ABSTRACT

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Prostaglandins exert pleiotropic effects throughout the body including maintenance of homeostasis and orchestration of inflammatory disease. Specifically, prostaglandin E_2 (PGE_2) is one of the most potent inflammatory mediators and is implicated in inflammatory bowel disease, atherosclerosis, and arthritis. Arthritis is especially important in equine athletes, as it is a leading cause of reduced performance. Nonsteroidal anti-inflammatory drugs (NSAIDs) limit inflammation by inhibiting PGE_2 synthesis, however are also associated with multiple side effects in horses due to non-specific prostanoid blockade. Thus, safer mechanisms of targeting the PGE_2 pathway are needed. We have investigated two novel strategies of targeting the PGE_2 pathway in equine leukocytes, including (1) selective inhibition of PGE_2 production, and (2) selective targeting of PGE receptors.

The first strategy investigated was inhibition of the terminal enzyme in the PGE_2 synthesis cascade, microsomal prostaglandin E synthase-1 (mPGES-1), which mediates elevated levels of PGE_2 in inflammatory states. Inhibition of mPGES-1 led to a concentration-dependent decrease in lipopolysaccharide (LPS)-induced PGE_2 synthesis by equine leukocytes, while levels of other critical prostaglandins, PGI_2 and TXA_2, were unaffected. In contrast, a nonselective NSAID and a COX-2 specific NSAID decreased levels of PGE_2, PGI_2, and TXA_2 and mimicked conditions seen in vivo that elicit deleterious side effects. Thus, our data indicates that mPGES-1 inhibition is selective for induced PGE_2 production in horses and is a potentially safer strategy for decreasing PGE_2 production compared to NSAIDs.

The second novel PGE_2 targeting mechanism evaluated was use of a prostaglandin E

analog, misoprostol, to inhibit leukocyte functions. PGE_2 is highly pleiotropic and can produce anti-inflammatory effects in particular cell types, including leukocytes, which is attributed to E-prostanoid (EP) receptor distribution and signaling. PGE_2 binds to four different EP receptors, including the pro-inflammatory EP1 and EP3, and the anti-inflammatory EP2 and EP4. EP2 receptors are predominantly expressed on leukocytes, and thus mediate the anti-inflammatory effects of PGE_2 on these cells. Misoprostol is a specific EP2, EP3, and EP4 agonist leading to elevation of intracellular cyclic AMP (cAMP) and inhibition of many leukocyte functions. While misoprostol is currently used to treat NSAIDinduced gastrointestinal injury, it has not yet been evaluated as an anti-inflammatory in horses.

In this study, misoprostol pretreatment inhibited equine neutrophil adhesion, chemotaxis, and ROS production in response to multiple stimulants in vitro. Furthermore, application of misoprostol before or after LPS stimulation potently inhibited TNFα, IL-6, and IL-1β mRNA and protein production. The effect of oral misoprostol administration on TNFα production in an ex vivo equine inflammation model was also determined. LPS stimulation of equine leukocytes collected both before and after a single oral dose of misoprostol induced significant TNFα mRNA production that was not significantly affected by oral misoprostol treatment.

In conclusion, multiple novel anti-inflammatory methods of targeting the PGE_2 pathway exist in vitro in equine leukocytes and remain to be explored in vivo. Inhibition of mPGES-1 selectively reduces PGE_2 production while leaving levels of other essential prostanoids intact. This data indicates the mPGES-1 inhibition is a potentially safer route of limiting PGE_2 synthesis in horses when compared to traditional NSAIDs. Additionally, this

work demonstrates that misoprostol inhibits equine leukocyte adhesion, chemotaxis, respiratory burst, and pro-inflammatory cytokine production in vitro. While a single oral dose of misoprostol did not effect ex vivo LPS-stimulated TNFα production, further testing of misoprostol dosing strategies that are effective in human and rodent models remain to be tested.

Identifying Novel Mechanisms of Targeting the PGE_2 Pathway in Horses

by
Emily Gray Medlin Martin

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APPROVED BY:

Samuel L. Jones Committee Chair	Kenneth B. Adler
Shila K. Nordone	Jeffrey A. Yoder

DEDICATION

This dissertation is dedicated to the following people for all that they do and for being who they are: Mary Medlin, Bob Medlin, Meredith Medlin, Nikki Sewell, Meredith Burgess, and most importantly, Tim Martin for loving me and reminding me that I can do anything.

BIOGRAPHY

Emily was born on September 13th, 1987 in Raleigh, NC to a family of NC State Wolfpack fans. Her mother, Mary Medlin, graduated from NC State University in 1975, and her father, Bob Medlin, graduated 2 years later in 1977. Her younger sister, Meredith, is also a 2015 NC State graduate.

Emily grew up in Raleigh riding her beloved horse (who interestingly shares a name with her father). It was Bob (the horse) who instilled in her a love of equine medicine. As such, Emily began attending NC State in 2005 with plans to become a veterinarian. During her time at NC State, Emily developed life-long friendships with fellow NC State students Nikki (Stephenson) Sewell and Meredith Burgess, and began dating a wonderful guy named Tim Martin. In 2009, Emily graduated from NC State with a bachelor's degree in animal science.

Since graduation (7 years later), Emily has been a student at the NC State University College of Veterinary Medicine studying to become an equine veterinarian and researcher. As she has 2 additional years of vet school to complete, it is quite possible that she holds the record for most consecutive years spent as an NC State student in history, with a grand total of 13.

In 2015, Emily married the man of her dreams, Tim Martin, who graduated from NC State with a master's degree in Biomechanical Engineering in 2012. Together, they own a dog named Annie, who is the only member of Emily's family that holds no degrees from NC State. Go Wolfpack!

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CHAPTER I

PGE_2 Pathophysiology and Targeting in Inflammatory Orthopedic Disease: A Perspective on Horses as a Translational Model

Emily G. Martin[1]

[1]Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27607 USA

1. Abstract

PGE_2 is pleiotropic lipid mediator of health and disease in musculoskeletal tissues. PGE_2 is critical for maintaining regular bone metabolism under homeostatic conditions. However in diseases states, PGE_2 mediates inflammation and degradation of equine and human orthopedic tissues including synovial structures, articular cartilage, and bone. Mechanisms of decreasing PGE_2 synthesis effectively limit inflammation in orthopedic disease, yet are associated with adverse effects in horses and humans. Thus, further investigation is needed to reveal novel PGE_2-targeting strategies in both species. Horses have emerged as an excellent translational model of inflammatory orthopedic research. The musculoskeletal systems of horses and humans share many similar anatomical structures, environmental and athletic stressors, and naturally occurring disease pathologies. In this review, we will discuss the overlapping roles of PGE_2 in equine and human orthopedic disease, as well as review current PGE_2-targeting mechanisms and the use of horses as a translational model for inflammatory orthopedic disease research.

2. Introduction

Prostaglandins are lipid mediator molecules that orchestrate diverse paracrine and autocrine signaling mechanisms in multiple tissues. These molecules are formed through a multi-step process of arachadonic acid metabolism that produces five major bioactive prostanoids: prostaglandin E_2 (PGE_2), prostacyclin (PGI_2), prostaglandin D_2 (PGD_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and thromboxane (TXA_2). These prostanoids are synthesized ubiquitously via a cascade of prostaglandin synthetic enzymes within multiple cell types. Different patterns of expression of these synthetic enzymes leads to production of specific prostaglandin profiles that are dependent on the cell type and state of cellular activation. In quiescent cells, small amounts of prostaglandins are produced that serve homeostatic roles in multiple organs, including the gastrointestinal (Ferrer & Moreno, 2010; Mohajer & Ma, 2000), renal (Hao & Breyer, 2008), cardiovascular (Sellers et al., 2010; Swan & Breyer, 2011), hematologic (North et al., 2007), respiratory (Chung, 2005; Knight et al.,1994), reproductive (De Rensis et al., 2012; Rossitto et al., 2014), and musculoskeletal systems (Hikiji et al., 2008). However upon cellular activation, prostanoid production changes dramatically to aid in response to inflammatory insult. PGI_2 is a major mediator of increased blood flow, vasodilation, and subsequent edema formation and pain at sites of tissue injury (Williams, 1979). TXA_2 is associated with vasoconstriction and platelet aggregation, and acts in concert with PGI_2 to maintain vascular homeostasis and blood flow. Inflammatory PGD_2 is produced mainly by mast cells and other leukocytes to orchestrate acute allergic responses (Lewis et al., 1982; Murray et al., 1986). Elevated $PGF_{2\alpha}$ is associated with multiple inflammatory responses and

is detected at increased levels in patients suffering from arthritis (Basu et al., 2001). Finally, of these five native prostanoids, PGE_2 has been shown to be the most potent mediator of inflammation. PGE_2 induces vasodilation, increased blood flow, increased vascular permeability and edema at sites of tissue injury (Morimoto et al., 2014; Raud, 1990). Additionally, PGE_2 promotes tissue influx of innate immune cells (Morimoto et al., 2014), mediates T cell differentiation (Betz & Fox, 1991), elicits fever, and increases nerve sensitization and pain (Saper et al., 2012). Elevated and dysregulated PGE_2 levels are associated with multiple inflammatory conditions, including inflammatory bowel disease (Sheibanie et al., 2007), arthritis (Hikiji et al., 2008; McCoy et al., 2002), atherosclerosis, and thrombosis (Gómez-Hernández et al., 2006; Gross et al., 2007). Interestingly, in addition to acting as a pro-inflammatory mediator, PGE_2 has also been demonstrated to regulate anti-inflammatory response in certain cell types such as leukocytes. PGE_2 dampens innate immune cell chemotaxis (Armstrong, 1995), respiratory burst (Hong, 1996; Talpain et al., 1995), aggregation (Wise, 1996), and cytokine production (Meja et al., 1997). Furthermore, PGE_2 inhibits the activation of Ag-specific T cells (Sreeramkumar et al., 2011), and thus modulates both innate and adaptive arms of the immune response.

Specifically in tissues of the musculoskeletal system, which share many similarities between horses and humans, PGE_2 is implicated in both homeostasis and inflammatory pathology. Under homeostatic conditions, PGE_2 regulates bone remodeling and turnover and anabolic bone responses to mechanical strain and loading (Blackwell et al., 2010; Li et al., 2006). Additionally, PGs have been implicated in the process of bone healing and fracture repair (Blackwell et al., 2010; Xie et al., 2008). However, multiple models have also

established a deleterious role for PGE_2 in inflammatory orthopedic disease. Rodent models implicate PGE_2 in the pathophysiology of rheumatoid arthritis (Kamei et al., 2004; McCoy et al., 2002), bone resorptive disorders (Saegusa et al., 2003; Suzawa et al., 2000), exercise-induced muscle inflammation and damage (Lapointe et al., 2002; Prisk & Huard, 2003), and inflammatory bone loss (Hara et al., 2010; Inada et al., 2006). In humans, PGE_2 has been shown to play a role in degradation of cartilage proteoglycan and synovial inflammation in osteoarthritis (Attur et al., 2008a; Eymard et al., 2014; Hardy et al., 2002; Li et al., 2009), as well as increases mediators of bone metabolism and resorption in human OA chondrocytes (Moreno-Rubio et al., 2010). Importantly, horses have been identified as an excellent translational model for studying human inflammatory orthopedic disorders such as osteoarthritis and osteochondrosis. Equine athletes encounter many of the same mechanical stressors and injuries as human athletes, and naturally develop many of the same orthopedic diseases. Additionally, anatomy of the equine joint shares many similarities with that of humans, including subchondral bone and articular cartilage thickness that allow recapitulation of human orthopedic pathologies (McIlwraith et al., 2011). Thus, horses serve as an important translational model for human musculoskeletal injury and disease. PGE_2 is significantly elevated in joints of horses suffering from both experimental and naturally-occurring arthritis (Bertone et al., 2001; Frisbie et al., 2008; Lucia et al., 2013). Additionally, cartilage and synovial explants from horses with osteoarthritis and subchondral cystic lesions spontaneously secrete higher levels of PGE_2 when compared to healthy tissues and potentially increases osteoclast activity (Rechenberg et al., 2000a; Rechenberg et al., 2000b).

