Expression of cyclooxygenase genes in the jejunum of horses during low-flow ischemia and reperfusion

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Objective—To determine expression of cyclooxygenase (COX) genes 1 and 2 (also called prostaglandin-endoperoxide synthases 1 and 2) and stability of housekeeping gene expression during low-flow ischemia and reperfusion in the jejunum of horses.

Animals—5 healthy adult horses.

Procedures—Horses were anesthetized, and two 30-cm segments of jejunum were surgically exteriorized. Blood flow was maintained at baseline (untreated) values in 1 (control) segment and was decreased to 20% of baseline (low-flow ischemia) for 75 minutes, followed by 75 minutes of reperfusion, in the other (experimental) segment. Biopsy samples were collected from experimental segments at baseline (T0), after 75 minutes of ischemia (T1), and after 75 minutes of reperfusion (T2); samples were collected from control segments at T0 and T2. Horses were euthanized 24 hours after induction of ischemia (T3), and additional samples were collected. Samples were evaluated histologically. Total RNA was extracted; expression of COX genes and stability of 8 housekeeping genes were determined via quantitative real-time PCR assays.

Results—COX-1 and COX-2 genes were constitutively expressed in baseline samples. Low-flow ischemia resulted in significant upregulation of COX-2 gene expression at each subsequent time point, compared with baseline values. The most stably expressed reference genes were β-actin and hypoxanthine phosphoribosyltransferase, whereas glyceraldehyde 3-phosphate dehydrogenase and β-2 microglobulin were the least stably expressed.


Reduction in arterial blood flow to a region of intestine results in alterations in normal cellular physiologic functions, including depletion of energy reserves and oxygen.1 Following resumption of normal mesenteric circulation, additional injury develops due to liberation of accumulated inflammatory products and oxygen-derived free radicals.1

The membrane-anchored enzyme COX (also called prostaglandin-endoperoxide synthase) catalyzes the conversion of arachidonic acid, liberated by phospholipase from cell membranes during ischemia-reperfusion injury and other cellular injury, into prostaglandins and prostanoids.1,2 Cyclooxygenase has 2 distinct iso-
matory conditions, there is evidence to suggest that expression of COX-1 increases in mononuclear cells of the lamina propria with increasing severity of gastric ulceration in humans and, in conjunction with COX-2, is increased following ischemia and reperfusion in the jejunum of horses. Although COX-2 is upregulated during acute and chronic inflammation as well as after ischemia, constitutive expression of COX-2 has also been reported in the gastric mucosa, proximal colon, and ileocecal junction in rodents and humans and in the jejunum of horses.

As the roles of COX in intestinal health and disease are further investigated, the need for accurate laboratory techniques becomes increasingly important. Quantitative assays designed to detect changes in COX gene expression in tissues typically use housekeeping gene transcripts; the presumed stable expression of such products allows quantification by comparison to an internal standard. However, studies have revealed that commonly used housekeeping genes such as ACTB and GAPDH have variable stability under different experimental conditions. As a result, studies that rely on the stable expression of a single reference gene against which to measure expression of a gene of interest may have erroneous results.

The objectives of the study reported here were to determine expression of COX genes 1 and 2 (gene symbols, PTGS1 and PTGS2, respectively) and to evaluate stability of various housekeeping genes during low-flow ischemia and reperfusion in the jejunum of horses to identify an optimal reference gene for this purpose. The in vivo model of ischemia and reperfusion used in the study reported here was used previously in a study to evaluate a solution designed during acute and chronic inflammation as well as after ischemia, constitutive expression of COX-2 has also been reported in the gastric mucosa, proximal colon, and ileocecal junction in rodents and humans and in the jejunum of horses.

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Materials and Methods

Horses—Five healthy horses of various breeds (2 mares and 3 geldings; mean age, 3.6 years [range, 3 to 4 years]) were enrolled in the study. The horses were randomly selected from a group of university-owned horses housed at a nearby research facility. Horses were fed a diet of alfalfa and oat hay with free access to water, and feed was not withheld on the day of the experiment.Anthelmintics were administered according to a standard schedule, and the horses had no signs of systemic or gastrointestinal disease for 2 weeks prior to study initiation. Horses were deemed healthy on the basis of results of physical examination and a CBC. The study protocol was approved by the Institutional Animal Care and Use Committee at the University of California-Davis.

