Prostaglandins are 20-carbon fatty acid derivatives that serve numerous physiologic and pathophysiologic roles. The cascade of prostaglandin synthesis starts with cPLA2 catalyzing the release of arachidonic acid from the phospholipid bilayer of the cell membrane. The subsequent conversion of arachidonic acid to prostaglandin G2 and, eventually, prostaglandin H2 is controlled by cyclooxygenase, which exists in at least 2 isoforms. Cyclooxygenase 1 is constitutively expressed in most tissues and is involved in the homeostasis of several physiologic processes, such as ovulation, initiation of labor, bone metabolism, nerve growth and development, wound healing, kidney function, blood vessel tone, mucosal protection, and platelet aggregation. Although COX-2 has some roles in homeostasis, its promotor site has a TATA box and binding sites for several transcription factors that enable induction in the presence of proinflammatory stimuli, such as bacterial endotoxin. Importantly, prostaglandin H2 is metabolized by several enzymes, most notably mPGES1, also an inducible enzyme, culminating in the formation of PGE2. Thus, induction of COX-2 or mPGES1 gene expression ultimately results in production of PGE2, which contributes to the pathophysiology of inflammation through induction of fever and edema, vasodilation, and enhanced sensitivity of nocireceptors.

In recent years, it has also been discovered that COX-2 gene expression is upregulated in other disease states, such as inflammatory bowel disease, Alzheimer disease, diabetes, and cancer. 

Effect of firocoxib on cyclooxygenase 2, microsomal prostaglandin E2 synthase 1, and cytosolic phospholipase A2 gene expression in equine mononuclear cells

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OBJECTIVE
To validate primer sets for use in reverse transcription quantitative PCR assays to measure gene expression of cytosolic phospholipase A2 (cPLA2) and microsomal prostaglandin E2 synthase 1 (mPGES1) in equine mononuclear cells and determine the effects of firocoxib, a selective cyclooxygenase 2 (COX-2) inhibitor, on COX-2, cPLA2, and mPGES1 gene expression following incubation of mononuclear cells with lipopolysaccharide (LPS).

ANIMALS
8 healthy adult horses.

PROCEDURES
Peripheral blood mononuclear cells were isolated by density gradient centrifugation and incubated at 37°C with medium alone, firocoxib (100 ng/mL), LPS (1 ng/mL or 1 ug/mL), or combinations of firocoxib and both LPS concentrations. After 4 hours, supernatants were collected and tested for prostaglandin E2 (PGE2) concentration with an enzyme inhibition assay, and gene expression in cell lysates was measured with PCR assays.

RESULTS
Primer pairs for cPLA2 and mPGES1 yielded single products on dissociation curve analyses, with mean assay efficiencies of 102% and 100%, respectively. Incubation with firocoxib and LPS significantly decreased PGE2 supernatant concentrations and significantly reduced COX-2 and mPGES1 gene expression, compared with values following incubation with LPS alone.

CONCLUSIONS AND CLINICAL RELEVANCE
Primer sets for mPGES1 and cPLA2 gene expression in equine mononuclear cells were successfully validated. Firocoxib significantly decreased LPS-induced COX-2 and mPGES1 expression, suggesting that it may be useful in the control of diseases in which expression of these genes is upregulated. (Am J Vet Res 2015;76:1051–1057)
disease, primary open-angle glaucoma, ischemia, and certain cancers. In horses with intestinal ischemia, mucosal COX-2 gene expression is increased, and treatment with selective COX-2 inhibitors aids with mucosal restitution. Most notably, perhaps, COX-2 gene expression is induced in various neoplasms of epithelial cell origin, such as colorectal, lung, breast, gastric, cutaneous, and pancreatic carcinomas, with associated increased PGE2 expression in affected tissues. Given the data suggesting that COX-2 and PGE2 play important roles in oncogenesis, treatments have been explored with the goal of controlling expression of COX-2 and related enzymes, such as mPGES1 and cPLA2, and their metabolic products. Population-based and case-control studies and clinical trials provide supportive evidence that selective COX-2 inhibitors can reduce the risk of developing or aid in the treatment of cancers associated with increased expression of COX-2. However, the exact mechanism by which selective COX-2 inhibitors provide these benefits has not been fully elucidated.