While this data implicates PGE_2 in equine and human orthopedic pathologies, the exact mechanisms of action are not well understood. This review will outline what is known to date about (1) PGE_2 synthesis in orthopedic and inflammatory cells, (2) the role of PGE_2 in homeostasis and the pathophysiology of inflammatory orthopedic disease in humans and horses, (3) mechanisms of targeting the PGE_2 pathway to treat orthopedic pathology, and finally, (4) the utility of horses as translational orthopedic models.

3. PGE_2 Synthesis

Multiple steps are involved in the synthesis, secretion, signaling, and degradation of PGE_2. The enzymes involved in this cascade are common to most cells throughout the body, however are regulated differently to produce distinct prostaglandin profiles within particular tissues under different conditions. This section will give a brief, general overview of the basic steps of PGE_2 synthesis, secretion, signaling, and degradation in all cell types in both horses and humans. Additionally, Figure 1A-B gives an illustration of this general process.

3.1 Arachadonic Acid: The Substrate for Prostaglandin Synthesis.

Prostaglandins are synthesized from polyunsaturated fatty acid precursors, namely arachadonic acid (AA). AA is a highly mobile molecule that is an integral part of cellular membranes, aiding in their fluidity under a range of conditions. Cellular AA originates from dietary sources of polyunsaturated fatty acids, predominantly linoleic acid, which is converted to AA by desaturaes in the liver and transported to cells for incorporation into

cellular membranes (Leslie, 2004; Zhou & Nilsson, 2001). The structure of AA makes it highly susceptible to enzymatic and non-enzymatic interactions with molecular oxygen, leading to oxidative metabolic products such as PGE_2. In addition to the plethora of actions produced by its oxidative metabolites, AA itself exerts a range of effects within cells including activation of leukocyte NADPH oxidase and modulation of ion channels (Brash, 2001).

3.2 Phospholipases

Synthesis of PGE_2 from AA begins with cleavage of AA from cellular membranes of the endoplasmic reticulum, golgi, and nucleus (Funk, 2001). AA is cleaved by multiple classes of phospholipases, which are hydrolase enzymes that utilize water molecules to degrade phospholipids. Specifically, phospholipase A_2 (PLA_2) is the principle phospholipase responsible for liberating AA from cellular membrane phospholipids to act as a substrate for prostaglandin synthesis. In this sense, PLA_2 determines the amount of prostaglandin produced by controlling free AA levels within the cell (Park et al., 2006). Three major classes of PLA_2 exist, including secretory PLA_2 ($sPLA_2$), intracellular Group VI PLA_2 (GVI $iPLA_2$) and Group IV cytosolic PLA_2 (GIV $cPLA_2$). $sPLA_2$ enzymes catalyze calcium-dependent hydrolysis of fatty acids with no apparent fatty acid selectivity. $iPLA_2$ can function independent of calcium levels, and are involved in the "housekeeping" turnover of phospholipids in cellular membranes. In contrast, $cPLA_2$ has 3 different isoforms that are highly specific for AA, and is the only phospholipase that has been demonstrated to play a role in PGE production (Leslie, 2004; Park et al., 2006).

In quiescent cells, $cPLA_2$ is held in an inactive state within the cytosol. Upon cellular stimulation, increased intracellular calcium interacts with the $cPLA_2$ calcium-phospholipid-binding domain and promotes transport of $cPLA_2$ to cellular membranes for AA cleavage activity (Evans et al., 2001). Interestingly, $cPLA_2$ activity can also be induced by phosphorylation by multiple kinases, including mitogen-activated protein kinases (MAPKs) (Lin et al., 1993). It has also been shown that pro-inflammatory mediators, including cytokines, can upregulate $cPLA_2$ synthesis within cells to increase AA release from cellular membranes (Leslie, 2004)**.**

3.3 Cyclooxygenase Enzymes

Once AA is released from cellular membranes, it is funneled through one of multiple intracellular pathways for oxidative metabolism. One of these is the lipooxygenase pathway, which consists of 12-lipooxygenase, 15-lipoixygenase, and 5-lipoxygenase enzymes. Products of the lipooxygenase pathway, namely leukotriene B_4 (LTB_4), are involved in mediating inflammatory responses, leukocyte chemotaxis, and leukocyte activation *in vivo* (Korotkova & Jakobsson, 2014). Funneling of arachadonic acid through a different pathway, the cyclooxygenase (COX) pathway, produces substrates for the synthesis of prostaglandins. The two principle COX enzymes are COX-1 and COX-2. A third COX isoform has also been discovered, COX-3, which is a splice variant of COX-1 and has not been fully characterized (Chandrasekharan et al., 2002). The COX enzymes are predominantly localized to endoplasmic reticulum and nuclear membranes, and each enzyme displays some preference for one location over the other (Jackson et al., 2000; Morita et al., 1995). The principle

actions of COX enzymes are, (1) cyclization and oxidation of arachadonic acid to form the intermediate prostaglandin, PGG_2, and (2) reduction of PGG_2 to form PGH_2. PGH_2 is then used as the substrate for formation of multiple distinct downstream prostaglandins.

The COX enzymes share 61% amino acid homology with one another, however perform distinct functions within the cell (Park et al., 2006). COX-1 is generally recognized as a constitutively expressed enzyme, as the promoter region of COX-1 contains elements consistent with that of a "housekeeping" gene (Tanabe & Tohnai, 2002). COX-1 is ubiquitously expressed at low levels throughout the tissues, and plays homeostatic functions such as modulation of clotting and blood flow, vascular tone, and gastroprotection (Morita, 2002). COX-1 is expressed at high levels in certain tissues, including the kidney, heart, endothelium, platelets, and gastric mucosa, but is largely insensitive to pro-inflammatory stimuli (Jackson et al., 2000; Morita, 2002; Seibert et al., 1997). COX-1 is also inducible in some cell types including endothelial cells (Bryant et al., 1998), osteocytes (Forwood et al., 1998), and synovial cells (Morita, 2002), suggesting a role for COX-1 in pathologic processes in these systems. It has also been suggested that COX-1 plays a role in the resolution of inflammation.

In contrast to COX-1, COX-2 contains promoter sites for enhanced COX-2 synthesis upon cellular activation. COX-2 is generally not expressed in quiescent cells, but is highly induced by various pro-inflammatory stimuli including cytokines, bacterial components, growth factors, hormones, and mechanical stress (Seibert et al., 1997; Tanabe & Tohnai, 2002). This indicates that COX-2 plays a major role in inflammatory prostaglandin

production in multiple tissues. However like COX-1, several tissues also constitutively express COX-2 including the kidney (Tanabe & Tohnai, 2002).

3.4 Prostaglandin E Synthases

The unstable prostaglandin intermediate PGH_2 is converted to five stable prostanoids via prostaglandin synthase enzymes. Prostaglandin synthases are termed terminal synthases, as they are responsible for the final step in the prostaglandin synthesis cascade. PGD synthase converts PGH_2 to PGD_2 to mediate and allergy and inflammation (Lewis et al., 1982; Murray et al., 1986). PGF synthase is responsible for $PGF_{2\alpha}$ production to control bronchial and vascular smooth muscle tone, vasodilation and blood pressure (Basu et al., 2001; Watanabe, 2002). PGI and TXA synthase produce prostacyclin and thromboxane, respectively, to maintain vascular homeostasis through effects such as vasodilation (PGI_2) and vasoconstriction (TXA_2). Finally, PGE synthases convert PGH_2 to PGE_2, which exerts a myriad of pro- and anti-inflammatory effects in multiple tissues. Taken together, it has been shown that all prostaglandins and prostaglandin synthases are critical for maintenance of homeostasis and progression of disease.

There are three distinct isoforms of PGE synthases (PGES) leading to the formation of PGE_2. Cytosolic PGES (cPGES) is a constitutively expressed enzyme that is located predominantly within the cytoplasm and is generally unresponsive to inflammatory stimuli. Evidence suggests that cPGES preferentially couples with COX-1 for homeostatic PGE_2 production. However in some tissues, including the brain, cPGES is upregulated by inflammatory stimuli and might play a role in inflammation in this tissue (Park et al., 2006;

Tanioka et al., 2000). An additional constitutively expressed PGES is microsomal PGES-2 (mPGES-2), which is present predominantly in the brain and heart. mPGES-2 is produced in the golgi membrane before subsequent truncation and release into the cytosol where it can couple with either COX-1 or COX-2 for PGE production (Murakami et al., 2003).

A third isoform of PGES, mPGES-1, is a highly inducible enzyme that couples with COX-2 for enhanced production of PGE_2 during inflammation (Murakami et al., 2000). mPGES-1 is localized predominantly in the nuclear membrane for close association with COX-2, and both are coordinately induced by proinflammatory cytokines and bacterial products (Masuko-Hongo et al., 2004). Knockout or selective inhibition of mPGES-1 has been shown to decrease signs of acute and chronic inflammation in multiple models, indicating that mPGES-1 is essential for inflammatory PGE_2 production in multiple systems (Hara et al., 2010; Kamei et al., 2004; Trebino et al., 2003; Xu et al., 2008). Specifically, mPGES-1 expression is induced by pro-inflammatory mediators in multiple orthopedic tissues and has been implicated in osteoarthritis (Hardy et al., 2002; Kojima et al., 2004; Masuko-Hongo et al., 2004).

3.5 PGE_2 Cellular Release

PGE_2 is primarily synthesized within the endoplasmic reticulum, the cellular location of COX-2 and mPGES-1 enzymes (Morita, 2002). Synthesized PGE_2 can exit the cell via two main mechanisms: simple diffusion and active transport. Simple diffusion of PGE_2 is a relatively slow process when compared to active transport. Active transport of PGE_2 occurs via the multidrug resistance protein 4 (MRP4), which is interestingly inhibited by NSAIDs

and thus presents an additional route through which these drugs decrease extracellular PGE_2 levels (Reid et al., 2003). Both pathways have been shown to play a role in PGE_2 efflux across cellular membranes under homeostatic and inflammatory conditions.

3.6 Prostaglandin E Receptors

PGE_2 targets 4 distinct receptors: EP1, EP2, EP3, and EP4. EP1 signaling increases intracellular calcium and plays a role in pain responses and hyperalgesia (Moriyama et al., 2005). Both EP2 and EP4 couple to adenylate cyclase enzymes within the cell and lead to a robust increase in intracellular cyclic AMP (cAMP). Increased cAMP is associated with many anti-inflammatory properties in leukocytes, including dampening of various effector functions (Armstrong, 1995; Hong, 1996; Meja et al., 1997; Talpain et al., 1995). EP4 has also been implicated in PGE_2-induced bone resorption, with minor contribution from EP2 (Suzawa et al., 2000). The main mechanism of action of EP3 signaling is inhibition of adenylate cyclase function, and in turn, decreased intracellular cAMP. PGE_2-EP3 signaling has been implicated in many of the cardinal signs of inflammation, such redness, edema, and swelling, through actions on mast cells. Signaling through EP3 induces vasodilation, increased vascular permeability, and increased leukocyte influx into tissues (Morimoto et al., 2014).

3.7 PGE_2 Degradation.

15-PG dehydrogenase (15-PGDH) is a prostaglandin degradative enzyme that is ubiquitously present in cells and tissues. 15-PGDH reversibly oxidizes PGE_2 to form the inactive

metabolite, 15-keto PGE_2. Deletion of 15-PGDH results in decreased PGE_2 *in vivo,* indicating the importance of this enzyme in regulating PGE_2 levels (Coggins et al., 2002).

4. Roles of PGE_2 in Homeostasis

Almost every cell within the body is capable of producing PGE_2 to exert pleiotropic effects within tissues. These effects are varied in different cell and tissue types due in part to targeting of distinct EP receptors and variability of tissue PGE_2 concentrations. Many tissues produce constitutive levels of PGE_2 needed to maintain optimal function. Specifically, PGE_2 is involved in maintaining homeostasis of the gut (Dey et al., 2006; Ferrer & Moreno, 2010; Mohajer & Ma, 2000), kidney (Hao & Breyer, 2008), cardiovascular (Swan & Breyer, 2011), hematologic (North et al., 2007), immune (Harizi et al., 2008), respiratory (Chung, 2005; Knight et al., 1994), reproductive (Waclawik, 2011), and musculoskeletal systems (Hikiji et al., 2008). This section will give a brief overview of PGE_2's homeostatic role in each of these tissues.

