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Inhibition of microsomal prostaglandin E-synthase-1 (mPGES-1) selectively suppresses PGE₂ in an *in vitro* equine inflammation model

Emily M. Martin^a and Samuel L. Jones^{a,b,*}

^aDepartment of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC, USA

^bComparative Medicine Institute, College of Veterinary Medicine, North Carolina State University, Raleigh, NC, USA

Abstract

Inhibition of prostaglandin E₂ (PGE₂) production effectively limits inflammation in horses, however nonspecific prostaglandin blockade via cyclooxygenase (COX) inhibition elicits deleterious gastrointestinal side effects in equine patients. Thus, more selective PGE₂ targeting therapeutics are needed to treat inflammatory disease in horses. One potential target is microsomal prostaglandin E-synthase-1 (mPGES-1), which is the terminal enzyme downstream of COX-2 in the inducible PGE₂ synthesis cascade. This enzyme has yet to be studied in equine leukocytes, which play a pivotal role in equine inflammatory disease. The objective of this study was to determine if mPGES-1 is a PGE₂-selective anti-inflammatory target in equine leukocytes. To evaluate this objective, leukocyte-rich plasma (LRP) was isolated from equine whole blood collected via jugular venipuncture of six healthy adult horses of mixed breeds and genders. LRP was primed with granulocyte-monocyte colony-stimulating factor (GM-CSF) and stimulated with lipopolysaccharide (LPS) in the presence or absence of an mPGES-1 inhibitor (MF63), a COX-2 inhibitor (NS-398), or a nonselective COX inhibitor (indomethacin). Following treatment, mPGES-1 and COX-2 mRNA and protein levels were measured via qPCR and western blot, respectively, and PGE₂, thromboxane (TXA₂) and prostacyclin (PGI₂) levels were measured in cellular supernatants via ELISA. This study revealed that LPS significantly increased mPGES-1 mRNA, but not protein levels in equine LRP as measured by qPCR and western blot, respectively. In contrast, COX-2 mRNA and protein were coordinately induced by LPS. Importantly, treatment of LPS-stimulated leukocytes with indomethacin and NS-398 significantly reduced extracellular concentrations of multiple prostanoids (PGE₂, TXA₂ and PGI₂), while the mPGES-1 inhibitor MF63 selectively inhibited PGE₂ production only. mPGES-1 inhibition also preserved higher basal levels of PGE₂ production when compared to either COX inhibitor, which might be beneficial in a clinical setting. In conclusion, this work identifies mPGES-1 as a key regulator of PGE₂ production and a PGE₂-selective target in equine leukocytes. This study demonstrates that

*Corresponding author at: North Carolina State University College of Veterinary Medicine, 1060 William Moore Drive, Raleigh, NC 27607, USA. sam_jones@ncsu.edu (S.L. Jones).

Conflicts of interest disclosure

The authors declare no conflict of interest.

mPGES-1 is a potentially safer and effective therapeutic target for treatment of equine inflammatory disease when compared to traditional non-steroidal anti-inflammatory drugs.

Keywords

Prostaglandin E₂; Prostaglandin E-synthase; Leukocyte; Anti-inflammatory; NSAID; Horse

1. Introduction

Leukocytes are a significant source of tissue injury in many equine and human inflammatory diseases. Some of the most potent regulators of injurious leukocyte functions are prostanoids, which are produced at low levels in healthy tissues to maintain homeostasis, but become significantly elevated under inflammatory conditions to orchestrate leukocyte responses. Of these prostanoids, including prostaglandin E₂ (PGE₂), PGF_{2α}, PGD₂, prostacyclin (PGI₂) and thromboxane A₂ (TXA₂), PGE₂ is the most potent inflammatory mediator. PGE₂ plays a major role in osteoarthritis, muscle injury, and in horses is significantly elevated in inflammatory conditions such as joint disease (Bertone et al., 2001; Li et al., 2005; Prisk and Huard, 2003). Thus, many anti-inflammatory therapies in equine athletes target PGE₂. Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit PGE₂ formation by targeting isoforms of cyclooxygenase (COX) enzymes, which are required for synthesis of all prostanoids. COX-1 is constitutively expressed and regulates prostanoid synthesis for platelet aggregation, gastrointestinal (GI) mucosal protection, and renal electrolyte homeostasis. COX-2 is inducible and governs production of prostanoids under inflammatory conditions. Non-selective NSAIDs target COX-1 and COX-2, leading to global reduction of prostanoid production and adverse effects including renal and GI toxicity. COX-2 inhibitors selectively inhibit inflammatory prostanoid synthesis, however are associated with dangerous cardiovascular effects in humans (Sharma and Jawad, 2005) that could translate to equine diseases including laminitis. Thus, more specific and consequently safer therapeutics that selectively inhibit inflammatory PGE₂ production are needed in horses.