Surgical procedures—A 14-gauge, 3.25-inch IV catheter was placed in the left jugular vein following aseptic preparation of the insertion site. Horses were premedicated with xylazine hydrochloride (0.4 mg/kg, IV), and general anesthesia was induced with guaiacol (50 mg/kg, IV, to effect) and ketamine hydrochloride (2 mg/kg, IV). Following orotracheal intubation, a surgical plane of anesthesia was maintained with isofluorane vaporized in oxygen. Horses were placed in dorsal recumbency, and the ventral midline was clipped and aseptically prepared with 2% chlorhexidine solution. The ventral midline was incised along the linea alba; the cecum was exteriorized, and the ileocecal band was traced to the ileum. The small intestine was examined in an oral direction until a 30-cm segment of jejunum supplied by 1 major mesenteric vessel was located. A second 30-cm jejunal segment, separated from the first segment by ≥1 mesenteric arcade, was selected oral to the first segment. One randomly selected segment served as the untreated control segment, and the other segment (experimental segment) was used for the arterial low-flow ischemia and reperfusion experiment. Full-thickness biopsy samples were obtained under various blood flow conditions.

Flow probes were placed around the mesenteric vessels of control and experimental jejunal segments, and lubricating gel was applied around the probe to ensure good contact with the blood vessels. The mesenteric artery and vein supplying the selected experimental segment of intestine were bluntly dissected, and lidocaine hydrochloride (2% solution) was applied topically to the surface of each vessel to minimize vasospasm. After baseline blood flow rates were measured, vascular clamps were placed around the mesenteric vessels and tightened to occlude the vessels sufficiently to reduce the blood flow to 20% of the baseline value for 75 minutes. Collateral circulation into the bowel segment was minimized by occlusion of peripheral blood vessels with vascular bulldog clamps. Penrose drains placed circumferentially around the serosal surface of the bowel at the margin of each segment further prevented collateral circulation. Following this period of arterial low flow, arterial clamps were removed and full arterial flow was restored for 75 minutes. Blood flow rates were continuously monitored.

The serosal surface of the tissue was periodically laved with warm (37.5°C) lactated Ringer’s solution in both treatment and control segments of jejunum. The ventral abdomen was covered with a large plastic drape to minimize desiccation and keep the tissue warm. The surgical sites were closed routinely, and horses were allowed to recover from surgery after the experimental procedure; maintenance crystalloid fluids were administered via continuous rate infusion of lactated Ringer’s solution (3 mL/kg/h, IV), and butorphanol tartrate (0.01 mg/kg, IV, q 6 h) was administered. Horses were euthanized with an overdose of sodium pentobarbital 24 hours after initiation of ischemia.

Sample collection—Biopsy samples (2 x 2 cm, full thickness) were collected during anesthesia from experimental segments of the jejunum at baseline (T0), after 75 minutes of ischemia (T1), and after 75 minutes of reperfusion (T2); samples were collected from...
control segments at T0 and T2. Full-thickness samples were also collected from control and experimental segments of the jejunum of each horse immediately after euthanasia (T3). Samples were processed for histologic examination and for evaluation of gene expression.

**Histologic examination**—Biopsy samples were mounted on balsa wood with 23-gauge needles and fixed in neutral-buffered 10% formal saline solution for ≥48 hours. Samples were routinely processed in paraffin blocks, cut into 4-µm sections on a rotary microtome, and stained with H&E. Sections were evaluated histologically in a minimum of 4 fields at 40× magnification by 1 observer (PFM) who was unaware of the identification of the source of the biopsy sample (control vs experimental treatment segment).

Sample processing for evaluation of COX-1 and COX-2 gene expression—Biopsy samples for evaluation of gene expression were collected at the same time points as those used for histologic evaluation. These biopsy samples were placed in 2-mL plastic tubes, immediately frozen in liquid nitrogen, and stored at –80°C until further processing.