Gastrointestinal System. In the GI tract, PGE_2 has been implicated in regulation of gastric acid, bicarbonate, and mucous secretion (Ding et al., 1997; Larsen et al., 2005; Takahashi et al., 1999), increasing GI motility (Grasa et al., 2006), and providing additional cytoprotective mechanisms in GI cells (Dey et al., 2006). Inhibition of homeostatic PGE_2 production has been implicated in many of the deleterious GI side effects that are observed with nonspecific

NSAID therapy, including gastric ulceration and colitis in horses (Marshall & Blikslager, 2011).

Kidney. In the kidney, PGE_2 is important for maintaining renal blood flow and glomerular filtration rate in patients with reduced arterial blood volume. In these instances, PGE_2 acts to vasodilate afferent arterioles to maintain adequate glomerular filtration rate and maintain salt balance to modulate blood pressure (Hao & Breyer, 2008).

Vasculature, Hemostasis, and Leukocytes. PGE_2 has been shown to regulate systemic blood pressure as both a vasopressor and vasodepressor, depending on EP receptor expression. However most reports indicate that PGE_2 elicits anti-hypertensive effects (Swan & Breyer, 2011). PGE_2 has been shown to enhance hematopoietic stem cell numbers in zebrafish and mice (North et al., 2007), indicating a proliferative role for PGE_2 in hematopoiesis. PGE_2 also modulate maturation, activation, and MHC II expression on dendritic cells, decrease cytokine secretion by macrophages, inhibits activation and proliferation of T cells, and suppress natural killer cell functions to balance immune responses (Harizi et al., 2008).

Respiratory System. PGE_2 relaxes smooth muscles of the respiratory system and prevents bronchoconstriction induced by multiple agents. PGE_2 also limits airway response to allergens by reducing inflammation and mast cell activation (Chung, 2005; Knight et al., 1994).

Reproduction. PGE_2 plays multiple roles in reproduction, including leutoprotective mechanisms such as progesterone secretion, promotion of uterine function and conceptus development, and increased vascular permeability and angiogenesis upon conceptus implantation (Waclawik, 2011).

Musculoskeletal System. Importantly in the musculoskeletal system, PGE_2 regulates bone remodeling through osteoclast activation and bone resorption, as well as bone-forming activities in osteoblasts. Additionally, PGE_2 induces expression of bone resorbing factors such as receptor activator of NF-κβ ligand (RANKL), IL-6, IL-1β and proteinases, as well as bone-forming agents including bone forming protein-2 and collagen (Hikiji et al., 2008). The roles of PGE_2 in regulating cells of the musculoskeletal system will also be discussed in greater detail below.

5. Roles of PGE_2 in Inflammatory Orthopedic Disease

Inflammatory orthopedic diseases, especially those of the joint, are among the most common and debilitating disorders in horses and humans. While multiple factors contribute to the pathogenesis of inflammatory orthopedic disease, four main etiologic categories exist: trauma/exercise-induced, developmental, infectious, and autoimmune. Trauma or exercise-induced pathologies are one of the most common causes of lameness in horses, and encompasses both acute trauma and repeated load-bearing "wear-and-tear" of the joints over time (Peat & Kawcak, 2015). Developmental joint diseases in both species most

predominantly include disturbances in endochondral ossification, termed osteochondrosis, which can lead to lesion fracture and subsequent fragmentation, synovitis, and joint inflammation (osteochondritis dessicans) (Olstad et al., 2015). Infectious causes of joint disease are numerous, including seeding during systemic bacteremia, spread of pathogens from neighboring infected structures (such as osteomyelitis), or penetrating injury or iatrogenic introduction (Morton, 2005; Shirtliff & Mader, 2002). Autoimmune orthopedic diseases are very rare in horses (Carter et al., 1995), but in humans include diseases such as rheumatoid arthritis. In addition to these categories, laminitis is an inflammatory orthopedic disease of interest in the equine hoof, in which inflammation and PGE_2 production have been proposed to play a role.

In this section, subsection 5.1 will discuss the pathogenesis and pathophysiology of selected examples from each category of orthopedic pathobiology. Following an introduction of selected disorders, subsection 5.2 will review cell types and tissues that contribute to PGE_2 production in these disorders. Finally, subsection 5.3 will focus on the role that PGE_2 plays in the individual pathologic components that contribute to the pathophysiology of these inflammatory orthopedic diseases.

5.1 Review of Select Orthopedic Diseases in Horses and Humans

Before the specific roles of PGE_2 are discussed, we will give a brief overview of the etiology and pathophysiology of selected orthopedic diseases of interest that plague both horses and humans. Additionally, diseases that are of serious concern in just one of either species (including autoimmune joint diseases and laminitis) will also be reviewed.

5.1.1 Trauma/Exercise-Induced Orthopedic Disease: Osteoarthritis.

Osteoarthritis (OA) is the most prevalent arthritic condition in people, affecting nearly 35% of the population over 65 years of age (Scanzello & Goldring, 2012). In horses, lameness associated with OA is one of the major causes of equine wastage, often due to poor performance or a need for early retirement. One study found that as many as 33% of performance horses have OA in at least one joint, which is similar to statistics found in humans (Schlueter & Orth, 2004). OA can affect all synovial joints, and is characterized by loss of articular cartilage integrity and function, formation of osteophytes along the joint margins (or bone spavin in horses), increased remodeling and sclerosis of subchondral bone, joint effusion, inflammation of the synovial membrane (synovitis), pain, and reduced joint mobility (Schlueter & Orth, 2004; Sellam & Berenbaum, 2010). Multiple factors influence the development of OA, most notably abnormal or excessive biomechanical forces exerted on articular cartilage during athletic performance, acute trauma, or abnormal loading due to poor conformation (Schlueter & Orth, 2004). In the following section, we will overview various pathologic processes that contribute to the progression of OA.

Inflammation of soft tissue and bone structures surrounding the joint, including the synovial membrane, subchondral bone, meniscus, tendons, and musculature, heavily contribute to the pathophysiology of OA. The synovial membrane is particularly important in the pathophysiology of OA, as it has critical roles in maintaining joint homeostasis and mediating inflammation and cartilage destruction. The presence and degree of synovitis within a joint has been shown to correlate with the severity of chondropathy in human OA patients and is associated with altered cartilage metabolism and increased production of

proteolytic enzymes, cytokines, prostaglandins, and reactive oxygen species (Ayral et al., 1999; Palmer, et al., 1996; Sellam & Berenbaum, 2010). Synovitis in OA joints is an active inflammatory process, as indicated by inflammatory cell infiltration of the synovial tissues and pro-inflammatory cytokine production. One study found that macrophages made up approximately 65% of synovial inflammatory cell infiltrate, followed by T cells (predominantly CD4+ T cells of the Th1 subtype), which made up approximately 26% (Pessler et al., 2008). Additionally, increased levels of pro-inflammatory cytokines including IL-1β and TNFα have been detected in synovial tissues from OA patients when compared to healthy tissues (de Lange-Brokaar et al., 2012). These cytokines elicit multiple degenerative effects within the joints, including prostaglandin synthesis, cartilage degeneration, bone resorption, angiogenesis, and augmentation and perpetuation of the inflammatory response.

Degeneration and lesion formation within articular cartilage is a hallmark of OA. Major mediators of OA pathophysiology and cartilage destruction include pro-inflammatory cytokines such as IL-1β and TNFα, which are increased in OA affected joints in horses and humans (Kamm et al., 2010; Kapoor et al. 2010). The major cell types that produce IL-1β and TNFα in equine and human OA are activated macrophages, synoviocytes, and chondrocytes (de Lange-Brokaar et al., 2012; Sellam & Berenbaum, 2010; Kamm et al., 2010; Tetlow et al., 2001)**.** Receptors for IL-1β and TNFα (IL-1 receptor type I, and TNF receptor I, respectively) are increased on OA chondrocytes, indicating their importance in modulating cartilage synthesis and degradation in this disease (Kapoor et al., 2010). In both humans and horses, IL-1β is known to inhibit chondrocyte anabolic processes such as collagen and proteoglycan synthesis, as well as induce production of proteolytic enzymes

such as matrix metalloproteinases (MMPs) and ADAMTS (a disintegrin metalloproteinase with throbmospondin motif) (Goldring et al., 1988; Platt & Bayliss, 1994; Saklatvala, 1986; Mengshol et al., 2000; Richardson & Dodge, 2000; Ross et al., 2012; Tung et al., 2002b). Furthermore, IL-1β and TNFα can also stimulate cartilage reactive oxygen and nitrogen species production, which can elicit oxidative stress in chondrocytes and induce MMP production to elicit cartilage degradation (Murrell et al., 1995). IL-1β also upregulates equine and human chondrocyte production of cPLA2, COX-2, and mPGES-1, leading to increased PGE_2 formation within the joint that is associated with cartilage degradation (Farley et al., 2005; Kapoor et al., 2010; Martel-Pelletier et al., 2003; Moses et al., 2001). In addition to direct effects on cartilage, IL-1β and TNFα can also undergo a positive feedback loop by inducing additional synovial synthesis of proinflammatory cytokines IL-6, IL-8, monocyte chemotactic protein-1 (MCP-1), and RANTES that exacerbate synovial inflammation and augment IL-1β and TNFα synthesis (Kapoor et al., 2010).

In addition to the relatively well-defined roles of IL-1β and TNFα, the specific role of induced IL-6 in OA development and cartilage destruction is controversial. Studies have shown that in combination with IL-1β, IL-6 increases synthesis of MMPs and enhances cartilage breakdown (Rowan et al., 2001), as well as reduces type II collagen expression (Poree et al., 2008). Additionally, IL-6 can induce osteoclast differentiation and bone resorption in subchondral bone (Kotake et al., 1996; Steeve et al., 2004). However, IL-6 has also been shown to reduce cartilage destruction in some arthritis models (van de Loo et al., 1997).

In humans, increased subchondral bone remodeling, turnover, and loss are associated with early-stage OA, while sclerosis and densification of subchondral bone and slowing of bone turnover are hallmarks of late-stage disease (Burr & Gallant, 2012). In horses, OA is associated with increased bone remodeling, decreased bone density, and decreased bone volume, as is seen in early human OA (Lacourt et al., 2012). Equine OA alone does not seem to induce an increase in bone density or sclerosis as seen in late stage human OA, however increased training and load on these joints can lead to bone thickening and even pathological sclerosis (Kawcak et al., 2001; 2011). These changes in subchondral bone can play a critical role in OA progression through effects not only on bone integrity, but also on other joint structures. A correlation between subchondral bone changes and cartilage lesions has been established in equine OA, suggesting that cross-talk between cartilage and subchondral bone plays a role in progressive tissue degradation (Lacourt et al., 2012). During cartilage destructive processes, chondrocytes produce pro-inflammatory mediators such as IL-6, TGFβ, PGE_2, and MMPs that stimulate subchondral bone resorption and remodeling (Hayami et al., 2004; 2006) In return, OA subchondral bone produces significantly increased levels of IL-1β, IL-6, TGFβ, and PGE_2 and induces release of glycosaminoglycan from articular cartilage explants (Massicotte et al., 2002; Westacott et al., 1997). This creates a positive feedback loop through which bone and cartilage potentiate inflammation and degradation in OA joints.

Conversely, subchondral bone has also been shown to secrete chondroprotective osteoprotegerin (OPG), indicating that a balance of anabolic and catabolic signals occur between cartilage and bone (Kadri et al., 2008). OPG provides chondroprotective effects by

serving as a decoy receptor for RANKL. RANKL is a transmembrane TNF receptor superfamily protein that is expressed on osteocytes and chondrocytes following pro-inflammatory stimulation. RANKL binding to the RANK receptor on osteoclasts stimulates osteoclast differentiation, maturation, and activation leading to increased bone remodeling. OPG produced by osteoblasts serves as a competitive inhibitor of RANKL binding and modulates its bone-resorptive effects. Interestingly, OPG, RANK, and RANKL proteins are also expressed on chondrocytes and serve a role in mediating cartilage degradation, potentially through signaling on the subchondral bone (Nakashima et al., 2011; Moreno-Rubio et al., 2010).