Given the cardinal roles of PGE2 in the pathogenesis of inflammatory and neoplastic diseases, pharmacological control of its synthetic pathway is a prime interest. The discovery of the COX-2 isozyme and its link to PGE2 led to an era of drug testing to identify safe and effective COX-2 inhibitors. Selective COX-2 inhibitors bind noncovalently to the active site of COX-2, thereby functionally blocking enzymatic activity and reducing PGE2 synthesis. Ex vivo studies have shown that firocoxib, the only selective COX-2 inhibitor approved by the US FDA for use in equids, is highly effective in decreasing LPS-induced PGE2 concentrations. However, it is not known whether there are mechanisms independent of COX-2 inhibition, such as downregulation of COX-2 gene expression or the expression of other enzymes involved in the eicosanoid pathway, that may account for firocoxib’s effects on PGE2 concentration. Given evidence of coregulation of genes expressing proteins involved in the prostaglandin enzymatic pathway, exploring the effect of firocoxib on key upstream and downstream genes involved with prostaglandin synthesis is merited. Thus, the purposes of the study reported here were to validate primer sets for use in RT-qPCR assays to measure gene expression of cPLA2 and mPGES1 in equine mononuclear cells and to determine the effects of firocoxib on LPS-induced COX-2, mPGES1, and cPLA2 gene expression. Our working hypothesis was that firocoxib would downregulate LPS-induced COX-2, mPGES1, and cPLA2 gene expression.

Materials and Methods

Blood collection

Blood was sterilely collected by means of jugular venipuncture into 60-mL syringes containing 2 mL of 100µM EDTA from 8 adult light breed or warmblood horses determined to be healthy on the basis of results of a complete physical examination. All blood samples were collected the morning of the experiment, and samples were immediately transported to the laboratory for separation of mononuclear cells. Study methods were approved by the University of Georgia Institutional Animal Care and Use Committee (proposal A2013 06-008), and horses were cared for according to guidelines stated in the Animal Use Protocol developed and approved by the university’s Department of Animal Resources.

Mononuclear cell separation

Mononuclear cells were separated by means of density gradient centrifugation, as previously described. Mononuclear cells were suspended in RPMI-1640 medium with 10% commercial equine serum and 1% penicillin-streptomycin. Aliquots (1 × 107 cells) were placed in 50-mL polypropylene conical tubes for 30 minutes before further testing.

Incubation

Cells were incubated, in duplicate, for 4 hours at 37°C in 5% CO2 in medium alone, medium to which O55:B5 LPS had been added at a final concentration of 1 µg/mL or 1 ng/mL, medium to which firocoxib (100 ng/mL) had been added, and medium to which both firocoxib (100 ng/mL) and LPS (final concentration, 1 µg/mL or 1 ng/mL) had been added. All tubes were manually swirled by gentle rotation after 2 hours of incubation. After 4 hours of incubation, cell viability was determined by means of trypan blue exclusion for cells from 3 horses incubated, in duplicate, in medium alone, or in medium to which firocoxib (100 ng/mL) had been added.

For all other tubes, after 4 hours of incubation, cells were pelleted in a refrigerated centrifuge (4°C). The supernatant was removed and stored at –80°C for later determination of PGE2 concentration by means of an enzyme inhibition assay. Cell pellets were resuspended and washed with cold PBS solution, and the final cell pellet was homogenized with RNA cell lysis solution supplemented with β-mercaptoethanol according to the manufacturer’s instructions and stored at –80°C.

Measurement of gene expression

Total RNA was extracted from homogenized cell pellets with a commercial kit according to the manufacturer’s protocol with the inclusion of a DNase I treatment to eliminate genomic DNA contamination. Oligonucleotide primers for COX-2 previously validated for equine mononuclear cells were used for measurement of COX-2 gene expression. For measurement of mPGES1 and cPLA2 gene expression, primers were designed with software on the basis of equine sequences available from the GenBank Database (Appendix).