Potential PGE₂-specific therapeutic targets are prostaglandin E-synthase enzymes (PGES), which act downstream of COX in the PGE₂ biosynthetic pathway. Cytosolic PGES (cPGES) is a constitutively-expressed PGES isoform that couples with COX-1 to mediate basal PGE₂ synthesis (Tanioka et al., 2000). In contrast, inducible microsomal PGES-1 (mPGES-1) couples with COX-2 for inflammatory PGE₂ production (Murakami et al., 2000). mPGES-1 is essential for development of pain, edema, and leukocyte tissue influx in rodent models (Trebino et al., 2003), and is significantly increased in human orthopedic disease (Li et al., 2005). Furthermore, mPGES-1 inhibition reduces acute and chronic inflammation and demonstrates GI tolerability in animal models (Xu et al., 2008). mPGES-1 has not been evaluated as a PGE₂-specific anti-inflammatory target in horses; thus, the objectives of this study were to characterize mPGES-1 expression and determine if mPGES-1 inhibition selectively decreases PGE₂ production in equine leukocytes. We hypothesized that lipopolysaccharide (LPS) would increase mPGES-1 mRNA and protein production in equine leukocytes, that mPGES-1 is essential for leukocyte PGE₂ production, and that inhibition of

mPGES-1 would selectively reduce leukocyte PGE₂ production without affecting levels of regulatory prostanoids, PGI₂ and TXA₂.

2. Materials and Methods

2.1. Reagents

Experimental reagents were obtained from the following sources: equine recombinant granulocyte-monocyte colony stimulating factor (GM-CSF) was obtained from Kingfisher Biotech (Saint Paul, MN, USA); lipopolysaccharide (LPS) from *E. coli* 055:B5, bovine serum albumin (BSA), sodium dodecyl sulfate (SDS), sodium deoxycholate, NP-40, sodium pyrophosphate, sodium fluoride, phenylmethylsulfonyl fluoride (PMSF), and diisopropylfluorophosphate (DFP) were obtained from Sigma-Aldrich (St. Louis, MO, USA); goat polyclonal anti-mPGES-1, anti-COX-1, and anti-COX-2 antibodies and HRP-conjugated donkey anti-goat secondary antibodies were purchased from Santa Cruz Biotechnologies (Dallas, TX, USA); mouse monoclonal anti-cPGES antibody, MF63, NS-398, and indomethacin were from Cayman Chemical (Ann Arbor, MI, USA); rabbit polyclonal anti-β-Actin antibody and HRP-conjugated anti-rabbit and anti-mouse secondary antibodies were from Cell Signaling (Danvers, MA, USA).

2.2. Equine donors and leukocyte isolation

All procedures were approved by the Institutional Animal Care and Use Committee at North Carolina State University. All horses were fed and housed in similar conditions at the Teaching Animal Unit at the university and deemed healthy. Horses did not receive any anti-inflammatory medications while being used in this study. Leukocytes were isolated as previously described (Eckert et al., 2007). Briefly, 30–60cc of equine whole blood was sterilely collected into heparinized syringes via jugular venipuncture of male and female horses ages 5–25 years. Whole blood settled at room temperature for 1 h and the leukocyte-rich plasma (LRP) supernatant layer was collected for experiments. The predominant leukocyte populations within LRP include innate immune cells such as neutrophils, monocytes, eosinophils, and basophils, and adaptive immune cells such as T-cells. Once LRP was collected, cells remained within their intrinsic plasma and cell numbers and native ratios were not altered, in order to more accurately reflect the unique *in vivo* interactions between leukocyte populations in individual horses.

2.3. Equine leukocyte priming and stimulation

Equine leukocytes in LRP were first primed for 30 min at 37 °C using granulocyte-monocyte colony-stimulating factor (GM-CSF) at a final concentration of 1 ng/mL, which was found to be optimal for cell priming in our experiments (data not shown), or the vehicle control (sterile PBS). Following priming, cells were stimulated with LPS from *E. coli* 055:B5 at a final concentration of 100 ng/mL or vehicle control (sterile PBS) at 37 °C for the indicated time periods. Cell viability was evaluated via trypan blue exclusion and was routinely > 95%.

2.4. RNA isolation and first-strand cDNA synthesis

All RNA isolation materials were obtained from Qiagen (Valencia, CA, USA). Equine leukocytes in 1 ml aliquots of LRP were primed and stimulated as described. RNA was isolated from cells using an RNeasy Mini Kit per manufacturer's protocol with homogenization using a QIAshredder. Two DNase digestions were performed using an RNase-free DNase Set. RNA was then cleaned up using the RNeasy Mini Kit per manufacturer instruction and quantified using a NanoDrop. First-strand cDNA synthesis of equal quantities of RNA was performed using the Superscript III Reverse Transcription System with random hexamers (50 ng/μl) per manufacturer's protocol (Invitrogen, Thermo Fischer Scientific, Grand Island, NY, USA).

2.5. Real-Time PCR

Real-time PCR was performed using a MyIQ Single-Color Real-Time PCR Detection System (Biorad, Hercules, CA, USA). PCR reactions were prepared with 10 ng cDNA, Taqman Gene Expression Master Mix, Taqman primers and probes, and RNase/DNase-free water to a final reaction volume of 25 μl (Invitrogen). Taqman primers and probes were obtained from Invitrogen's proprietary database of pre-designed Taqman Gene Expression Assays for equine mPGES-1 (Ec04321097), COX-2 (Ec03467558) and COX-1 (Ec03469511). Primers and probes for equine cPGES were designed using Invitrogen's proprietary Custom Taqman Assay Design Tool. For all assays, the company identifies the NCBI reference sequence used for primer and probe design, the 25-nucleotide probe binding location, and the amplicon length. In preliminary experiments, all products were run on a 2% agarose gel and visualized using EZ Vision Three DNA Dye (Amresco, Solon, OH, USA) to verify specificity of the PCR product. No-reverse-transcriptase and no-template controls were included to verify the absence of genomic DNA and DNA contamination, respectively. Amplification cycle conditions for all reactions were as follows: 50 °C for 2 min, once; 95 °C for 10 min, once; 95 °C for 15 s, followed by 60 °C for 1 min (with data-collection and real-time enabled), 40 times. Data analysis was performed using the C_t method using equine β_2 Microglobulin (Ec03468699) as the housekeeping gene for normalization. The housekeeping gene was chosen by evaluating expression stability of three common housekeeping genes using protocols established by Radonic et al. (data not shown) (Radoni et al., 2004).