Changes in bone density have also been implicated in fragmentation or fracture of subchondral bone and its associated cartilage (together, termed osteochondral tissue) in horses that are undergoing strenuous physical activity (Kawcak et al., 2001). These fragments elicit osteoarthritis through stimulation of severe synovitis and direct production of proinflammatory mediators IL-6, high mobility group box protein-1 (HMGB-1), and PGE_2 (Ley et al., 2009; May et al., 1988)**.** Osteochondral fractures are associated with areas of bone resorption and osteoclast activity in equine subchondral bone, however the exact pathogenesis of how osteochondral fragmentation occurs in horses has yet to be determined (Kawcak et al., 2001).

Pannus formation and angiogenesis are also implicated in the pathogenesis of OA and are intimately associated with chronic synovitis. Pannus formation is characterized by fibrous granulation tissue covering the articular surfaces of inflamed joints. Pannus tissues secrete pro-inflammatory cytokines and have been shown to spontaneously produce proteases that

break down cartilage (Furuzawa-Carballeda et a., 2008; Yuan et al., 2004). Pannus can be either hypo- or hyper-vascular, but is commonly associated with robust angiogenesis (Ghosh, 2003). Angiogenesis occurs as endothelial cells are stimulated to proliferate in inflamed OA synovium. Paired with regression of existing vasculature, these processes lead to a new mapping of immature synovial vasculature. Inflammatory cytokines upregulate endothelial adhesion molecule expression on immature vasculature, leading to increased leukocyte tissue infiltration and edema formation (Bonnet, 2005; Elshabrawy et al., 2015). In addition to the synovium, angiogenesis also occurs in the cartilage of OA patients supplied by vasculature in the subchondral bone. Angiogenesis of the cartilage is associated with increased inflammatory cell infiltrate, MMP and ADAMTS expression, and cartilage erosion (Bromley et al., 1985; Burr & Gallant, 2012).

5.1.2 Developmental Orthopedic Disease: Osteochondritis Dessicans (OCD)

Multiple equine and human orthopedic diseases manifest as a result of osteochondrosis. Osteochondrosis is characterized by failure of endochondral ossification during development. While the etiology of osteochondrosis is not completely defined, it believed to occur as a result of reduced vascular supply within epiphyseal cartilage. Epiphyseal cartilage is an area of growth tissue beneath the articular cartilage that is replaced by bone during the ossification front. Repeat or unusual biomechanical loading in developing joints, most predominantly the femoropatellar, tarsocrural, and metacarpophalangeal joints, can disrupt microvasculature and lead to decreased vascular supply, chondrocyte necrosis, and ischemic necrotic lesions within the epiphyseal cartilage during ossification (Olstad et al., 2015; Peat & Kawcak,

2015). Necrotic regions are inferior weight bearing structures when compared to healthy tissue, and thus often result in fracture or collapse of overlying cartilage (McCoy et al., 2013). This can lead to a number of different disorders in growing horses termed juvenile osteochondral conditions (JOCC), and most commonly in athletic animals develops into osteochondrosis dessicans. Osteochondrosis dissecans occurs when a fissure develops in the overlying articular cartilage, dissects into the necrotic endochondral cartilage below, and fragments into the joint to elicit profound synovitis (McCoy et al., 2013; Olstad et al., 2015). In humans, the pathogenesis of OCD has not been elucidated, as end-stage disease is most often observed. However the characteristics of human end-stage disease are common to those seen in older horses and thus are likely similar between species. Many of the advances made in human osteochondrosis research to date have involved horses as a translational disease model.

OCD presents in horses and humans as pain and joint effusion, especially following athletic performance. Early stages of the disease in young horses are characterized by increased collagen II cleavage and elevated levels of cartilage protease pro-MMP-2 within the joint (Laverty et al., 2002; Trumble et al., 2001). However, little evidence for increased cartilage turnover is apparent in older horses *in vivo* and suggests that cartilage degradation occurs primarily in the early stages of osteochondrosis (de Grauw et al., 2006). One factor that is likely to be involved in joint pain and swelling at later stages of OCD is synovitis. Osteochondral fragments within synovial joints are associated with synovial effusion and increased synovial fluid mononuclear cell infiltration and nitrous oxide levels (Carmona et al., 2009; de Grauw et al., 2006). Elevated levels of IL-1β, TNFα, and PGE_2 are also detected

within equine OCD joints and are involved in the induction of synovitis, joint effusion, and pain (May et al., 1988; Owens et al., 1996; Trumble et al., 2001). Furthermore, boney fragments themselves directly produce proinflammatory mediators IL-6 and HMGB-1 *in vitro* and can further promote synovial inflammation (Ley et al., 2009). As previously discussed, synovial inflammation is a major mediator of cartilage breakdown in inflamed joints. As such, degenerative joint diseases such as OA are a common sequela of OCD lesions.

5.1.3 Autoimmune Orthopedic Disease: Rheumatoid Arthritis.

Autoimmune reactivity is very low in equine joints, and thus autoimmune mechanisms are unlikely to play a major role in equine joint disease (Carter et al., 1995). In contrast, rheumatoid arthritis (RA) is a relatively common autoimmune condition in humans that is characterized by inflammation and edema of synovial structures, destruction of cartilage and subchondral bone, and production of autoantibodies such as anti-rheumatoid factor and anti-citrullinated protein antibody (ACPA). While the inciting factors of RA remain elusive, genetics, environmental factors, and certain infectious agents appear to play a role (McInnes & Schett, 2011).

RA synovial inflammation is initiated by activation of the vascular endothelium, increased endothelial expression of adhesion molecules and chemokines, and infiltration of innate and adaptive immune cells. This is accompanied by neoangiogenesis to aid leukocytes in trafficking to synovial tissues, and formation of articular granulation tissue, or pannus. RA granulation tissue is composed of activated fibroblasts and leukocytes that secrete a number

of cytokines and prostaglandins. These mediators activate osteoclast activity, bone destruction, and production of destructive cartilage mediators by synoviocytes (Wright et al., 2014; Sabeh et al., 2010). During the progression of RA, the inflamed synovium becomes hyperplastic and beings to invade neighboring cartilage (McInnes & Schett, 2007). Pro-inflammatory cytokines produced by the synovium stimulate fibroblast-like synoviocytes to produce MMPs that degrade cartilage collagen matrix (Sabeh et al., 2010). Additionally, synovial cytokines promote apoptosis of chondrocytes and destruction of the cartilage surface (McInnes & Schett, 2011). Bone erosion is also a characteristic of RA, and expression of synovial cytokines such as macrophage-colony stimulating factor (M-CSF), RANKL, TNFα, and IL-1β promote osteoclastogenesis, osteoclast activity, and irreversible erosion of subchondral bone into the articular cartilage (McInnes & Schett, 2011).

Immune cells such as neutrophils and macrophages play a particularly important role in the progression of RA. Neutrophils are activated in the pannus and synovial fluid by autoantibody immune complexes, leading to release of neutrophil extracellular traps (NETs), proteases, and reactive oxygen species that elicit further cartilage degradation and oxidative tissue injury (Khandpur et al., 2013; Robinson et al., 1992). RA neutrophils also produce RANKL, which stimulates ostoclastogenesis and bone resorption in RA patients (Chakravarti et al., 2009). Macrophages have also been found in large numbers in the synovial tissues of RA patients, where they produce pro-inflammatory cytokines, reactive oxygen species, proteases, prostaglandins, and present antigens for T cell activation (Haringman, 2005).

The role of the adaptive immune response in RA is not completely defined. T and B cells are present in excess in the joints of patients with RA, and a role for costimulation

between T cells, B cells, and follicular dendritic cells in RA synovium has been demonstrated. Interactions between these cells leads to increased expression of autoantibodies that form immune complexes that activate and amplify destructive immune cell functions (Humby et al., 2009; McInnes & Schett, 2011). Of the T cell subsets present within the RA joint, Th17 cells have been shown to play an important role in RA. Th17 cells produce IL-17, a hallmark Th17 cytokine that induces neutrophil influx and increases pro-inflammatory cytokine and MMP production in synovial tissues. Th17 cells have also been shown to induce RANKL expression on osteoblasts to stimulate osteoclastogenesis and increased bone destruction in RA (Komatsu & Takayanagi, 2015; Miossec et al., 2009). Due to this pivotal role, IL-17 has been shown to be predictive of disease progression in RA (Kirkham et al., 2006).

TNFα has also been shown to be predictive of RA disease progression (Kirkham et al., 2006). TNFα is produced by almost all inflammatory and articular cells within RA joints and stimulates release of cytokines, prostaglandins, MMPs, and reactive oxygen species in monocytes and neutrophils. Additionally, TNFα stimulates endothelial cell expression of adhesion molecules that aid in leukocyte tissue influx. Because of these prominent roles, anti-TNFα therapeutics have been developed that effectively treat pain and inflammation associated with RA. IL-1β shares many of these characteristics with TNFα, and is produced predominantly by monocytes, B cells, chondrocytes, and fibroblasts in RA joints. Furthermore, IL-18 is another critical cytokine in RA that is produced by multiple innate immune cells and endothelial cells. IL-18 leads to T cell differentiation, angiogenesis, and activation of monocytes, neutrophils and natural killer cells (McInnes & Schett, 2007).

5.1.4 Infectious Orthopedic Disease: Septic Arthritis

Septic arthritis in horses and humans can occur as a result of seeding during systemic bacteremia, penetrating trauma, iatrogenic introduction, or the spread of bacterial osteomyelitis. Multiple bacterial pathogens can be involved, the most common of which are *Staphylococcus aureus* in humans and adult horses, and multiple gram-negative bacterial species in foals (Morton, 2005; Shirtliff & Mader, 2002). Once within the joint, bacteria adhere and colonize under low-sheer conditions and are aided by production of extracellular matrix proteins produced in traumatized joints. The result of bacterial colonization is a robust inflammatory response characterized by increased leukocyte infiltration, proteolytic enzyme synthesis and activity, and increased pro-inflammatory cytokine production within the joint (Morton, 2005; Shirtliff & Mader, 2002; Trumble et al., 2001). This response also leads to histologic changes including vasodilation, fibrin accumulation, and pannus formation on articular tissues (Madison et al., 1991).

Bacterial components including peptidoglycan, unmethylated DNA CpG motifs, and alpha-hemolysin are responsible for the robust articular inflammatory response (Deng et al., 1999; Gemmell et al., 1997). These bacterial products induce macrophage and synovial cell production of pro-inflammatory mediators, including IL-1β, IL-6, and TNFα, and promote infiltration and activation of leukocytes. In acute phases, neutrophils are the predominant infiltrating leukocytes within the synovium, and act together with monocytes and macrophages to contain infection (Morton, 2005; Osiri et al., 1998) However during the course of inflammation, these leukocytes elicit tissue damage by adhering to articular cartilage and releasing proteases that degrade cartilage extracellular matrices and stromal

cells (DiCarlo & Kahn, 2011). In cases in which the immune system cannot eliminate the pathogen, an amplified and prolonged inflammatory response occurs and leads to many of the same destructive properties seen in RA. Elevated cytokine levels lead to increased MMP production and subsequent cartilage degeneration, as well as prolonged neutrophil activation, production of reactive oxygen species, and secretion of proteases that can further damage cartilage and subchondral bone (Morton, 2005; Shirtliff & Mader, 2002). In addition to these direct effects on cartilage, pro-inflammatory cytokines orchestrate T cell responses in later stages of the disease, including activation of CD4+ T cells that have been shown to play a destructive role in pathophysiology of septic arthritis (Abdelnour et al., 1994) At advanced stages of septic arthritis, the synovial lining often becomes covered with granulation tissue and hematoma and accumulates fibrin. Additionally, the underlying subchondral bone ranges from sclerotic, to hemorrhagic and necrotic with neutrophilic infiltration if adjacent osteomyelitis has developed (DiCarlo & Kahn, 2011; Morton, 2005).