Frozen biopsy samples were pulverized in liquid nitrogen by use of a mortar and pestle. Total RNA was isolated by use of a commercial kit and quantified by use of a spectrophotometer. The RNA was transformed from 65° to 95°C in 0.5°C increments with a dwelling time at each temperature of 10 seconds during which fluorescence was continuously monitored. All samples were run in triplicate, and nontemplate negative controls were included. To determine stability of reference gene expression, PCR reactions were performed by use of the 8 housekeeping genes in 14 experimental and control samples. The most stably expressed genes were selected by use of commercially available software as previously described. The software calculates a gene expression stability measure (M value) for a reference gene as the mean pairwise variation for that gene, compared with all other tested reference genes. Stepwise exclusion of the gene with the highest M value allows ranking of the tested genes according to their expression stability. From the 2 most stably expressed housekeeping genes, the gene with the lowest Cp value was selected as the reference gene for use in the study.

**Real-time quantitative PCR**—Primers for COX-1 and COX-2 target gene cDNA were designed in the same region as published human primers after sequencing the PCR product. Efficiencies for the 8 housekeeping genes were calculated by use of PCR assays with known serial dilutions of cDNA from one of the collected samples. Resulting crossing point cycles (Cp values) were plotted against the logarithm of the cDNA concentration, and a regression line was calculated. The slope of the reactions was determined, and amplification efficiencies were calculated on the basis of dilutions by use of the following equation:

\[
\text{efficiency} = (10^{-1/	ext{slope}}) - 1
\]

Real-time PCR assays were performed by use of a real-time detection system to detect dye intercalation. Reaction samples had a final volume of 10 µL, containing 5 µL of a reaction mix containing SYBR green, 0.5 µM of each primer, 0.2 U of uracyl glucosylase, 1 µL of cDNA, and DNase-free water. Amplification conditions were as follows: 37°C for 10 minutes and 95°C for 5 minutes, followed by 45 cycles of 95°C for 5 seconds, 55°C for 10 seconds, and 72°C for 6 seconds. To ensure specificity of amplifications and to detect primer-dimer formation, dissociation curves of the melting temperature were created and evaluated by heating the samples from 65° to 93°C in 0.5°C increments with a dwelling time at each temperature of 10 seconds during which fluorescence was continuously monitored. All samples were run in triplicate, and nontemplate negative controls were included. To determine stability of reference gene expression, PCR reactions were performed by use of the 8 housekeeping genes in 14 experimental and control samples. The most stably expressed genes were selected by use of commercially available software as previously described. The software calculates a gene expression stability measure (M value) for a reference gene as the mean pairwise variation for that gene, compared with all other tested reference genes. Stepwise exclusion of the gene with the highest M value allows ranking of the tested genes according to their expression stability. From the 2 most stably expressed housekeeping genes, the gene with the lowest Cp value was selected as the reference gene for use in the study.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Tm (°C)</th>
<th>Size (bp)</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTB</td>
<td>5′-CCAGACATGAAAGATCAAG-3′</td>
<td>5′-GTGAGAATGAGCCAGAT-3′</td>
<td>84.34</td>
<td>86</td>
<td>1.83</td>
</tr>
<tr>
<td>BM</td>
<td>5′-GTCACGATCCGCTGAGAT-3′</td>
<td>5′-GAGGCTTCCAGATAGAGT-3′</td>
<td>84.58</td>
<td>182</td>
<td>1.54</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5′-AAGTGAGGATTGTGCCCATCAAT-3′</td>
<td>5′-AAGTTGCAATGGTGAATC-3′</td>
<td>79.71</td>
<td>86</td>
<td>1.83</td>
</tr>
<tr>
<td>HRPT1</td>
<td>5′-GGCAAAATGCAAAGGCTTT-3′</td>
<td>5′-CAAGGGCATATCTCATGCAAA-3′</td>
<td>81.43</td>
<td>163</td>
<td>2.31</td>
</tr>
<tr>
<td>MRPS-9</td>
<td>5′-ACTAGGCTGAGGAGGTGCC-3′</td>
<td>5′-TGCCGTCTCCCTCTCAG-3′</td>
<td>86.89</td>
<td>133</td>
<td>1.79</td>
</tr>
<tr>
<td>SDHA</td>
<td>5′-GCTGACCAGGTGATAGAG-3′</td>
<td>5′-CATACGATGATCTGACATC-3′</td>
<td>78.20</td>
<td>84</td>
<td>1.7</td>
</tr>
<tr>
<td>TUBA-4A</td>
<td>5′-GCCCTAATCCATGATCTG-3′</td>
<td>5′-ATGCTTCTATTGACGACTA-3′</td>
<td>83.82</td>
<td>78</td>
<td>1.83</td>
</tr>
<tr>
<td>UBB</td>
<td>5′-GCAAGACCTATATGCTTTGA-3′</td>
<td>5′-CTGACGCTGAGATCCAGAC-3′</td>
<td>86.74</td>
<td>206</td>
<td>1.71</td>
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<tr>
<td>COX-1*</td>
<td>5′-GAATGTTCAAGAGGAGAGA-3′</td>
<td>5′-GAGAAGGCAATGCTTGG-3′</td>
<td>83.94</td>
<td>118</td>
<td>1.82</td>
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<tr>
<td>COX-2†</td>
<td>5′-AGAATGTTCAAGGAGAGA-3′</td>
<td>5′-CTGACGCTGAGATCCAGAC-3′</td>
<td>86.74</td>
<td>206</td>
<td>1.71</td>
</tr>
</tbody>
</table>