2.6. Cell lysis and western blot

Equine leukocytes in 1 ml aliquots of LRP were primed and stimulated as described. Cells were then lysed using 2X concentrated RIPA lysis buffer (0.2% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate, 2% NP-40, 10 mM sodium pyrophosphate and 100 mM sodium fluoride), containing protease inhibitors (1 mM PMSF, and 1 Roche EDTA-free protease inhibitor cocktail tablet). Cells were placed on ice with agitation for 20 min, sonicated briefly, and centrifuged. Lysate protein concentrations were determined by bicinchoninic acid (BCA) Assay (Thermo Fischer Scientific). Equal concentrations of protein (85–100 μg) were loaded onto precast 4–12% bis-tris gels for electrophoresis (Invitrogen). Protein was transferred to Immobilon-P PVDF Membranes (EMD Millipore, Billerica, MA, USA) and blocked for 1 h at room temperature using 5% skim milk.

Membranes were then incubated overnight at 4 °C with primary antibodies at the following concentrations: mPGES-1 (1:200)ⁱ, COX-2 (1:500)ⁱ, COX-1 (1:500)ⁱ, cPGES (1:2000)^j, β -actin (1:5000). Next, membranes were incubated in appropriate HRP-conjugated secondary antibodies in 5% skim milk for 2 h at room temperature prior to development using Clarity Western ECL Substrate (Biorad). Membranes were visualized using the ChemiDoc MP System, and Image Lab Software was used for image normalization and quantification (Biorad).

2.7. Inhibitor studies

Equine leukocytes in 100 μ L aliquots of LRP were primed for 30 min with 1 ng/mL GM-CSF. Following priming, 100 ng/mL LPS was added to cells simultaneously with various concentrations of selective inhibitors of mPGES-1 (MF63), COX-2 (NS-398), and a non-selective COX inhibitor (Indomethacin), or an inhibitor vehicle control of 0.04% DMSO. Vehicle control did not exert a significant effect in any assay (data not shown). Cells were incubated for 18 h at 37 °C, centrifuged, and supernatant was collected for ELISA. Cell viability was evaluated via trypan blue exclusion and was routinely > 95% following incubation with inhibitors.

2.8. Elisa

Plasma PGE₂, a stable metabolite of TXA₂ (TXB₂), and a stable metabolite of PGI₂ (6-keto-Prostaglandin F_{1 α}) were measured via ELISA per manufacturers protocol (Cayman Chemical). Serum-matrix effects for equine plasma in each ELISA kit were evaluated using serial plasma dilutions. The minimum dilution that did not exert an effect was determined when less than a 20% difference was detected between two consecutive serial dilutions. All samples were prepared, at a minimum, to this dilution using EIA buffer (supplied with ELISA kit) prior to analysis.

2.9. Statistical analysis

Statistical analysis was performed using Sigmaplot Version 12.0 (Systat Software Inc., San Jose, CA, USA). All data achieved equality of variance ($p < 0.05$). Data were assessed for normality via the Shapiro-Wilk test ($p < 0.05$), and data that were not normally distributed were log transformed to achieve normality prior to statistical analysis (noted in figure legends). Log transformed data were back-transformed for presentation, and thus all data are expressed as mean $\pm$ SEM. Statistically significant differences in treatments were determined using one-way analysis of variance (One Way ANOVA) with Holm-Sidak multiple comparisons *post hoc* testing, or via two-tailed *t*-test where noted. A p value < 0.05 was considered statistically significant.

3. Results

3.1. LPS stimulation following GM-CSF priming increases mPGES-1, but not cPGES mRNA in equine leukocytes

mPGES-1 is strongly inducible in most tissue types, but is also constitutively expressed in a number of tissues where it plays a role in normal cellular physiology, including the kidney, gastric mucosa, and spleen (Boulet et al., 2004). A lack of knowledge of PGES kinetics in

equine leukocytes led us to characterize the effect of pro-inflammatory mediator stimulation on mPGES-1 and cPGES mRNA levels in our model. Equine leukocytes were primed with 1 ng/mL GM-CSF followed by stimulation with 100 ng/mL LPS, and mRNA was extracted for real-time PCR. mPGES-1 mRNA was not detectable in GM-CSF-primed or unprimed leukocytes prior to LPS stimulation (data not shown). However, mPGES-1 RNA levels were significantly higher in leukocytes following priming and LPS stimulation when compared to unprimed, control-treated (PBS) cells (Fig. 1A). mPGES-1 mRNA was approximately 15-fold higher than time-matched controls after 2 h of LPS stimulation, and slowly declined over the following 18 h (Fig. 1A). In contrast, cPGES mRNA levels did not change over the 18-h period, regardless of treatment. The kinetics of mPGES-1 mRNA closely mimicked those of COX enzymes in our system. COX-2 mRNA was significantly increased in primed cells by LPS treatment and peaked after 6 h of stimulation, whereas COX-1 mRNA levels remained unchanged (Fig. 1A).

