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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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Abstract

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 µg/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.

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.¹ The subsequent conversion of arachidonic acid to prostaglandin G₂ and, eventually, prostaglandin H₂ is controlled by cyclooxygenase, which exists in at least 2 isoforms.

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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.² 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.³ Importantly, prostaglandin H₂ is metabolized by several enzymes, most notably mPGES1, also an inducible enzyme, culminating in the formation of PGE₂.⁴ 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 nociceptors.⁵

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, primary open-angle glaucoma, ischemia, and certain cancers.³ In horses with intestinal ischemia, mucosal COX-2 gene expression is increased, and treatment with selective COX-2 inhibitors aids with mucosal restitution.^{6,7} 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.^{3,8} 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.^{1,4,9,10} 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-2.¹¹ 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 isoform 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,¹² is highly effective in decreasing LPS-induced PGE₂ concentrations.^{13,}^a 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

^a.McCann M, Andersen D, Brideau C, et al. In vitro activity and in vivo efficacy of a novel COX-2 inhibitor in the horse (abstr). *J Vet Intern Med* 2002;16:789.

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 100 μ M 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.¹⁴ Mononuclear cells were suspended in RPMI-1640 medium with 10% commercial equine serum^b and 1% penicillin-streptomycin. Aliquots (1×10^7 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^c had been added at a final concentration of 1 μ g/mL or 1 ng/mL, medium to which firocoxib^d (100 ng/mL) had been added, and medium to which both firocoxib (100 ng/mL) and LPS (final concentration, 1 μ g/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.^e Cell pellets were resuspended and washed with cold PBS solution, and the final cell pellet was homogenized with RNA cell lysis solution^f supplemented with β -mercapto-ethanol according to the manufacturer's instructions and stored at –80°C.

^bHyclone, Logan, Utah.

^cList Biological Laboratories Inc, Campbell, Calif.

^dMerial, Duluth, Ga.

^eCayman Chemical, Ann Arbor, Mich.

^fRNeasy Plus, QIAGEN, Germantown, Md.

Measurement of gene expression

Total RNA was extracted from homogenized cell pellets with a commercial kit^f 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 cells¹⁴ were used for measurement of COX-2 gene expression. For measurement of mPGES1 and cPLA₂ gene expression, primers were designed with software^g 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,^h with 18S rRNA serving as the endogenous control as previously described.¹⁴ A control sample that did not contain any template (RNase-free 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 $2^{(-CT)}$ method, wherein the cycle threshold for the unstimulated control sample served as the calibrator and was therefore assigned an expression of 1.¹⁵

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 μ L water) of cDNA template for each gene from LPS-stimulated mononuclear cells. Efficiency was calculated by use of the equation $E = [(10^{-1/\text{slope}}) - 1] \times 100$, where E is PCR assay efficiency. Assay efficiency was considered acceptable if it was between 95% and 105%.¹⁴ 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 .¹⁴

Statistical analysis

Data are expressed as mean $\pm$ 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 1-sample *t* test was used to determine whether the mean $2^{(-CT)}$ 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 2-way ANOVA for repeated measures 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 PGE₂ concentration were found to be normally distributed with equal variances and therefore were

^g-Primer Express, Foster City, Calif.

^h-Life Technologies, Grand Island, NY.

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.ⁱ

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). For 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 ($P = 0.987, 0.981, \text{ and } 0.279$, 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 $\mu\text{g/mL}$ 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 differ significantly ($P = 0.853, 0.398, \text{ and } 0.409$, respectively, for COX-2, mPGES1, and cPLA₂) 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 cPLA₂). When cells were incubated with the higher concentration of LPS, the addition of firocoxib reduced mean relative COX-2, mPGES1, and cPLA₂ 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 cPLA₂ 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 cPLA₂ ($P = 0.067$) gene expression.