Physical changes within the joint can also exacerbate the effects of septic arthritis by altering joint biomechanics and function. Joint effusion leads to fluid accumulation in the synovial space, and in horses has been associated with reduced blood flow to synovial structures (Hardy et al., 1996; Morton, 2005). Pannus formation also leads to isolation of bacteria and nonviable tissue on the joint surface to act as a nidus for continued infection and inflammation. Pannus also serves to isolate the synovium to deny access to nutrition and antibiotic therapies (Morton, 2005).

5.1.5 Other Orthopedic Diseases of Interest: Laminitis.

Laminitis is a disease that is unique to hooved animals and is characterized by inflammation, degradation, and breakdown of laminar structures within the hoof. The pathophysiology of laminitis is not completely understood, but has been noted to display many similarities to acute organ injury in human sepsis including local vascular endothelial activation, leukocyte activation and tissue influx, and production of pro-inflammatory mediators that amplify the immune response (Belknap et al., 2009). Laminitis can be divided into multiple phases, including developmental, acute, and chronic stages. Initiation and progression of the disease can be driven by multiple factors such as inflammation, metabolic disturbances, vascular dysfunction, or trauma. For the purposes of this review, we will focus on the contribution of inflammation to the pathogenesis and pathophysiology of equine laminitis.

While the pathophysiologic mechanisms of laminitis have not been full elucidated, multiple experimental laminitis models have been developed in horses to aid in understanding this disease. The two most common laminitis models are carbohydrate overload (CHO) and administration of black walnut extract (BWE). Excessive carbohydrate intake in horses, as occurs in the CHO model, leads to a shift in the hindgut bacterial populations and subsequent bacterial lysis, especially of *streptococcal* species. This is accompanied by a drop in gastrointestinal pH, degeneration of gut epithelial cells, and reduced epithelial barrier function (Milinovich et al., 2008; Weiss et al., 1998). It has been hypothesized that these events lead to an efflux of endotoxin (lipopolysaccharide, LPS) from the damaged gut into systemic circulation, as evidenced by increased plasma endotoxin levels following experimental carbohydrate overload. Circulating LPS can activate platelets,

leukocytes, and vascular endothelial cells through the p38 MAPK pathway, leading to increased leukocyte-endothelial adherence and vascular disturbances that may contribute to the pathogenesis of laminitis (Bailey et al., 2009; Brooks et al., 2009). Induction of p38 MAPK is also essential for equine leukocyte pro-inflammatory cytokine synthesis and chemotaxis, and thus LPS could also stimulate pro-inflammatory leukocyte effector functions in laminar tissues (Eckert et al., 2007; Neuder et al., 2009). Equine keratinocytes, major cellular components of the equine laminar structure, have also been shown to be highly responsive to gram negative bacterial components such as LPS by releasing the pro-inflammatory cytokines IL-1β, IL-6, and CXCL8. Interestingly, this study also established that keratinocytes are not responsive to gram-positive bacterial components, indicating a potentially distinct role for different bacterial toxins in the pathogenesis of laminitis (Leise et al., 2010). Black walnut extract has also been shown to induce experimental laminitis, however the mechanism through which this happens is less well defined. Studied have shown that black walnut can induce formation of reactive oxygen species in water and activate caspase and cytokine production in equine monocytes (Hurley et al., 2011a; 2011b), which potentially play a role.

The histopathologic progression of laminitis generally includes a loss of laminar collagen and laminin, degradation of the endothelial basement membrane, and accompanying laminar edema, hemorrhage, red blood cell and thrombus vascular occlusion, and leukocyte tissue influx (Hood et al., 1993; Visser & Pollitt, 2011). In the CHO model, increased leukocyte influx has been shown to correlate with the onset of lameness during the progression of laminitis, and thus this event has been hypothesized to play a role in laminar

breakdown and separation of epithelial cells from the basement membrane (Faleiros et al., 2010). This is further supported by the progression of laminitis in the BWE model, in which signs of leukocyte infiltration and laminar stress precede signs of laminar epithelial breakdown (Faleiros et al., 2009). Influx of leukocytes into tissues begins with activation of the vascular endothelium by mediators such as LPS and damage associated molecular patterns (DAMPs). LPS and DAMPs upregulate laminar endothelial adhesion molecule expression and chemoattractant mediator production, leading to leukocyte homing, arrest, and extravasation into laminar tissues (Fiuza, 2002; Fries et al., 1993; Loftus et al., 2007). Once within the tissues, leukocytes produce a battery of pro-inflammatory mediators including reactive oxygen species, pro-inflammatory cytokines IL-1β, IL-6, IL-8, and increased COX-2 expression that damage tissues and augment inflammatory cell influx. In addition, activated endothelial and epithelial laminar cells contribute to the production and accumulation of pro-inflammatory cytokines within the lamina to further drive inflammation and disease progression (Belknap et al., 2007; Leise et al., 2010; Loftus et al., 2007; Yin et al., 2009).

It has been proposed that leukocytes themselves are the drivers of basement membrane breakdown through production of degradative enzymes including MMP-9 (Loftus et al., 2009), ADAMT-4 (Coyne et al., 2009), myeloperoxidase (Riggs et al., 2007), and neutrophil elastase (la RebiÃ re de Pouyade et al., 2010). However this theory is controversial, as some groups have shown that influx of leukocytes occurs simultaneously with expression of stress-related proteins in endothelial cells at the onset of lameness, and thus could be caused by other inciting factors (Faleiros et al., 2010) Therefore, the exact

involvement of leukocytes in inducing laminar tissue breakdown has yet to be fully elucidated.

Additional mechanisms that potentially contribute to laminar breakdown include dysregulation of adhesion molecule expression and activity, apoptosis of laminar cells, oxidative tissue injury, and changes in the laminar vasculature. Integrin adhesion molecules attach epithelial cells of the stratum internum of the hoof wall, to the basement membrane of the laminar corium of the distal phalanx. Integrin expression is disregulated in response to proinflammatory mediators, and is specifically down regulated on epithelial cells in response to PGE_2 (Noguchi et al., 2000). The loss of integrin expression can lead to breakdown and eventual separation of the laminar structures. Apoptosis could also play a role in basement membrane breakdown, as it has been shown that laminar epithelial cells and keratinocytes undergo increased apoptosis during the course of laminitis (Faleiros et al., 2004). Equine laminar tissues are also exquisitely sensitive to oxidative injury, as these tissues lack superoxide dismutase (SOD). SOD is a potent antioxidant that decreases the destructive effects of leukocyte reactive oxygen species (Loftus et al., 2010). Increased reactive oxygen products have been identified in the black walnut, but not starch-overload models of laminitis, and thus their role in the pathophysiology of naturally occurring laminitis has yet to be determined (Burns et al., 2011; Yin et al., 2009). Vascular changes have also been implicated in laminitis progression in multiple models. Developmental stages of laminitis are characterized by vasoconstriction, decreased blood flow, and increased vascular pressure. This increase in pressure is associated with formation of laminar edema and potentially leads to pressure ischemia of the tissues (Eades et al., 2006; Eaton et al., 1995). Multiple

vasoconstrictive compounds have also been identified in laminar tissue including 5-hydroxytyptamine, which can be released by activated platelets to play a role in vascular dysfunction and ischemia (Bailey et al., 2009). The endothelium can also directly secrete vasoconstrictors such as endothelin-1, leading to enhanced vasoconstriction and decreased blood supply (Peroni, 2005). It is also possible that an inability of laminar tissues to promote vasodilatory effects could play a role in development of decreased blood flow in laminitis.

5.2 Induction of PGE_2 synthesis in Orthopedic Cells and Tissues

Throughout the course of these inflammatory orthopedic diseases, PGE_2 is produced by a number of cells in response to pro-inflammatory cytokines, DAMPS, and bacterial products. Here, we will discuss the specific cell types and mechanisms of cellular activation that contribute to PGE_2 production in orthopedic tissues.

Multiple tissues contribute to the production of PGE_2 in orthopedic disease, including cartilage, meniscus, synovial membrane, and osteophytic fibrocartilage (Hardy et al., 2002). Individual cell types that play a role in PGE_2 production in humans include synoviocytes (Kojima et al., 2002; Stichtenoth et al., 2001; Westman et al., 2004), synovial fibroblasts (Hardy et al., 2002; Westman et al., 2004), chondrocytes (Hardy et al., 2002; Kojima et al., 2004; Li et al., 2005; Masuko-Hongo et al., 2004; Shimpo et al., 2009), tenocytes and tendon stem cells (Zhang et al., 2013)**,** osteoblasts (Inada et al., 2006; Saegusa et al., 2003)**,** and infiltrating immune cells such as monocytes and synovial macrophages (Hardy et al., 2002; Westman et al., 2004)**.** Specifically in horses, there is evidence that multiple orthopedic cells

and tissues produce PGE_2 in response to pro-inflammatory stimulation, including cartilage and cartilage chondrocytes (Briston et al., 2009; Moses et al., 2001; Rechenberg et al; 2000b; Ross-Jones et al., 2015; Takafuji et al., 2002; Byron et al., 2008; Landoni et al., 1996; May et al., 1991; Tung et al., 2002b), and synovium and synoviocytes (Rechenberg et al., 2000b; Ross-Jones et al., 2015; Briston et al., 2009; Byron et al., 2008; Frean, 1999). Production of PGE_2 in these cells and tissues is mediated through upregulation of enzymes involved in the PGE_2 synthesis cascade. cPLA2 expression is highly upregulated by pro-inflammatory mediators in chondrocytes and synovial fibroblasts (Hulkower et al., 1994; Leistad et al., 2011). Additionally, COX-2 and mPGES-1 are highly expressed in infiltrating leukocytes, chondrocytes, synovial lining cells, fibroblasts, fibroblast-like cells, and vascular endothelial cells that play a critical role in the development of inflammatory orthopedic disease. (Hulkower et al., 1994; Kojima et al., 2004; Korotkova & Jakobsson, 2011; Westman et al., 2004).

Interestingly, diseased orthopedic cells and tissues produce increased levels of PGE_2 both at rest, and following pro-inflammatory stimulation. Cartilage explants from human patients with OA produce greater amounts of COX-2, mPGES-1, and PGE_2 at rest than do healthy tissues (Amin et al., 1997). Additionally, COX-2 expression can also be induced in multiple OA cell types following pro-inflammatory cytokine stimulation (Hardy et al., 2002; Kojima et al., 2004; Li et al., 2005; Masuko-Hongo et al., 2004; Shimpo et al., 2009). Furthermore, human RA synovial cells including synoviocytes, synovial macrophages, and synovial fibroblasts produce increased levels of COX-2, mPGES-1, and PGE_2 with and without pro-inflammatory stimulation (Kojima et al., 2002; Stichtenoth et al., 2001;

Westman et al., 2004). In tendons, pro-inflammatory cytokines, mechanical loading, and wounding can upregulate COX-1, COX-2, mPGES-1, and PGE_2 levels, and EP3 receptor genes in tenocytes and tendon stem cells (Eliasson et al., 2012; Y. Zhang et al., 2013). Mechanical loading has also been demonstrated to increase COX-2 and mPGES-1 mRNA, and PGE_2 levels in mouse cartilage explants (Gosset et al., 2008).

Multiple pro-inflammatory mediators induce expression of enzymes involved in the PGE_2 synthesis cascade, including LPS (Briston et al., 2009; Frean, 1999; Landoni et al., 1996; Lucia et al., 2013; Moses et al., 2001), IL-1β (Farley et al., 2005; Kojima et al., 2004; Takafuji et al., 2002), TNFα (Kojima et al., 2004), IL-17 (Faour et al., 2003), and mechanical stimulation and trauma (Gosset et al., 2008). A positive-feedback loop also exists in which PGE_2 itself can increase the stability of COX-2 mRNA and expression of mPGES-1 to induce additional PGE_2 production (Faour, 2001; Kojima et al., 2004). Many of these mediators induce expression of PGE_2-synthetic enzymes through the p38 MAP kinase (MAPK) pathway. p38 MAPK activation is required for upregulation and stabilization of COX-2 mRNA, induced COX-2 protein expression, and PGE_2 production in response to LPS-induced toll-like receptor 4 (TLR4) signaling in equine leukocytes (Eckert et al., 2007; Saklatvala et al., 2004). In embryonic murine chondrocytes, p38 MAPK is also required for LPS-induced upregulation of COX-2. Furthermore in osteoblasts, LPS-TLR4 signaling induces COX-2 and mPGES-1 production and results in inflammatory bone loss in mouse disease models (Inada et al., 2006; Saegusa et al., 2003).