LPS stimulation alone (without GM-CSF priming) significantly increased mPGES-1 and COX-2 mRNA levels over controls after 2 h of stimulation (Fig. 1B). Additionally, GM-CSF priming significantly augmented the LPS-stimulated increase in COX-2 mRNA (Fig. 1B).

3.2. MPGES-1 and cPGES proteins are constitutive in equine leukocytes and are not increased by GM-CSF/LPS treatment

Equine leukocytes were primed with GM-CSF followed by LPS stimulation, and protein was extracted for evaluation via western blot. Surprisingly, we found that mPGES-1 protein was constitutively present in equine leukocytes prior to priming and LPS stimulation (at time 0, T0). Despite significantly increased mPGES-1 mRNA in our system, mPGES-1 protein levels remained unchanged across all treatment groups over 18 h (Fig. 2A and B). In contrast, COX-2 protein levels were significantly increased in leukocytes following priming and 6 h of LPS treatment when compared to controls (PBS only) and GM-CSF treatment alone. These levels remained significantly elevated over the 18-h incubation period (Fig. 2A and C). cPGES and COX-1 protein were constitutive, as both proteins were present at baseline and remained unchanged under all treatment conditions (Fig. 2A).

3.3. GM-CSF priming followed by LPS stimulation significantly increases equine leukocyte extracellular PGE₂ concentrations

To test the hypothesis that mPGES-1 is essential for PGE₂ production in equine leukocytes, we first sought to characterize the effect of GM-CSF priming and LPS stimulation on PGE₂ levels in our model. 100 µl of freshly isolated equine leukocytes were primed with 1 ng/mL GM-CSF followed by stimulation with 100 ng/mL LPS. Supernatants were collected and analyzed for PGE₂ via ELISA. Prior to priming and stimulation, PGE₂ was present in the plasma at a concentration of 265.83 ± 59.63 pg/mL, which is similar to previously reported data in equine whole blood (Brideau et al., 2001). GM-CSF priming alone did not significantly increase PGE₂ plasma concentration at any time point. LPS stimulation in the absence of priming did increase PGE₂ concentration in plasma, but this effect was not significant beyond 6 h of stimulation. However, GM-CSF priming followed by LPS stimulation led to a significant increase in extracellular PGE₂ concentrations by 6 h when compared to time matched controls (PBS only) and primed cells without LPS stimulation.

These levels continued to increase and were significantly elevated throughout the 24-h period of assessment (Fig. 3).

3.4. Simultaneous treatment with LPS and the mPGES-1 inhibitor, MF63, selectively decreases extracellular PGE₂ concentrations in GM-CSF primed equine leukocytes

An important advantage of selective mPGES-1 inhibition, is the ability to block induced PGE₂ production without decreasing synthesis of other prostanoids. Therefore, we evaluated the ability of a selective mPGES-1 inhibitor, MF63 (Xu et al., 2008), to specifically decrease PGE₂ levels in our equine inflammation model. This was compared to the efficacy of both a non-selective COX inhibitor (indomethacin) and a COX-2 selective inhibitor (NS-398).

Equine leukocytes were primed with 1 ng/mL GM-CSF for 30 min, followed by simultaneous application of inhibitors and 100 ng/mL LPS for 18 h. These stimulation conditions resulted in this highest PGE₂ plasma concentration in prior experiments (Fig. 3) and closely mimic clinical situations in which leukocytes have often been exposed to priming agents *in vivo* before treatment can be administered. Supernatant was harvested and analyzed for PGE₂, PGI₂, and TXA₂ via ELISA. We found that MF63, indomethacin, and NS-398 all significantly decreased extracellular PGE₂ concentrations in our model (IC₅₀ = 0.1147 μ M, 0.0159 μ M, and 0.0528 μ M, respectively (Fig. 4)). Maximal concentrations of indomethacin and NS-398 led to more complete inhibition of PGE₂ to 5.24% and 4.66% of controls, respectively, when compared to MF63-mediated PGE₂ inhibition to 12.50% of controls (Fig. 5). MF63 was a selective inhibitor of PGE₂ in our model, as concentrations of 0.1 μ M MF63 and above significantly decreased PGE₂, but not PGI₂, and TXA₂ levels (Fig. 5A). In contrast, indomethacin and NS-398 significantly inhibited all three prostanoids evaluated at inhibitor concentrations of 1 and 0.1 μ M, respectively, and above (Fig. 5B–C).

Shunting of the PGH₂ precursor to other PG synthases has been reported in some models following mPGES-1 inhibition or deletion (Xu et al., 2008). While application of MF63 led to increased PGI₂ and TXA₂ levels to a maximum of 118.63% and 118.50% of controls, respectively, this trend was not statistically significant (Fig. 5A). Thus, we conclude that no significant shunting to other prostanoids was found in our system.