Gene expression was determined by use of a 2-step RT-qPCR assay incorporating SYBR green detection, with 18S rRNA serving as the endogenous control as previously described. A control sample that did not
Assay efficiency for mPGES1 and cPLA2 gene expression was determined from the slope of a standard curve generated with serial 1/2 logarithmic dilutions (at least 5 dilutions ranging from 0.01 to 10 ng cDNA/10 µL water) of cDNA template for each gene from LPS-stimulated mononuclear cells. Efficiency was calculated by use of the equation $E = \left(\frac{10^{-1/m (p-1)}}{1}\right) \times 100$, where $E$ is PCR assay efficiency. Assay efficiency was considered acceptable if it was between 95% and 105%.

Equivalent amplification efficiencies of the target gene and 18S rRNA were determined by plotting the difference in cycle threshold (ie, cycle threshold for the target gene minus cycle threshold for 18S mRNA) versus the logarithm of the cDNA dilution. Amplification efficiency was considered equivalent if the slope was $< 0.1$.

**Statistical analysis**

Data are expressed as mean ± SE. Normality of the data and equality of variances were assessed by means of the Shapiro-Wilk and Levene tests, respectively. Gene expression data were subjected to base 10 logarithmic transformation to achieve normal distributions. For each gene of interest, a 2-way ANOVA was used to determine whether the mean 2(–$\Delta\Delta$CT) value for cells incubated with firocoxib alone was significantly different from 1 (ie, the value for cells incubated in medium alone). For each gene of interest, a 1-sample $t$ test was used to assess whether LPS concentration (high vs low), treatment with firocoxib (yes vs no), or the interaction between LPS concentration and treatment with firocoxib had a significant effect on gene expression. When warranted, pairwise comparisons were performed with the Student-Newman-Keuls test. Data for PGE$_2$ concentration were found to be normally distributed with equal variances and were therefore analyzed by means of an ANOVA for repeated measures, followed by the Student-Newman-Keuls test for comparisons between groups.

For all analyses, values of $P < 0.05$ were considered significant. Statistical analyses were performed with commercial software.

**Results**

**Primer validation**

Primer pairs for mPGES1 and cPLA2 yielded single products on dissociation curve analyses, and assay efficiencies for mPGES1 and cPLA2 were 100% and 102%, respectively (Figures 1 and 2). The mPGES1 and cPLA2 assays, the slopes of the curves for the difference in cycle threshold (ie, cycle threshold for the target gene minus cycle threshold for 18S mRNA) versus the logarithm of the cDNA dilution were 0.054 and 0.069, respectively, indicating equivalent amplification efficiencies.

**Cell viability**

After 4 hours of incubation, mean viability of cells from 3 horses incubated in medium alone was 99.1% (range, 98% to 100%), and mean viability of cells from 3 horses incubated in medium to which firocoxib (100 ng/mL) had been added was 98.8% (range, 98% to 100%).

**Gene expression**

Cyclooxygenase 2, mPGES1, and cPLA2 gene expressions did not differ significantly ($P = 0.987, 0.981,$ and $0.279$, respectively) between equine mononuclear cells incubated in medium alone and cells incubated in medium with firocoxib. Compared with gene expression when cells were incubated in medium alone (relative gene expression of 1), addition of LPS at a final concentration of 1 µg/mL or 1 ng/mL increased mean relative COX-2 gene expression by 7.1- and 6.3-fold, respectively (Figure 3); increased mean relative mPGES1 gene expression by 12.8- and 15.7-fold, respectively (Figure 4); and increased mean relative cPLA2 gene expression by 3.1- and 1.3-fold, respectively (Figure 5). Mean relative gene expression did not change in medium alone when 10 µL water was included with every assay run. All reactions were performed in triplicate in wells on 384-well microtitration plates. Changes in gene expression were calculated by means of relative quantification versus 18S rRNA with the 2$^{-\Delta\Delta$CT} method, wherein the threshold for the unstimulated control sample served as the calibrator and was therefore assigned an expression of 1.

Findings associated with $P < 0.05$ are considered statistically significant.

**Figure 1**—Validation graph for an RT-qPCR assay developed to measure mPGES1 gene expression in cultured equine mononuclear cells; the graph was generated by assaying serial dilutions of cDNA template from LPS-stimulated mononuclear cells. The solid line represents the linear regression equation ($y = -3.31x + 27.554; R^2 = 0.982$). Assay efficiency was calculated as 100%.