*Also called prostaglandin-endoperoxide synthase 1. †Also called prostaglandin-endoperoxide synthase 2. E = Efficiency, Size = PCR assay product size, Tm = Melting temperature.
spanning 2 exons to prevent genomic DNA amplification; published equine sequences for the targets (Genbank accession Nos. DQ246452.1 and AF027335) were used as the basis for primer design. The PCR assays were performed as described for housekeeping genes; specificity of the reactions was monitored by analysis of the melting curves and by separation and examination of the PCR products on an ethidium bromide–labeled 2% agarose gel. Samples were run in triplicate and in the presence of a nontemplate negative control and a calibrator (control cDNA). Quantification relative to the most stably expressed housekeeping gene was performed by use of commercial software6 with a technique for gene-expression analysis as previously described.26 The method used produces accurate relative quantification data by compensating for differences in target- and reference-gene amplification according to specific gene efficiencies.

Statistical analysis—Statistical analysis of differences in gene expression between control and experimental segments of jejunum was performed by use of commercially available software.63 Data were analyzed via repeated-measures ANOVA. When significant differences were detected, the least significant difference post hoc test was used to determine difference from baseline. Values of $P < 0.05$ were accepted as significant.

Results

Histologic examination—Arterial low-flow ischemia resulted in mild submucosal hemorrhage in 2 of 5 samples and submucosal edema and lacerate dilation in all biopsy samples collected at T1. Mild submucosal edema was present in 2 of 5 samples following reperfusion at T2; no other pathological changes were identified in the samples evaluated. There was no evidence of epithelial denudation in any of the samples evaluated.

Reference gene expression—Results of expression stability analysis of the 8 evaluated housekeeping genes indicated that ACTB and HRPT-1 were the most stably expressed genes, followed (in order of decreasing stability) by TUBA-4A, UBB, MRPS-9, SDHA, B2M, and GAPDH. We selected ACTB as the reference gene for use in real-time PCR assays to analyze target (COX-1 and COX-2) gene expression. Analysis of the melting curves that resulted from PCR reactions of the target genes and the reference gene showed that only a single product was amplified for each (Figure 1). For every assay of our target genes, a single amplicon of the expected size (153 bp for COX-1 cDNA and 118 bp for COX-2 cDNA) was generated without primer-dimer formation.

COX gene expression—The COX-1 and COX-2 genes were constitutively expressed in the jejunum of horses at baseline. Expression of the COX-2 gene was significantly upregulated after 75 minutes of ischemia (T1), after ischemia followed by 75 minutes of reperfusion (T2), and 24 hours after the initiation of ischemia-reperfusion (T3), compared with baseline values (Figure 2). There was an increase in COX-1 expression after 75 minutes of ischemia followed by 75 minutes of reperfusion; however, the increase was not significantly different from baseline values.