4. Discussion

The aim of this study was to investigate mPGES-1 as a potential novel anti-inflammatory therapeutic target in equine leukocytes. mPGES-1 inhibition significantly and selectively decreased PGE₂ production in our model without decreasing synthesis of other essential prostanoids (Fig. 5A), consistent with reports in human whole blood (Xu et al., 2008). Additionally, this study provides the first evidence that selective mPGES-1 inhibitors developed for human use are selective for equine mPGES-1 and display similar efficacy (IC₅₀ = 0.8 μ M in human whole blood (Xu et al., 2008), 0.11 μ M in equine LRP (Fig. 4A)). While there are currently no mPGES-1 inhibitors commercially-available, there has recently been increasing effort into development of these drugs; we show that these therapies have the potential to translate effectively to equine medicine. *In vivo* rodent studies demonstrate that mPGES-1 inhibition induces dose-dependent analgesia, decreased hyperalgesia, decreased signs of acute and chronic inflammation, and increased gastrointestinal

tolerability compared to NSAIDs (Xu et al., 2008). This data is complemented in mPGES-1 knockout animals, which display decreased pain, edema, and inflammation (Trebino et al., 2003) and a more favorable cardiovascular profile than COX-2 knockout animals (Cheng, 2006). Taken together, our data suggests that mPGES-1 is an excellent candidate for investigation as an effective, PGE₂-specific, anti-inflammatory therapeutic target in horses against which drugs that are currently being developed will be effective.

For this study, we designed an inflammation model utilizing GM-CSF-primed equine leukocyte-rich plasma. This model better represents *in vivo* conditions in horses where individual leukocyte types interact with one another, the plasma environment, and endogenously-released priming factors to mount an inflammatory response. Using this model, we demonstrated that pro-inflammatory stimulation with LPS increases mPGES-1 mRNA in a mixed population of equine blood leukocytes, consistent with data in leukocytes from other species (St-Onge et al., 2007). Additionally, we found that cPGES mRNA is constitutively present in equine leukocytes (Tanioka et al., 2000), and is the first evidence of cPGES expression in blood leukocytes of any species. Interestingly, mPGES-1 mRNA levels peaked slightly before those of COX-2 (Fig. 1A), suggesting independent regulation of mPGES-1 and COX-2 in equine leukocytes and that selective targeting of each might have differing effects *in vivo*.

Interestingly, while COX-2 mRNA and protein levels were coordinately inducible (Fig. 1 and 2), mPGES-1 protein was constitutively expressed in equine leukocytes regardless of stimulation (Fig. 2A–B). Discrepancy in mPGES-1 mRNA and protein levels has been observed previously in human neutrophils (St-Onge et al., 2007), which make up a significant portion of the leukocyte population and provide one of the first lines of defense against infection *in vivo*. Thus, neutrophils might maintain a stable, ready-made source of mPGES-1 protein for rapid PGE₂ production in early immune responses in humans and horses. The mechanism dictating lack of coordinate mPGES-1 mRNA and protein induction remains elusive. Enzymatic activity of multiple intracellular enzymes, including COX, produce reactive oxygen species that damage surrounding intracellular proteins (Morgan and Liu, 2010), potentially accelerating protein degradation and increasing protein turnover in inflammatory states. Thus, we hypothesize that mPGES-1 protein turnover might be accelerated during inflammation, and increased mPGES-1 mRNA transcription functions to quickly replenish mPGES-1 protein stores to maintain rapid PGE₂ production.

In contrast to neutrophils, mPGES-1 protein has been found to be inducible in isolated human monocytes (Mosca et al., 2007). Differences found in our mixed equine leukocyte population may be explained by the high ratio of neutrophils to monocytes in equine whole blood (43–60% vs 0–7%, respectively). Thus, insensitive western blotting techniques might not detect minute increases in mPGES-1 protein contributed by the monocyte population. Additionally, it is possible that both monocytes and neutrophils serve as a ready-made source of mPGES-1 protein in the horse.

Inhibition of PGE₂ production is a major goal of NSAID therapies; however, preservation of basal PGE₂ levels is also critical for maintaining gastroprotection and homeostatic renal function in a clinical setting. In this study, a non-selective COX inhibitor (indomethacin), a

COX-2 selective inhibitor (NS-398), and an mPGES-1 specific inhibitor (MF63) all significantly decreased LPS-induced PGE₂ production. However, both the specific and non-specific COX inhibitors more completely reduced PGE₂ levels (about 95%, Fig. 5) compared to mPGES-1 inhibition alone. Selective mPGES-1 targeting had a slightly reduced inhibitory effect on PGE₂, inhibiting approximately 88% of PGE₂ production (Fig. 5A); this is likely due to COX enzyme function which remains following selective mPGES-1 inhibition, and the ability of COX-1 and COX-2 to couple with constitutive cPGES (Tanioka et al., 2000) and/or mPGES-2 enzymes (Murakami et al., 2003). Thus, this study indicates that inhibition of inflammatory PGE₂ levels with simultaneous preservation of critical homeostatic PGE₂ production is likely more achievable with selective mPGES-1 inhibition compared to COX inhibitors.