In addition to bacterial components, pro-inflammatory cytokines such as IL-1β are also potent inducers of p38 MAPK activity and COX-2 and mPGES-1 expression. IL-1β

activates p38 MAPK in human osteoarthritic chondrocytes, leading to increased NF-κβ activity, COX-2 and mPGES-1 mRNA and protein synthesis, and PGE_2 production (Shimpo et al., 2009). Additionally, IL-1β has also been shown to induce COX-2 and mPGES-1 expression and increase PGE_2 production specifically in equine chondrocytes (Farley et al., 2005). Furthermore, PGE_2 has been demonstrated to augment IL-1β-induced mPGES-1 expression in chondrocytes, suggesting that PGE_2 forms a positive feedback loop in cartilage (Shimpo et al., 2009). In synovial fibroblasts, IL-1β increases COX-2 mRNA stability and induces COX-2 mRNA and protein expression through p38 MAPK signaling (Faour, 2001). In addition to p38 MAPK, the protein tyrosine kinase Src has also been shown to mediate IL-1β-induced COX-2 mRNA production and PGE_2 synthesis is mouse osteoblasts (Park et al., 2004).

Additional cytokines that induce PGE_2 formation in orthopedic cells include TNFα and IL-17. In bovine chondrocytes, TNFα induces PGE_2 production by increasing COX-2 protein activity (Morisset et al., 1998). In human chondrocytes, IL-17 induces expression of COX-2 through a p38 MAPK and NF-κβ-dependent mechanism (Shalom-Barak et al., 1998). Additionally, IL-17 utilizes stress-activated protein kinase-2 (SAPK2) to increase COX-2 mRNA production and stabilization in chondrocytes, synovial fibroblasts, and macrophages (Faour et al., 2003).

5.3 Role of PGE_2 in Pathophysiologic Processes of Orthopedic Disease

Evidence for the involvement of PGE_2 in inflammatory orthopedic disease is present in current treatment regimens utilized for these patients. Therapeutics that inhibit PGE_2

production, such as COX inhibitors, effectively limit pain and multiple signs of inflammation in these disorders. In equine models of arthritis, COX inhibitors decrease PGE_2 synovial fluid concentrations, synovial edema, leukocyte tissue influx, and lameness when compared to control-treated horses (Morton et al., 2005; Owens et al., 1996). In addition, deletion or inhibition of mPGES-1 in rodent models inhibits PGE_2 production and reduces the severity of experimentally induced arthritis and pain (Kamei et al., 2004; Trebino et al., 2003; Xu et al., 2008). Taken together, this indicates that PGE_2 plays a prominent role in the development of inflammatory orthopedic disease in multiple species. In this section, we will explore the known roles of PGE_2 in specific pathophysiological processes that are implicated in equine and human inflammatory orthopedic diseases discussed in section 5.1.

5.3.1 Synovial Inflammation and Cytokine Production

Synovitis has been implicated in the etiology and development of multiple arthritides. Evidence for the role of PGE_2 in synovitis progression comes from mPGES-1 knockout mice, which demonstrate decreased synovial inflammation and leukocyte tissue infiltration in arthritis models (Trebino et al., 2003). Cartilage breakdown products in arthritic joints, such as low molecular weight hyaluronic acid (LMW HA), tenascin-C, and biglycan, serve as pro-inflammatory DAMPSs that activate synoviocytes and incite inflammation. DAMPs directly simulate toll-like receptor 2 (TLR2) and TLR4 signaling on synoviocytes, monocytes, and macrophages, leading to enhanced NF-κβ activity, expression of cPLA2, COX-2, and mPGES-1 enzymes, and subsequent PGE_2 synthesis within the synovium (Bondeson et al., 2006; Frean, 1999; Landoni et al., 1996; Midwood et al., 2009; Moses et al., 2001; Schaefer

et al., 2005; Sokolowska et al., 2015; Sommerfelt et al., 2015; Van Loon et al., 2010). In addition, signaling through TLR2 and TLR4 on synovial macrophages also induces production of pro-inflammatory cytokines such as IL-1β, TNFα, and IL-6 which further induce PGE_2 synthesis within the synovial tissues (Benito, 2005; Eymard et al., 2014; Schaefer et al., 2005). PGE_2 produced by the synovium can act in an autocrine manner on fibroblast-like synoviocytes to induce additional pro-inflammatory cytokine production and create a positive-feedback loop of PGE_2 and pro-inflammatory cytokine synthesis (Eymard et al., 2014; Martel-Pelletier et al., 2003). Moreover, as PGE_2 has been shown to elicit cartilage breakdown, PGE_2 further contributes to DAMP production and activation of synovial cells to promote additional PGE_2 and pro-inflammatory cytokine synthesis. Taken together, the pro-inflammatory actions of PGE_2 act in concert with cytokines to drive synovitis and exacerbate inflammation within the synovium.

5.3.2 Effusion

Joint effusion is caused by edema produced in inflamed synovial tissues that results in accumulation of exudate in the synovial space. In human patients, joint effusion is highly correlated with the presence of synovitis and OA within synovial joints (Ayral et al., 1999; Roemer et al., 2010). As previously discussed, inflammation within the synovium leads to increased production of PGE_2, which is a critical mediator of edema and swelling. PGE_2 is strikingly elevated in the synovial fluid of horses with OA (Gibson et al., 1996; Kirker-Head et al., 2000), and is correlated with increased joint effusion in canine OA (Trumble et al.,

2004). Additionally, deletion of mPGES-1 reduces edema formation in RA models (Trebino et al., 2003).

As demonstrated in mouse models, PGE_2 mediates edema formation by inducing vascular hyperpermeability and neutrophil extravasation in an EP3-dependent manner (Claudino, 2006; Morimoto et al., 2014). This has been specifically linked to EP3 receptors on mast cells. Mast cell PGE_2-EP3 receptor signaling leads to PI3K activation and a PLC-dependent increase in intracellular calcium, which induces mast cell degranulation, histamine release, IL-6 secretion, and subsequent vascular hyperpermeability (Morimoto et al., 2014). In addition, PGE_2 signaling through EP2 and EP4 receptors in rodent ear tissue has been shown to increase intracellular cAMP and leads to increased local blood flow and vascular diameter (Omori et al., 2014).

5.3.3 Angiogenesis

Angiogenesis is closely associated with chronic joint inflammation and is characterized by growth of new vasculature from an existing vascular network. Angiogenesis involves degradation of the basement membrane in existing vasculature, detachment, migration, and proliferation of activated endothelial cells, formation of new capillary tubes and basement membranes, and anastomosis of new vessels (Bonnet, 2005). This process is aided by multiple factors, including prostaglandins, proteolytic enzymes, and growth factors. To begin the process, activated endothelial cells secrete MMP enzymes to degrade existing basement membrane and induce release of angiogenic factors that promote endothelial migration. PGE_2 has also been shown to induce proteolytic MMP expression in certain cell types, including

murine cementoblasts, which are collagen producing dental cells that share similar functions with fibroblasts and osteoblasts (Sanchavanakit et al., 2015). Thus, PGE_2 could potentially play a role in breakdown of the vascular basement membrane during angiogenesis.

Multiple growth factors are also necessary for development of new vasculature, including platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF). Endothelial derived PDGF attracts supporting cells, such as pericytes, to newly forming vasculature for integration into the basement membrane (Bonnet, 2005). VEGF is a critical regulator of endothelial cell proliferation and migration, and has been shown to induce COX-2 expression and PGE_2 synthesis. In return, PGE_2 induces production of VEGF through EP2-cAMP-PKA activation in fibroblasts, osteoblasts, and monocytes, thus establishing a PGE_2-VEGF positive feedback loop (Ben-Av et al., 1995; Harada et al., 1995; Hoper et al., 1997; Tsujii et al., 1998). Increased VEGF production induces endothelial cells to form three-dimensional tube-like structures *in vitro,* which is a critical step in angiogenesis (Tsujii et al., 1998). PGE_2 can also directly act on endothelial cells to induce tube formation during angiogenesis, as evidenced by increased numbers of intracellular junctions formed between PGE_2-stimulated endothelial cells *in vitro* (Tamura, Sakurai, & Kogo, 2006). Taken together, this indicates that PGE_2 plays a critical role in multiple pathways that mediate angiogenesis. This is further evidenced in mPGES-1 knockout mice that demonstrate reduced angiogenic responses *in vivo* (Kamei et al., 2004).

5.3.4 Pannus Formation

Pannus formation is a common sequela to joint inflammation in multiple arthritides. Pannus is a non-osseous, fibrous-like granulation tissue that covers joint surfaces and can invade nearby structures. In RA and OA, this tissue is made up of macrophages, fibroblast-like cells, and other inflammatory cells that originate at the junction of the synovium and cartilage (Furuzawa-Carballeda et al., 2008; Shibakawa et al., 2003). Pannus tissue is often hypervascular and expresses multiple pro-angiogenic factors (Ghosh, 2003), and has been shown to produce elevated levels of pro-inflammatory cytokines such as TNFα, IL-1β, and IL-8, and spontaneously produce collagenolytic enzymes such as MMPs that elicit articular cartilage destruction (Yuan et al., 2004).

PGE_2 has been shown to play a critical role in pannus and granulation tissue formation *in vivo*. COX-2 and mPGES-1 are upregulated in murine granulation tissues and are associated with increased levels of PGE_2 that preceded angiogenesis and pannus formation. Furthermore, knockout of mPGES-1 reduces granulation tissue formation, tissue angiogenesis, and macrophage infiltration in mouse models (Kamei et al., 2004). Lastly, PGE_2 has also been shown to increase levels of VEGF and histidine decarboxylase (HDC, an enzyme required for synthesis of histamine), both of which are critical to formation of granulation tissue and angiogenesis (Ghosh, 2003; Kamei et al., 2004).

5.3.5 Cartilage Metabolism

PGE_2 is a potent mediator of chondrocyte metabolism. Arthritic cartilage explants spontaneously produce COX-2 and mPGES-1 proteins, leading to increased cartilage PGE_2

production *ex vivo* (Amin et al., 1997; Kojima et al., 2004). Additionally, pro-inflammatory mediators such as IL-1β, TNFα and bacterial LPS are potent inducers of COX-2 and mPGES-1 expression and subsequent PGE_2 synthesis in equine and human chondrocytes (Briston et al., 2009; Byron et al., 2008; Kojima et al., 2004; Takafuji et al., 2002; Tung, Fenton, et al., 2002b). TLR2 and TLR4 are increased in OA cartilage lesions, and stimulation via LPS or DAMPS enhances chondrocyte PGE_2 synthesis through MAPK and NF-κβ activation (H. A. Kim et al., 2006).

Deletion of mPGES-1 reduces proteoglycan loss in mouse models of RA, suggesting a catabolic role for PGE_2 in cartilage matrix regulation (Trebino et al., 2003). PGE_2-EP4 receptor signaling on cartilage tissue inhibits synthesis of extracellular matrix components such as proteoglycan and collagen, and induces type II collagen and aggrecan fragmentation by increasing MMP-13 and ADAMTS-5 activity. PGE_2 can also augment the catabolic activity of IL-1β on cartilage tissue by enhancing IL-1β-induced MMP-13 synthesis and inhibiting synthesis of matrix proteoglycan (Attur et al., 2008). PGE_2 itself can also induce IL-1β expression in chondrocytes to amplify inflammation and cytokine-induced cartilage destruction (Martel-Pelletier et al., 2003). In OA chondrocytes, PGE_2 also enhances RANKL expression and transport to cell membranes in an EP2 and EP4-dependent manner, which subsequently stimulates RANK receptors on osteoclasts to induce subchondral bone resorption (Martínez-Calatrava et al., 2012). Interestingly, PGE_2 has also been shown to enhance chondrocyte synthesis of OPG, an inhibitor of RANKL function, however to a smaller magnitude compared to RANKL (Moreno-Rubio et al., 2010).