Discussion

The study reported here was performed to investigate expression of COX genes in the jejunum of horses under control conditions and under conditions of arterial low-flow ischemia and reperfusion. In addition, we examined expression of 8 housekeeping genes to determine their expression stability under control and experimental conditions. Our results showed that COX-2 was significantly upregulated after 75 minutes of low-flow ischemia and following ischemia and reperfusion in the described in vivo experiments. The traditional
housekeeping genes, ACTB and HRPT-1, were found to be the most stably expressed reference genes under control and experimental conditions in the present study. The finding of a significant increase in COX-2 gene expression following arterial low-flow ischemia and reperfusion in the jejunum of healthy horses is in agreement with the results of several previous studies, in which investigators reported upregulation of COX protein expression following ischemic intestinal damage in horses and other species. Although expression of the COX-1 gene also appeared to be upregulated after ischemia and reperfusion in the present study, this increase was not significant. This finding is in contrast to the results of other studies, in which both COX isoforms were significantly upregulated after intestinal injury. Although this finding may be reasonably explained if it is accepted that COX-1 is a constitutive enzyme with a primary role in intestinal physiology and that COX-2 is an inducible isoenzyme upregulated secondary to cellular injury, current research suggests increasingly complex roles for these enzymes. Constitutive expression of COX-2 has been described in several tissues, and upregulation of COX-1 has been reported secondary to tissue injury in various species. The lack of a significant increase in COX-1 gene expression in the present study may reflect differences in the severity of ischemic damage produced by different experimental methods for induction of intestinal ischemia. Whereas arterial flow to selected segments of the jejunum was reduced to 20% of baseline for 75 minutes in the present study, investigators in other studies have used complete arterial occlusion for longer periods to model intestinal lesions. Therefore, expression of the COX-1 gene may increase with severe intestinal compromise but may change only to a lesser (nonsignificant) degree when the damage induced is moderate. Our results and those from previous studies suggest that nonselective NSAIDs may be more likely than selective COX-2 NSAIDs to produce undesirable effects in the mucosa of horses with small intestinal injury.

Upregulation of COX-2 gene expression in the study reported here was detected during ischemia, after 75 minutes of reperfusion, and 24 hours after initiation of ischemia and reperfusion. Peak expression of the gene was detected after 75 minutes of reperfusion. This finding is consistent with the rapid initiation of ischemic damage reported in previous studies. Peak gene expression was detected following resumption of normal arterial flow, indicating that the experimental methods employed successfully induced reperfusion injury. Inflammation, as evidenced by an increase in COX expression, induced secondary to ischemia and reperfusion, in the absence of severe histologic changes that develop following complete arterial occlusion, indicates that the low-flow experimental model of ischemia accurately reflects changes that develop at the periphery of strangulating lesions or secondary to marked intestinal distension. The presence of COX-2 mRNA was detected at baseline in the jejunum of horses in the present study, suggesting constitutive expression of the enzyme in this tissue. This finding is in agreement with several studies, in which investigators detected COX-2 protein in the gastrointestinal tracts of various species in the absence of detectable inflammation. Although it has been suggested that COX-2 plays a role in the regulation of gastrointestinal motility, its exact physiologic function is not known and is likely dependent on the coexpression of a series of prostaglandin synthases. Further work directed at pharmacological inhibition of specific COX isoenzymes and prostaglandin synthases is required to elucidate their specific functions as they pertain to intestinal function in health and disease.

Several techniques that allow quantification of mRNA for a gene of interest rely on comparison with mRNA of 1 or more genes that are synthesized in all cell types. These so-called housekeeping or reference genes are considered essential for cell survival, and as a result, their expression is thought to remain stable. Recently, however, the results of several studies have suggested that expression of these genes may vary under some conditions. Thus, relative quantification of a gene of interest against only 1 reference gene may lead to inaccurate conclusions. Investigators in previous studies have suggested that expression of ACTB was the most stable among several examined housekeeping genes and that expression of GAPDH was the least stable of these under the described experimental conditions. This finding does not necessarily invalidate the findings of previous studies, nor does it suggest that ACTB should be used as a reference gene in future studies that explore the effect of intestinal ischemia; expression of these genes may differ under various ischemic or inflammatory conditions. Rather, this result demonstrates the need for accurate determination of reference gene expression stability under each specific experimental protocol used.

In the study reported here, we investigated the expression of COX genes in jejunum of healthy horses; we did not evaluate the production of COX proteins. As a result, we cannot definitively rule out the influence of a posttranslational mechanism that modulates the translational rate, protein concentrations, mRNA half-life, or the intracellular location and molecular associations of the protein products of expressed genes. Analysis of immunohistochemical investigations could be used to confirm the presence of protein products and to determine localization of the proteins. This would be of particular interest in regard to the constitutive expression of COX-2 because it may provide information to suggest a physiologic function for this enzyme.