Additionally, indomethacin and NS-398 significantly inhibited multiple prostanoids, including PGE₂, TXA₂, and PGI₂, while MF63 specifically inhibited PGE₂ (Fig. 5). Non-specific prostanoid inhibition is associated with unwanted and potentially lethal side effects in horses (Cook and Blikslager, 2014; Marshall and Blikslager, 2011). Inhibition of both COX-1 and COX-2-mediated prostanoid production can cause gastrointestinal inflammation, ulceration and bleeding, renal dysfunction, and disturbances in cardiovascular homeostasis. While coxibs display improved gastrointestinal safety, they pose increased risk of adverse cardiovascular events in humans, possibly as a result of differential effects on the prothrombotic and antithrombotic prostanoids TXA₂ and PGI₂ (Sharma and Jawad, 2005). Consistent with data in other species (Xu et al., 2008), our data supports that mPGES-1 inhibition is selective for PGE₂ in horses, significantly reducing extra-cellular PGE₂ concentrations without altering TXA₂ and PGI₂ levels (Fig. 5). Additionally, mPGES-1 inhibition did not cause significant shunting of the PGH₂ precursor to increase production of other prostanoids in our model, as has occurred in some studies (Xu et al., 2008). In the current study, mPGES-1 inhibition in equine leukocytes led to a significant decrease in PGE₂ only, while TXA₂ and PGI₂ levels remained consistent.

Previous *in vivo* studies have demonstrated that when compared to non-selective and COX-2 selective NSAIDs, pharmacologic inhibition of mPGES-1 is highly tolerated in organ systems that rely on constitutive PGE₂ production for hemostasis, including the gastrointestinal tract (Xu et al., 2008), and the renal system (Salazar et al., 2014). Additionally, knockout of mPGES-1 does not alter vascular homeostasis (*i.e.* thrombogenesis or blood pressure) in mice (Cheng, 2006). Therefore, the side effects of mPGES-1 in these body systems are hypothesized to be minimal in horses. Taken in context with the results of our study, selectivity of PGE₂ inhibition coupled with maintenance of basal PGE₂ levels in equine leukocytes suggests that therapeutic mPGES-1 inhibition in horses would have similar efficacy to traditional and COX-2 selective NSAIDs, but display a safer gastrointestinal, renal, and cardiovascular profile. *In vivo* efficacy and safety studies of MF63 are needed in horses to test this hypothesis.

MF63 is an optimized phenanthrene imidazole inhibitor that is highly potent in human whole blood and displays favorable oral pharmacokinetics in rodents. Following 30 mg/kg oral administration in guinea pigs, MF63 reaches systemic concentrations of 3.0 µM within 1 h, 4.1 µM within 2 h, and remains at 3.2 µM at 6 h post-dosing; these concentrates are well

above the MF63 IC₅₀ for LPS-induced PGE₂ production in human whole blood. Additionally, MF63 becomes even more concentrated (up to 20 µM) in certain organs, such as the brain syntheses (Cote et al., 2007). MF63 also displays 1000-fold selectivity for mPGES-1 over other prostaglandin and PGE synthases (Cote et al., 2007). Interestingly, MF63 is ineffective against native mouse and rat mPGES-1 (Xu et al., 2008), potentially due to differences in size or availability of the mPGES-1 active site between species (Koeberle and Werz, 2015); this has potentially prohibited advancement of phenanthrene imidazole inhibitor studies and clinical trials. Our data is the first to suggest that mPGES-1 inhibitors of the phenanthrene imidazole type are active against equine mPGES-1. There are currently no mPGES-1 inhibitors available for medical use in human or veterinary species, however our data suggests that horses are an excellent model to study these drugs moving forward, and may provide a platform on which to advance bioavailability and safety studies of MF63 and other phenanthrene imidazole derivatives in naturally occurring inflammation models.

In conclusion, this study establishes proof of principle for evaluating mPGES-1 as an anti-inflammatory therapeutic target in horses. This data could lead to exciting, novel therapeutics for inflammatory diseases in horses that avoid the unwanted adverse effects of NSAIDS. Our data also establish that mPGES-1 inhibitors developed for use in humans will inhibit equine mPGES-1, suggesting that they will be effective for clinical use in horses.

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References

- Bertone AL, Palmer JL, Jones J. Synovial fluid cytokines and eicosanoids as markers of joint disease in horses. *Vet Surg.* 2001; 30:528–538. DOI: 10.1053/jvet.2001.28430 [PubMed: 11704948]
- Boulet L, Ouellet M, Bateman KP, Ethier D, Percival MD, Riendeau D, Mancini JA, Methot N. Deletion of microsomal prostaglandin E2 (PGE2) synthase-1 reduces inducible and basal PGE2 production and alters the gastric prostanoid profile. *J Biol Chem.* 2004; 279:23229–23237. DOI: 10.1074/jbc.M400443200 [PubMed: 15016822]
- Brideau C, Van Staden C, Chan CC. In vitro effects of cyclooxygenase inhibitors in whole blood of horses, dogs, and cats. *Am J Vet Res.* 2001; 62:1755–1760. [PubMed: 11703020]
- Cheng Y. Cyclooxygenases, microsomal prostaglandin E synthase-1, and cardiovascular function. *J Clin Invest.* 2006; 116:1391–1399. DOI: 10.1172/JCI27540 [PubMed: 16614756]
- Cook VL, Blikslager AT. The use of nonsteroidal anti-inflammatory drugs in critically ill horses. *J Vet Emerg Crit Care.* 2014; 25:76–88. DOI: 10.1111/vec.12271
- Cote B, Boulet L, Brideau C, Claveau D, Ethier D, Frenette R, Gagnon M, Giroux A, Guay J, Guiral S, Mancini J, Martins E, Masse F, Methot N, Riendeau D, Rubin J, Xu D, Yu H, Ducharme Y, Friesen RW. Substituted phenanthrene imidazoles as potent, selective, and orally active mPGES-1 inhibitors. *Bioorg Med Chem Lett.* 2007; 17:6816–6820. [PubMed: 18029174]
- Eckert RE, Neuder LE, Bell JL, Trujillo JC, Jones SL. The role of p38 mitogen-activated kinase (MAPK) in the mechanism regulating cyclooxygenase gene expression in equine leukocytes. *Vet Immunol Immunopathol.* 2007; 118:294–303. DOI: 10.1016/j.vetimm.2007.06.001 [PubMed: 17614138]