**Figure 2**—Validation graph for an RT-qPCR assay developed to measure cPLA2 gene expression in cultured equine mononuclear cells; the graph was generated by assaying serial dilutions of cDNA template from LPS-stimulated mononuclear cells. The solid line represents the linear regression equation ($y = -3.26x + 27.576; R^2 = 0.994$). Assay efficiency was calculated as 102%.
not differ significantly ($P = 0.853, 0.398, \text{and} 0.409$, respectively, for COX-2, mPGES1, and cPLA$_2$) between cells incubated with the higher concentration of LPS and cells incubated with the lower concentration, and there was no significant interaction between LPS concentration (high vs low) and treatment with firocoxib (yes vs no) on relative gene expression ($P = 0.192, 0.119, \text{and} 0.776$, respectively, for COX-2, mPGES1, and cPLA$_2$). When cells were incubated with the higher concentration of LPS, the addition of firocoxib reduced mean relative COX-2, mPGES1, and cPLA$_2$ gene expression by 66%, 46%, and 76%, respectively. When cells were incubated with the lower concentration of LPS, the addition of firocoxib reduced mean relative COX-2, mPGES1, and cPLA$_2$ gene expression by 54%, 41%, and 17%, respectively. The effect of treatment with firocoxib (yes vs no) was significant for COX-2 ($P = 0.004$) and mPGES1 ($P = 0.026$) gene expression, but not for cPLA$_2$ ($P = 0.067$) gene expression.

**PGE$_2$ concentration**

Cells incubated with the high or low LPS concentration had supernatant PGE$_2$ concentrations significantly ($P < 0.001$) higher than concentrations for cells incubated with medium alone or medium and firocoxib (Figure 6). Supernatant PGE$_2$ concentrations for cells incubated with firocoxib and LPS, at either the high or low concentration, were not significantly ($P > 0.9$) different from concentrations for cells incubated with medium alone or medium and firocoxib.

**Discussion**

The first step in the present study was validating the RT-qPCR assays used to measure mPGES1 and cPLA$_2$ gene expression. General criteria for validation of PCR assays that incorporate the comparative cycle threshold method include demonstrating the presence of a single, specific amplification product by means of dissociation curve analysis, high efficiency of replication, and equivalent amplification of target and housekeeping genes. These criteria were previously shown to be met for the COX-2 primer set used in the present study and were met for the cPLA$_2$ and mPGES1 primer sets designed for this study.

Our working hypothesis that firocoxib would downregulate LPS-induced expression of all 3 genes, although the effect was significant only for COX-2 and mPGES1 expression. Other studies have also shown that treatment with certain COX-2 inhibitors can downregulate COX-2 gene expression. Because COX-2 is constitutively expressed at very low levels under normal physiologic conditions, expression of COX-2 must be induced for the enzyme to be detected. In the present study, sig-
Significant inhibitory effects of firocoxib on LPS-induced COX-2 expression were identified with both concentrations of LPS that were used. Concurrently, firocoxib significantly decreased LPS-induced PGE2 concentrations in the cell culture supernatant. However, the study design did not allow us to determine whether the reduction in PGE2 concentration was a result of direct enzymatic inhibition versus a reduction in enzymatic gene expression.

Specific information on the effect of firocoxib on genes involved in the prostaglandin metabolic pathway is sparse. In 1 study,20 when firocoxib was given PO for 3 days to healthy dogs, COX-2 protein expression, as determined by western blot densitometry, in the pylorus and duodenum was not altered. A second study7 from the same laboratory showed that COX-2 protein expression was upregulated in the equine jejunum after ischemia, but there was no effect of pretreatment with firocoxib on COX-2 expression in the jejunal mucosa. The present study was unique in that, to the authors’ knowledge, this was the first study to find that treatment with firocoxib was associated with a reduction in LPS-induced COX-2 gene expression in equine mononuclear cells in vitro, as determined with an RT-qPCR assay. Although the molecular mechanisms responsible for this association were not investigated, depending on the type of stimulus and cellular context, COX-2 expression is regulated at both the transcriptional and posttranscriptional levels.