PGE_2 has also been shown to mediate chondrocyte apoptosis, which is critical to the pathogenesis of osteoarthritis and other degenerative diseases of the joint. One of the major mediators of chondrocyte apoptosis is nitric oxide (NO), which induces apoptosis through caspase 3, MAPK, and NF-κβ activation (Notoya et al., 2000; Pelletier et al., 2000). NO also stimulates PGE_2 production in chondrocytes, which in turn augments NO-induced chondrocyte apoptosis (Notoya et al., 2000). PGE_2 can also stimulate chondrocyte apoptosis directly in a cAMP-dependent manner (Miwa, 1999).

Interestingly, there is also evidence that PGE_2 can also exert anabolic effects on articular cartilage. PGE_2 has been shown to inhibit collagenase production in synovial fibroblasts in a cAMP-PKA dependent manner (DiBattista et al., 1994). Additionally, PGE_2 increases levels of cartilage glucocorticoid receptors that are normally reduced in OA patients and have been shown to aid in maintaining cartilage homeostasis (DiBattista et al., 1991). Additional studies in equine chondrocytes have shown that PGE_2 reduces IL-1β-induced MMP-1, MMP-3, and MMP-13 expression (Tung et al., 2002a). Furthermore, the PGE_1 analog, misoprostol, has been shown to reverse the inhibitory effects of IL-1β and NSAIDS on GAG synthesis in human chondrocytes (Dingle, 1993). However, in concert with reduced MMP expression in some models, PGE_2 has also been shown to reduce tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) expression, which is a critical endogenous inhibitor of MMP function (Tung et al., 2002a). Thus, it appears that the role of PGE_2 in cartilage metabolism and turnover is not straightforward, and that PGE_2 might play a regulatory role in this tissue rather than a strictly catabolic role.

5.3.6 Subchondral Bone Resorption

Increased resorption of subchondral bone is a hallmark of early OA pathology in humans and horses. PGE_2 has been shown to play a critical role in this process *in vivo*, as evidenced by mPGES-1 knockout mice that display reduced bone erosion in arthritis models (Trebino et al., 2003). *In vitro,* osteoarthritic osteoblasts spontaneously produce more PGE_2 than do normal osteoblasts (Hilal et al., 2001), and pro-inflammatory mediators such as IL-1β and LPS increase osteoblast COX-2 mRNA expression and subsequent PGE_2 synthesis (Suda et al., 2004). PGE_2 also stimulates additional osteoblast IL-1β production and osteocyte PGE_2 synthesis, forming a PGE_2-cytokine positive feedback loop that regulates osteoclastogenesis, osteoclast activity, and bone resorption (Park et al., 2004; Sakuma et al., 2004; Suda et al., 2004).

Osteoblast PGE_2 production is essential for LPS and IL-1β -induced osteoclastogenesis and bone resorption (Suda et al., 2004). Osteoblasts are the major bone cell type that produces PGE_2, and PGE_2 can act in an autocrine manner on these cells to induce osteoclast differentiation (Hikiji et al., 2008). Additionally, PGE_2-EP2 and PGE_2-EP4 receptor signaling on murine macrophages enhances their differentiation into osteoclasts (Kobayashi et al., 2005), and stimulates human bone marrow cells to differentiate into osteoclasts in the presence of M-CSF, TNFα, and IL-1β (Lader & Flanagan, 1998). PGE_2-EP2 and PGE_2-EP4 signaling also leads to increased osteoblast expression of RANKL, which binds RANK receptors on osteoclasts to stimulate osteoclast differentiation, maturation, and activation (Li et al., 2000; Suzawa et al., 2000). Additionally, PGE_2 suppresses osteoblast OPG mRNA expression to enhance RANKL-RANK signaling (Suda et al., 2004). PGE_2

stimulation of EP4 receptors also increases synthesis of MMP-2, MMP-13, and ADAMTS-1 in murine bone and murine bone explants, leading to increased bone resorption (Miles et al., 2000; Miyaura, 2000). Furthermore in horses, conditioned cell media from cultured equine subchondral bone cystic lesions were found to have increased levels of PGE_2 and induced recruitment osteoclasts *in vitro* and enhanced their activity (Rechenberg, Guenther, et al., 2000a).

5.3.7 Subchondral Bone Sclerosis.

In human OA patients, late-stage disease is associated with an uncoupling of bone-resorptive and bone-forming mechanisms that favors bone formation and increased subchondral bone volume, density, and sclerosis (Burr & Gallant, 2012). PGE_2 is a pleiotropic lipid that, in addition to bone catabolic mechanisms, can also mediate bone anabolism through EP2 and EP4 receptor signaling (Minamizaki et al., 2009). PGE_2 can stimulate osteoblast differentiation, increase osteoblast activity, and induce osteoblast expression of multiple bone-forming agents such as bone morphogenic proteins and extracellular matrix products (Choudhary et al., 2003; Arikawa et al., 2004; Tang et al., 2005).

Osteoblasts from sclerotic bone tissue display an altered phenotype that predisposes the bone to undergo thickening and sclerosis. This includes increased release of insulin-like growth factor-1 (IGF-1), and a blunted cAMP response to PGE_2 stimulation (Hilal et al., 1998). IGF-1 contributes to bone sclerosis by stimulating osteoblast differentiation, collagen type I production, and preventing MMP degradation of bone matrix (Martel-Pelletier et al., 1998). A strong correlation has been noted between IGF-1 and PGE_2 production in

subchondral sclerotic bone osteoblasts. Within these cells, PGE_2 has been shown to induce IGF-1 mRNA production and increase levels of IGF binding proteins (IGFBPs), which can prolong the half-life of IGF-1 and modulate its binding and signaling (Massicotte et al., 2006; Mohan et al., 1995). Additionally, inhibitors of COX, EP2, EP4, or cAMP-PKA signaling reduces IGF-1 production in sclerotic osteoblasts. This suggests that PGE_2 is involved in modulating osteoblast IGF-1 production to mediate processes of subchondral bone sclerosis (Massicotte et al., 2006). As PGE_2 is also involved in bone resorptive processes, it is likely that similar to its role in cartilage, PGE_2 plays a regulatory role in bone rather than a strictly catabolic or anabolic role.

5.3.8 Innate Immune Cell Regulation.

Innate immune cells play a critical etiologic and pathophysiologic role in acute and chronic inflammatory orthopedic disease. In acute onset synovitis, neutrophils are the most numerous cells present within the synovium, while macrophages predominate in chronic synovial inflammation (Bonnet, 2005). Additionally, neutrophils are one of the major cell types that contribute to inflammation and joint destruction in rheumatoid arthritis (Wright et al., 2014). PGE_2 orchestrates innate immune cell functions both indirectly through effects on the vasculature, as well as directly through signaling on innate immune cells.

In vivo, PGE_2 increases neutrophil tissue infiltration by increasing vascular hyperpermeability (Morimoto et al., 2014). Additionally, PGE_2-induced angiogenesis provides new avenues through which these cells can reach sites within synovial tissues. PGE_2 can also induce epithelial cells to produce IL-8, a potent neutrophil chemoattractant that

stimulates neutrophil homing to inflamed tissues (Yu & Chadee, 1998). Moreover, PGE_2 also stimulates mast cells to secrete monocyte chemoattractant porotein-1 (MCP-1), an important chemokine for monocyte and macrophage chemotaxis and tissue infiltration (Nakayama, 2005).

In contrast to these pro-extravasation effects, the direct effects of PGE_2 on innate immune cell activity and function are quite varied. These effects are orchestrated by differences in EP receptor expression and sensitivity on individual cell types. Many of these effects are anti-inflammatory in nature, as PGE_2 has been shown to dampen TNFα production in monocytes (Meja et al., 1997), IL-1β production in macrophages (Sokolowska et al., 2015), chemotaxis and respiratory burst of neutrophils (Armstrong, 1995; Ottonello et al., 1995), monocyte migration (Singhal et al., 2000) and expression of leukocyte adhesion molecules (Hong, 1996). However, additional studies reveal that PGE_2 can also induce production of IL-6 in monocytes and mast cells (Dendorfer et al., 1994; Gomi et al., 2000) and augment LPS-induced IL-1β formation in peripheral blood monocytes (Sung & Walters, 1991). Additionally, PGE_2 enhances IgE-induced mast cell degranulation (Gomi et al., 2000) and is a potent mast cell chemoattractant mediated by EP3 receptor signaling (Weller et al., 2007).

5.3.9 Adaptive Immune Cell Regulation.

Co-stimulatory mechanisms between T cells, B cells, and dendritic cells (DCs) have been demonstrated in inflamed RA synovial tissues and are hypothesized to play a role in RA progression (Humby et al., 2009). Similar to innate immune cells, PGE_2 can exert both pro-

and anti-inflammatory effects on these cells of the adaptive immune system. PGE_2-EP2 and PGE_2-EP4 signaling on DCs enhances CCR7 expression and production of MMP-9, both of which are necessary for DC migration to draining lymph nodes for T cell activation (Scandella, 2002; Yen et al., 2007). PGE_2 also promotes expression of DC co-stimulatory molecules that enhance their ability to activate and expand naïve T cells (Rieser et al., 1997). In contrast, PGE_2 blocks the ability of DCs to produce T cell chemotactic peptide CCL19, which is necessary for naïve T cell homing (Muthuswamy et al., 2010). Furthermore, DCs that were matured in the presence of PGE_2 together with IL-1β or TNFα display suppressed pro-inflammatory mediator IL-12 production and a biasing of T cell expansion towards the Th2 phenotype (Kalinski et al., 1997). In addition to shifting CD4+ T cell responses from the aggressive Th1 phenotype towards the milder Th2 phenotype (Betz & Fox, 1991), PGE_2 can also inhibit monocyte IL-12 production to further suppress Th1 cell responses (van der Pouw Kraan et al., 1995). PGE_2 also directly stimulates the activity of the suppressive T regulatory cells (Tregs) to dampen immune responses and maintain T cell tolerance (Baratelli et al., 2005). Furthermore, PGE_2 exerts suppressive effects on cytotoxic lymphocytes, including CD8+ T cells, by decreasing their secretory and adhesive capabilities and thus mitigating their destructive potential (Valitutti et al., 1993).

5.3.10 Pain.

Increased concentrations of PGE_2 within arthritic joints sensitizes nerves and increases nerve firing in response to stimuli (Bonnet, 2005). Inhibition of PGE_2-EP4 signaling on peripheral nociceptors suppresses inflammatory hyperalgesia and relieves joint pain in rodent models of

arthritis (Clark et al., 2008; Lin et al., 2006). Additionally, stimulation of PGE_2-EP1 signaling increases pain responses *in vivo* by activating PKA and PKC to enhance the activity of capsaicin receptor TRPV1 (Moriyama et al., 2005; Sachs et al., 2009). EP4 signaling has been shown to induce intracellular cAMP production in nociceptors, leading to activation of PKA and exchange proteins activated by cAMP (Epac). Epac signaling stimulates PKC activity via the Rap1 pathway to induce hyperalgesia and pain. PKA and PKC can also sensitize multiple downstream channels and receptors that mediate hyperalgesia. These include TTX-R sodium channels ($Na_v1.8$ and 1.9), T-type channels (including $Ca_v3.2$), and purrigenic P2X3 receptors on nociceptive sensory neurons (Kawabata, 2011).

6. Mechanisms of Inhibiting PGE_2

As previously discussed, PGE_2 is intimately involved in the pathophysiology of multiple inflammatory orthopedic processes; thus, inhibition of PGE_2 has long been recognized as an effective means of decreasing orthopedic inflammation and pain in horses and humans (Curiel & Katz, 2013; McIlwraith & Frisbie, 2015; Morton et al., 2005; Owens et al., 1996). Nonsteroidal anti-inflammatory drugs (NSAIDs) are defied as substances other than steroids that inhibit inflammation. More specifically, the term NSAID is generally associated with substances that inhibit prostaglandin production by interfering with one or more parts of the prostaglandin synthetic pathway. The major targets of current NSAIDS are the COX enzymes COX-1 and/or COX-2. While COX inhibitors are generally effective anti-inflammatories, the possibility of gastrointestinal (GI) and renal side effects limits their long-

term use in horses (McIlwraith & Frisbie, 2015). However, new PGE_2-targeting therapeutics are currently being developed that are as effective as COX inhibitors but are associated with fewer adverse effects. Interestingly, there is also data indicating PGE_2 and PGE analogs can also exert anti-inflammatory effects in multiple animal models. The following sections will overview current therapeutics and novel treatments to inhibit PGE_2 formation or signaling. Additionally, we will discuss the potential use of PGE analogs as anti-inflammatories. This information is summarized in Figure 1.