- Koeberle A, Werz O. Perspective of microsomal prostaglandin E2 synthase-1 as drug target in inflammation-related disorders. *Biochem Pharmacol.* 2015; 98:1–15. [PubMed: 26123522]
- Li X, Afif H, Cheng S, Martel-Pelletier J, Pelletier JP, Ranger P, Fahmi H. Expression and regulation of microsomal prostaglandin E synthase-1 in human osteoarthritic cartilage and chondrocytes. *J Rheumatol.* 2005; 32:887–895. [PubMed: 15868626]
- Marshall JF, Blikslager AT. The effect of nonsteroidal anti-inflammatory drugs on the equine intestine. *Equine Vet J.* 2011; 43:140–144. DOI: 10.1111/j.2042-3306.2011.00398.x
- Morgan, MJ., Liu, Z-G. Crosstalk of Reactive Oxygen Species and NF- κ B Signaling 21Nature Publishing Group; 2010103-115
- Mosca M, Polentarutti N, Mangano G, Apicella C, Doni A, Mancini F, De Bortoli M, Coletta I, Polenzani L, Santoni G, Sironi M, Vecchi A, Mantovani A. Regulation of the microsomal prostaglandin E synthase-1 in polarized mononuclear phagocytes and its constitutive expression in neutrophils. *J Leukoc Biol.* 2007; 82:320–326. DOI: 10.1189/jlb.0906576 [PubMed: 17505022]
- Murakami M, Naraba H, Tanioka T, Semmyo N, Nakatani Y, Kojima F, Ikeda T, Fueki M, Ueno A, Oh-ishi S, Kudo I. Regulation of prostaglandin E2 biosynthesis by inducible membrane-associated prostaglandin E2 synthase that acts in concert with cyclooxygenase-2. *J Biol Chem.* 2000; 275:32783–32792. DOI: 10.1074/jbc.M003505200 [PubMed: 10869354]
- Murakami M, Nakashima K, Kamei D, Masuda S, Ishikawa Y, Ishii T, Ohmiya Y, Watanabe K, Kudo I. Cellular prostaglandin E2 production by membrane-bound prostaglandin e synthase-2 via both cyclooxygenases-1 and -2. *J Biol Chem.* 2003; 278:37937–37947. DOI: 10.1074/jbc.M305108200 [PubMed: 12835322]
- Prisk V, Huard J. Muscle injuries and repair: the role of prostaglandins and inflammation. *Histol Histopathol.* 2003; 18:1243–1256. [PubMed: 12973691]
- Radoni A, Thulke S, Mackay IM, Landt O, Siegert W, Nitsche A. Guideline to reference gene selection for quantitative real-time PCR. *Biochem Biophys Res Commun.* 2004; 313:856–862. DOI: 10.1016/j.bbrc.2003.11.177 [PubMed: 14706621]
- Salazar F, Vazquez ML, Mansferrer JL, Mbalaviele G, Llinas MT, Saez F, Arhancet G, Salazar FJ. Renal effects induced by prolonged mPGES1 inhibition. *Am J Physiol Renal Physiol.* 2014; 306:F68–F74. [PubMed: 24197070]
- Sharma JN, Jawad NM. Adverse effects of COX-2 inhibitors. *Sci World J.* 2005; 5:629–645. DOI: 10.1100/tsw.2005.82
- St-Onge M, Flamand N, Biarc J, Picard S, Bouchard L, Dussault AA, Laflamme C, James MJ, Caughey GE, Cleland LG, Borgeat P, Pouliot M. Characterization of prostaglandin E2 generation through the cyclooxygenase (COX)-2 pathway in human neutrophils. *Biochim Biophys Acta (BBA) Mol Cell Biol Lipids.* 2007; 1771:1235–1245. DOI: 10.1016/j.bbalip.2007.06.002
- Tanioka T, Nakatani Y, Semmyo N, Murakami M, Kudo I. Molecular identification of cytosolic prostaglandin E2 synthase that is functionally coupled with cyclooxygenase-1 in immediate prostaglandin E2Biosynthesis. *J Biol Chem.* 2000; 275:32775–32782. DOI: 10.1074/jbc.M003504200 [PubMed: 10922363]
- Trebino CE, Stock JL, Gibbons CP, Naiman BM, Wachtmann TS, Umland JP, Pandher K, Lapointe JM, Saha S, Roach ML, Carter D, Thomas NA, Durtschi BA, McNeish JD, Hambor JE, Jakobsson PJ, Carty TJ, Perez JR, Audoly LP. Impaired inflammatory and pain responses in mice lacking an inducible prostaglandin E synthase. *Proc Natl Acad Sci U S A.* 2003; 100:9044–9049. [PubMed: 12835414]
- Xu D, Rowland SE, Clark P, Giroux A, Cote B, Guiral S, Salem M, Ducharme Y, Friesen RW, Methot N, Mancini J, Audoly L, Riendeau D. MF63 [2-(6-Chloro-1H-phenanthro[9, 10-d]imidazol-2-yl)-isophthalonitrile], a selective microsomal prostaglandin e synthase-1 inhibitor, relieves pyresis and pain in preclinical models of inflammation. *J Pharmacol Exp Ther.* 2008; 326:754–763. DOI: 10.1124/jpet.108.138776 [PubMed: 18524979]

Fig. 1.