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

PGE₂ concentration

Cells incubated with the high or low LPS concentration had supernatant PGE₂ concentrations significantly ($P < 0.001$) higher than concentrations for cells incubated with medium alone or medium and firocoxib (Figure 6). Supernatant PGE₂ 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 cPLA₂ 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.¹⁵ These criteria were previously shown to be met for the COX-2 primer set used in the present study¹⁴ and were met for the cPLA₂ and mPGES1 primer sets designed for this study.

Our working hypothesis that firocoxib would downregulate LPS-induced COX-2, mPGES1, and cPLA₂ gene expression was supported by results of the present study in that firocoxib reduced LPS-induced expression of all 3 genes, although the effect was significant only for COX-2 and mPGES1 expression. Other studies^{16–19} 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, 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.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 PGE₂ synthesis in the pathogenesis of diverse disease states including inflammation, ischemia, and neoplasia,^{5,6,8-11,22} and PGE₂ is known to regulate critical steps in tumorigenesis by stimulating cell proliferation and angiogenesis, preventing apoptosis, and inducing migration.⁸ Sixty-four percent of equine melanomas and 86% of equine squamous cell carcinomas express COX-2.²³ Given supportive evidence that COX-2 inhibitors may be protective in some neoplastic diseases in which COX-2 expression is upregulated,^{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 PGE₂ 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.^{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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Appendix

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

Gene	GenBank accession No.	Primer sequence (5' to 3')
COX-2	DQ480158	Forward CAG CAT AAA CTG CGC CTT TTC
		Reverse AGG CGG GTA GAT CAT TTC CA
mPGES1	NM 001081935	Forward CAC GCT GCT GGT CAT CAA GA
		Reverse CTC TCA GGC AAC GCT CCA C
cPLA ₂	NM 001081843	Forward TGG GTC CAA AGA AGA GTG AAG GCT
		Reverse ACA TGT ACC AGC TGG AGC CAG AAA

ABBREVIATIONS

COX-2	Cyclooxygenase 2
cPLA₂	Cytosolic phospholipase A ₂
LPS	Lipopolysaccharide
mPGES1	Microsomal prostaglandin E ₂ synthase 1
PGE₂	Prostaglandin E ₂
RT-qPCR	Reverse transcription quantitative PCR

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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%.

Figure 3—.

Mean relative COX-2 gene expression (fold change compared with expression in cells incubated for 4 hours in medium alone, with expression normalized to expression of 18S ribosomal DNA) in mononuclear cells from 8 horses; cells were incubated in a high (1 $\mu\text{g/mL}$; Hi) or low (1 ng/mL ; Lo) concentration of LPS, with firocoxib (100 ng/mL ; F) or without. Error bars represent SEM. *Values under the bracket were significantly ($P < 0.05$) different.

Figure 4—.

Mean relative mPGES1 gene expression (fold change compared with expression in cells incubated for 4 hours in medium alone, with expression normalized to expression of 18S ribosomal DNA) in mononuclear cells from 8 horses; cells were incubated in a high (1 $\mu\text{g/mL}$; Hi) or low (1 ng/mL ; Lo) concentration of LPS, with firocoxib (100 ng/mL ; F) or without. Error bars represent SEM. *Values under the bracket were significantly ($P < 0.05$) different.

Figure 5—.

Mean relative cPLA₂ gene expression (fold change compared with expression in cells incubated for 4 hours in medium alone, with expression normalized to expression of 18S ribosomal DNA) in mononuclear cells from 7 horses; cells were incubated in a high (1 µg/mL; Hi) or low (1 ng/mL; Lo) concentration of LPS, with firocoxib (100 ng/mL; F) or without firocoxib. Error bars represent SEM.

Figure 6—.

Mean PGE₂ concentration in the supernatant of cultured equine (n = 8 horses) mononuclear cells incubated for 4 hours in medium alone, medium and firocoxib (100 µg/mL; F), medium and a high concentration of LPS (1 mg LPS/mL; LPS Hi), medium and a low concentration of LPS (1 ng LPS/mL; LPS Lo), or combinations of LPS and firocoxib. Error bars represent SEM. Values with different letters were significantly ($P < 0.05$) different.