mucosa in pigs. Results are also in line with common knowledge that COX-1 is the constitutive isoform and COX-2 is the inducible isoform of COX in most tissues. Unexpectedly, however, COX-1 expression was significantly decreased in nonglandular ulcerated tissues, compared with expression in healthy ones. In fact, although COX-1 is known to be involved in the complex process of mucosal protection in glandular mucosa and increases in rats after experimentally induced glandular gastric ulceration,17 the role of this COX isoform in nonglandular mucosal homeostasis is unclear. A similar expression decrease in COX-1 was also observed in naturally occurring nonglandular gastric ulcers in pigs.22 As in horses, ulcers in swine are located in the nonglandular portion of the stomach. A possible explanation for the low COX-1 expression in equine and porcine nonglandular mucosal ulcers could be the loss of healthy submucosa with its COX-1–positive cells and its replacement by granulation tissue containing COX-2–expressing fibroblasts. Alternatively, it could result from negative feedback following the increase in COX-2, although no correlation was found between COX-1 and COX-2 expression in ulcerated gastric mucosal specimens in this study.

Cyclooxygenase-2 expression was significantly induced in the ulcerated squamous mucosa of adult horses. The same response was reported in pigs.22 Indeed, in naturally occurring porcine ulcers of the nonglandular portion of the stomach, COX-2 was also strongly expressed, compared with healthy mucosa in which COX-2 expression was absent or low.22 In the present study, COX-2–expressing cells were predominantly fibroblasts in the granulation tissue under the ulcer bed. The localization of COX-2 expression in the granulation tissue in equine ulcers suggests that the enzyme could be involved in the repair process of gastric ulcers in horses.

The COX-2 immunoreactivity was primarily located in fibroblasts at the ulcer base in this study. Similarly, in rats with chronic ulcers, the immunoreactivity of COX-2 is low in healthy gastric wall tissue and strongly increases in the tissue of the ulcer base,17 where it is identified in the cytoplasm of different cell types in regions of maximal repair activity. Immunoreactivity of COX-1 in rats is located mainly in the nonulcerated mucosa and was reduced after gastric ulceration in the mucosa adjacent to the ulcer crater. In the same rat study, it was determined that COX-1 immunoreactivity reappears from day 5 onward in the apical cytoplasm of the regenerative epithelial cells. Although the evolution of COX expression could not be assessed in the in vitro study reported here, observations from rodent studies suggest that in chronic ulcers, COX-1 and COX-2 may have different locations and different times of expression. If COX-2 is upregulated in chronic gastric ulcers and inhibitors of COX-2 prevent the healing of ulcers, then COX-2 may play an important role in acceleration of ulcer healing. Prostaglandins inhibit leukocyte adherence to the vascular endothelium,24 improving the resistance of the gastric mucosa to injury through the downregulation of inflammatory responses.25,26 Prostaglandin D2 derived from the COX-2 pathway decreases granulocyte infiltration in experimentally induced colitis in rats.31 The COX-2–derived prostaglandin D2 metabolite mediates neutrophil and macrophage apoptosis during resolution of acute inflammation.32 Findings in rats further support this hypothesis because selective COX-1 inhibition results in a reduction in gastric mucosal blood flow, although selective COX-2 inhibition increases leukocyte adherence in mesenteric venules, suggesting that the 2 COX isoforms differ in their biological activity.15 It is unknown if these mechanisms also apply to nonglandular ulcers.

Results of the present in vitro study suggested that, as found for glandular gastric tissue of rodents,34–36 COX-2 may play an important role in gastric ulcer healing in the nonglandular mucosa of horses. Further studies are required to elucidate the clinical relevance of these findings and the potential impact of the administration of selective COX-2 inhibitors (such as firocoxib) on gastric ulcer healing in horses.

References