LPS stimulation of primed equine leukocytes increases mPGES-1 and COX-2, but not cPGES or COX-1 mRNA levels.

Equine leukocytes were primed with GM-CSF (1 ng/mL) or control for 30 min, followed by stimulation with LPS (100 ng/mL) or control for indicated times. Levels of mPGES-1, cPGES, COX-2, and COX-1 mRNA were assessed via real-time PCR. Treatment designations are structured as priming agent/stimulating agent. (A) mRNA kinetics in GM-CSF/LPS treated leukocytes over 18 h. Data are expressed as mean fold change $\pm$ SEM over time-matched controls (PBS/PBS), $n = 3$. ** $p < 0.001$ and * $p < 0.05$ via two-tailed t -test compared to time-matched controls. (B) mRNA levels in leukocytes primed and stimulated for 2 h with indicated treatments. Data are expressed as in (A), $n = 3$. ** $p < 0.001$ and * $p < 0.05$ compared to enzyme-matched control and GM-CSF/PBS levels, and # $p < 0.05$ compared to enzyme-matched PBS/LPS levels via One Way ANOVA.

Fig. 2.

mPGES-1, cPGES, and COX-1 proteins are constitutive, and COX-2 protein is increased by LPS stimulation of primed equine leukocytes.

Equine leukocytes were primed with GM-CSF (1 ng/mL) or control for 30 min, followed by stimulation with LPS (100 ng/mL) or control for indicated times. Levels of mPGES-1, cPGES, COX-2, and COX-1 protein were assessed via western blot. Treatment designations are structured as in Fig. 1. (A) Representative western blots and, (B–C) quantification of mPGES-1 and COX-2 protein content from three independent experiments. Values are normalized to β-actin loading control and are expressed as mean relative intensity units ± SEM. ** $p < 0.01$ and * $p < 0.05$ compared to time-matched control (PBS/PBS) and GM-CSF/PBS, and # $p < 0.05$ compared to time 0 (T0) samples, via One Way ANOVA.

Fig. 3.

LPS stimulation significantly increases PGE₂ levels in GM-CSF primed equine leukocyte cell supernatants.

Equine leukocytes were primed with GM-CSF (1 ng/mL) or control for 30 min, followed by stimulation with LPS (100 ng/mL) or control for indicated times. PGE₂ levels were assessed via ELISA. Treatment designations are structured as in Fig. 1. Values represent mean PGE₂ production $\pm$ SEM in pg/mL, $n = 6$. * $p < 0.05$ compared to time-matched PBS/PBS and GM-CSF/PBS samples, via One-Way ANOVA. Data at 24 h only were log transformed for analysis, but back transformed for data presentation.

Fig. 4.

MF63, NS-398, and indomethacin decrease PGE₂ in equine leukocyte cell supernatants. Equine leukocytes were primed with GM-CSF (1 ng/mL), followed by stimulation with LPS (100 ng/mL) in the presence of a selective mPGES-1 inhibitor (MF63, A), a selective COX-2 inhibitor (NS-398, B), a nonselective COX inhibitor (indomethacin, C), or vehicle control (0.04% DMSO) for 18 h. PGE₂ production was assessed via ELISA. Data represent % inhibition of PGE₂ production $\pm$ SEM compared to cells stimulated in the presence of the vehicle for each inhibitor (0.04% DMSO), plotted against log₁₀ inhibitor concentration. IC₅₀ values were calculated for MF63 (0.1147μM), NS-398 (0.0528μM), and indomethacin (0.0159μM), $n = 3$.

Fig. 5.

Inhibition of mPGES-1 selectively decreases PGE₂, but not TXA₂ or PGI₂ in LPS-stimulated, GM-CSF primed equine leukocyte cell supernatants.

Equine leukocytes were primed with GM-CSF (1 ng/mL), followed by stimulation with LPS (100 ng/mL) in the presence of (A) an mPGES-1 inhibitor (MF63), (B) a COX-2 inhibitor (NS-398), (C) a nonselective COX inhibitor (indomethacin), or vehicle control (0.04% DMSO) for 18 h. PGE₂, TXB₂ (TXA₂ metabolite), and 6-keto-Prostaglandin F_{1α} (PGI₂ metabolite) levels were assessed via ELISA. Data represent % prostanoid formation ± SEM compared to stimulated cells in the presence of the vehicle for each inhibitor (0.04% DMSO), $n = 3$. ** $p < 0.001$ and * $p < 0.05$ PGE₂ secretion, ## $p < 0.001$ and # $p < 0.05$ TXA₂ secretion, and †† $p < 0.001$ and † $p < 0.05$ PGI₂ secretion compared to prostanoid-matched control, via One-Way ANOVA. In (C), PGI₂ and TXA₂ values were log transformed for analysis and back transformed for data presentation.