Cyclooxygenase 2 mRNA possesses a long 3′ untranslated region that mediates its stability by interacting with various RNA binding proteins.21 Recent evidence shows that 1 mechanism by which GL63, a novel COX-2 inhibitor, downregulates COX-2 gene expression is by destabilizing COX-2 mRNA through sequestration of the mRNA stabilizing protein, human antigen R.18 Similar to the case for COX-2, induction of mPGES1, with an associated increase in PGE2 concentration, has been demonstrated in several disease states, including arthritis, inflammatory bowel disease, hepatitis, Alzheimer disease, myositis, and numerous cancers.4,10 The PGE2 synthases are the terminal enzymes for the production of PGE2. There are 3 isoforms of the PGE2 synthases; 2 are membrane associated (mPGES1 and microsomal PGE2 synthase 2) and 1 is cytosolic (cytosolic PGE2 synthase). The latter is preferentially coupled with cyclooxygenase 1 expression. Microsomal PGE2 synthase 1 is located in proximity to COX-2 at the perinuclear membrane and endoplasmic reticulum, is functionally coupled with COX-2 expression, and is the only isoform that is inducible during inflammation.10 Thus, it might seem logical that downregulation of COX-2 expression by firocoxib should be associated with a similar reduction in mPGES1 expression, which was demonstrated in the present study. However, several other stimuli can induce mPGES1 expression without concurrent activation of COX-2 expression,10 suggesting that these 2 enzymes can also be independently regulated.
There is also evidence that COX-2 and cPLA2 are coregulated and that the selective COX-2 inhibitor flurbiprofen downregulates gene expression of both enzymes.\textsuperscript{19} In the present study, firocoxib did not have a significant effect on LPS-induced expression of cPLA2. However, the effect of firocoxib was tested only after incubation of mononuclear cells with LPS and firocoxib for 4 hours, and it is possible the lack of a significant effect of firocoxib on cPLA2 expression was due to weak induction of cPLA2 gene expression under these conditions. Compared with expression when cells were incubated with medium alone, COX-2 and mPGES1 expression increased between 6- and 15-fold with LPS. However, the LPS-induced response for cPLA2 expression was highly variable, and cPLA2 expression was increased by only approximately 1.5- to 3-fold with LPS. Without sufficient ability to induce cPLA2 expression, the effect of firocoxib on cPLA2 expression could not be fully elucidated.

Previous data strongly support an important role for the increased expression of COX-2 and upregulation of PGE\textsubscript{2} synthesis in the pathogenesis of diverse disease states including inflammation, ischemia, and neoplasia,\textsuperscript{5,6,8,11,22} and PGE\textsubscript{2} is known to regulate critical steps in tumorigenesis by stimulating cell proliferation and angiogenesis, preventing apoptosis, and inducing migration.\textsuperscript{8} Sixty-four percent of equine melanomas and 86\% of equine squamous cell carcinomas express COX-2.\textsuperscript{23} Given supportive evidence that COX-2 inhibitors may be protective in some neoplastic diseases in which COX-2 expression is upregulated,\textsuperscript{9,11} the potential use of affordable COX-2 inhibitors as ancillary treatment for neoplastic conditions in equids may warrant further investigation.

Results of the present study indicated that the COX-2 inhibitor firocoxib significantly decreased LPS-induced PGE\textsubscript{2} concentrations in the supernatant of cultured equine mononuclear cells and decreased LPS-induced COX-2 and mPGES1 expression by those cells. The concentration of firocoxib used in the present study was consistent with the steady-state plasma concentration achieved following oral administration of the drug to healthy horses at the recommended dosage of 0.1 mg/kg/d.\textsuperscript{13,24,25} Further study is needed to determine whether firocoxib is capable of downregulating COX-2 and mPGES1 gene expression in ex vivo or in vivo models of inflammation or in neoplastic equine cells.

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Footnotes


b. Hyclone, Logan, Utah.

c. List Biological Laboratories Inc, Campbell, Calif.

d. Merai, Duluth, Ga.

e. Cayman Chemical, Ann Arbor, Mich.

f. RNaseasy Plus, Qiagen, Germantown, Md.

g. Primer Express, Foster City, Calif.

h. Life Technologies, Grand Island, NY.

i. SigmaPlot, version 11.0, Systat Software Inc, San Jose, Calif.

References


**Appendix**

Primer sets used in RT-qPCR assays to determine COX-2, mPGES1, and cPLA2 gene expression in cultured equine mononuclear cells.

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