6.1 COX Inhibitors

COX-1 and COX-2 are intermediate enzymes in the PGE_2 synthesis pathway. COX-1 is constitutively expressed in most cells and contributes to homeostatic PGE_2 synthesis. In contrast, COX-2 is an inducible enzyme that is responsible for increased PGE_2 production following inflammatory insult. Because PGE_2 has been shown to play a potent role in inflammatory orthopedic disease, inhibition of COX enzymes is commonly used to treat orthopedic pain and inflammation (Frisbie et al., 2009; Morton et al., 2005).

Many COX inhibitors inhibit both COX-1 and COX-2, albeit usually with greater preference for one COX isoform over the other. Drugs that inhibit COX enzymes relatively equally are considered nonselective COX inhibitors. Nonselective COX inhibitors that are currently used in horses include flunixin meglumine (FM), ketoprofen, and most commonly, phenylbutazone (PBX) (McIlwraith & Frisbie, 2015). While PBZ and FM inhibit both COX-1 and COX-2 activity, these drugs show a slight preference for COX-1 inhibition (Beretta et al., 2005). Nonselective NSAIDS decrease serum PGE_2 levels and pain post-operatively in

horses (Cook et al., 2009a; Tomlinson et al., 2004), as well as decrease leukocyte influx and PGE_2 levels in equine models of LPS-induced synovitis (Morton et al., 2005). While these drugs effectively inhibit inflammation, they are also associated with multiple side effects that are attributed to inhibition of COX-1-mediated homeostatic PGE_2 production (Moses & Bertone, 2002). In horses, adverse effects of nonselective NSAIDS include right dorsal colitis, inhibition of GI mucosal barrier function and healing, increased flux of endotoxin across the GI mucosa, and gastric ulceration in predisposed patients (Cook & Blikslager, 2014; Cook et al., 2009a; Marshall & Blikslager, 2011; Monreal et al., 2004; Tomlinson et al., 2004). In human patients, nonselective COX inhibitors are associated with similar GI side effects including GI ulceration, colitis, and increased small intestinal inflammation, permeability, and malabsorption (Thiéfin & Beaugerie, 2005). These side effects are attributed to the role of PGE_2 in maintaining GI motility, blood flow, mucosal synthesis, and mucosal secretion. Other adverse effects of nonselective COX inhibitors include ischemia of the renal medulla and renal papillary necrosis in hypovolemic patients (Cook & Blikslager, 2014; Moses & Bertone, 2002). These adverse effects are caused in part by inhibition of basal PGE_2 production that is responsible for maintaining blood flow to the kidney. Additional adverse effects of nonspecific COX inhibitors in horses include bone sclerosis, cartilage erosion, and decreased cartilage proteoglycan synthesis *in vivo* (Beluche et al., 2001; Frisbie et al., 2009). Furthermore, nonspecific COX inhibitors have been shown to inhibit glycosaminoglycan (GAG) synthesis and potentially delay cartilage repair. This mechanism is potentially orchestrated by a loss of PGE_2 modulatory effects on IL-1β-induced cartilage damage (Dingle, 1993). While PGE_2 does elicit proinflammatory cytokine

production in many tissues, reports demonstrate that PGE_2 can also dampen cytokine secretion in certain cell types, leading to a potential balancing and modulating role of PGE_2 on IL-1β synthesis.

In an effort to avoid inhibition of COX-1-mediated homeostatic PGE_2 production, COX-2 specific inhibitors (or coxibs) have been developed that inhibit only COX-2 induced PGE_2 synthesis. The coxibs most commonly employed in horses include meloxicam and firocoxib. Etodolac is also used in horses and is considered a coxib, however shows some activity against COX-1 at higher doses (Tomlinson et al., 2004). Additionally, carprofen has been used in horses but is less selective for equine COX-2 than other coxibs such as meloxicam (Beretta et al., 2005). Meloxicam is a first-generation coxib that displays 5-12 times selectivity for COX-2 compared to PBZ or FM (Beretta et al., 2005; Burns et al., 2010). Meloxicam has been demonstrated to reduce synovial effusion and PGE_2 production in equine synovitis models, as well as reduce substance P, bradykinin, MMP activity, and signs of cartilage breakdown in equine synovial fluid (de Grauw et al., 2009). Additionally, meloxicam shows a better gastrointestinal safety profile when compared to FM, including increased recovery of GI barrier function following insult (Little et al., 2007). Firocoxib is a second-generation coxib that in horses is 265 times more selective for COX-2 than COX-1 (Burns et al., 2010). Firocoxib has been shown to be as effective as PBZ at decreasing pain, lameness, and swelling in naturally occurring equine OA, and is associated with decreased systemic PGE_2 levels, increased GI endothelial barrier recovery, and reduced flux of LPS across the GI mucosa (Cook et al., 2009a; Doucet et al., 2008; Orsini et al., 2012). A third

coxib, etodolac, has been shown to decrease leukocyte and PGE_2 levels in equine LPS-induced synovitis (Morton et al., 2005).

Overall, evidence suggests that coxibs show improved GI safety profiles in horses (Cook et al., 2009a; Little et al., 2007), with the exception of etodolac (Tomlinson et al., 2004). However, there have been reports that coxibs can elicit dangerous cardiovascular side effects in animal models and human patients. In mouse models, selective COX-2 inhibition or deletion elicits a pro-thrombotic state and elevates systemic blood pressure (Cheng, 2006). Additionally in human patients, the coxib rofecoxib has been shown to elicit more adverse cardiovascular events such as myocardial infarction when compared to nonspecific NSAIDs (Bombardier et al., 2000). This is believed to be due, in part, to an imbalance in blockade of pro-thrombotic TXA_2 and anti-coagulatory PGI_2. Platelets express primarily COX-1 and synthesize TXA_2 to enhance platelet aggregation and clotting. In contrast, COX-2 is predominantly expressed by endothelial cells and leukocytes and is the dominant COX isoform responsible for production of PGI_2. PGI_2 is a potent vasodilator and prevents platelet aggregation, and serves to balance TXA_2 functions in the vasculature. It has been proposed that singular inhibition of COX-2 leads to a selective decrease in PGI_2, causing a relative increase in TXA_2 compared to other prostanoids and induces a pro-thrombotic state (Cheng, 2006; Rao & Knaus, 2008). While these effects have yet to be observed in horses, caution should be used in patients in whom balanced coagulation pathways are critical, such as laminitis. Additionally, there is evidence that absolute inhibition of COX-2 expression might not be beneficial due to the homeostatic role of COX-2 in organs such as the kidney. Thus, it

is currently not recommended that coxibs be used as an absolute replacements for nonspecific COX inhibitors in horses (McIlwraith & Frisbie, 2015).

Finally, COX inhibitors that are more potent inhibitors of COX-1 than COX-2 include aspirin, indomethacin, and piroxicam. While these medications reduce PGE_2 levels, they are considered less beneficial in inflammatory disease than therapies that inhibit COX-2 (Goodrich & Nixon, 2006). However, studies by Dingle *et. al.* have shown that COX-1 inhibition using aspirin elicits fewer negative effects on cartilage GAG synthesis when compared to nonspecific NSAIDS, and potentially increases GAG synthesis in some patients (Dingle, 1993).

6.2 PGES Inhibitors

As an alternative to coxibs, inhibition of PGES enzymes provides selective blockade of PGE_2 while leaving levels of other essential prostanoids intact. Specifically, inhibition of the inducible mPGES-1 enzyme selectively inhibits induced PGE_2 synthesis *in vitro* and *in vivo* in human and animal models, respectively (Côté et al., 2007; Giroux et al., 2009; Leclerc et al., 2013; Xu et al., 2008). mPGES-1 inhibition is analgesic and reduces the severity of acute and chronic orthopedic inflammation in rodents. Furthermore, mPGES-1 inhibitors have been shown to decrease cartilage erosion, proteoglycan loss, and inflammatory cell influx in murine models of arthritis (Guerrero et al., 2009). In addition, knockout or inhibition of mPGES-1 is associated with fewer GI and cardiovascular side effects when compared to COX inhibitors (Cheng, 2006; Xu et al., 2008). Thus, selective inhibition of mPGES-1

appears to be as effective as COX inhibitors at decreasing PGE_2 levels, pain, and inflammation, with fewer GI, cardiovascular, and musculoskeletal side effects.

Tissue mPGES-1 expression is increased in human and equine inflammatory orthopedic disease (Farley et al., 2005; Kojima et al., 2004; Masuko-Hongo et al., 2004; Shimpo et al., 2009). Until recently, anti-inflammatory targeting of mPGES-1 had not been evaluated in horses. Recently, our lab demonstrated that the mPGES-1 inhibitor MF63, which was developed for use in humans, selectively inhibits equine mPGES-1 enzyme activity *in vitro* (see Chapter II). Pretreatment of equine leukocytes with MF63 led to a concentration-dependent decrease in LPS-stimulated PGE_2 production. This inhibition was selective for PGE_2, as levels of PGI_2 and TXA_2 were unaffected. In contrast, a COX-2 specific (NS-398) and a nonspecific COX inhibitor (indomethacin) significantly inhibited LPS-induced leukocyte production of PGE_2, PGI_2, and TXA_2. Additionally, higher basal levels of PGE_2 remained following MF63 treatment when compared to either COX inhibitor. Thus, we propose that inhibition of mPGES-1 would be a safe alternative to COX-2 inhibitors in horses, especially in patients with GI or vascular complications including colitis and laminitis.

Currently, no mPGES-1 inhibitors are commercially available for use in humans or horses. Furthermore, no advanced mPGES-1 inhibitor clinical trials have been reported (Koeberle & Werz, 2015). This is due to multiple factors, including differences in mPGES-1 inhibitor efficacy between humans and rodent models, reduced efficacy in whole blood due to high drug lipophilicity (required for mPGES-1 affinity), and shunting of the PGH_2 precursor to other prostanoids following mPGES-1 inhibition (Koeberle & Werz, 2015; Xu et

al., 2008). MK-886 was one of the first mPGES-1 inhibitory compounds discovered, and from this scaffold, phenanthrene imidazole inhibitors such as MF63 were developed. These inhibitors display 1000-fold selectively for mPGES-1 over other PGESs and are more lipophilic than its predecessors (Côté et al., 2007). However, these compounds are difficult to study due to a lack of efficacy in rodents, caused by differences in the amino acid sequence and 3D binding pocket of murine and human mPGES-1 (Korotkova & Jakobsson, 2013). Excitingly, our lab demonstrated that phenanthrene imidazole inhibitors are effective in equine cells, indicating that the equine mPGES-1 protein is more similar to the human mPGES-1 protein than that of rodents. Furthermore, this suggests that horses are better suited to serve as translational models of mPGES-1 inhibition in humans, specifically when studying phenanthrene imidazole derivative compounds (see Chapter II). In addition to MF63, other compounds that have been shown to inhibit mPGES-1 enzymatic function include various endogenous anti-inflammatory mediators, COX inhibitors and their derivatives, and novel chemical compounds (Koeberle & Werz, 2015). Of the latter, two recently developed compound derivatives of imidazoquinolines (Shiro et al., 2013) and benzoxazole piperidinecarboxamides (Arhancet et al., 2013) show better pharmacokinetic profiles and increased efficacy *in vivo* than previously developed drugs, and have recently entered early phases of clinical study.

6.3 Other Mechanisms of Inhibiting PGE_2 Activity

6.3.1 Piprants (EP Receptor Antagonists)

Piprants are a novel drug class that antagonizes EP receptors (Rausch-Derra et al., 2015). Thus far, the only FDA-approved piprant for use in animals is grapiprant, which has been approved for treatment of canine OA. Grapiprant is an EP4 receptor antagonist that binds to EP4 receptors with high affinity and reduces acute and chronic pain in murine models (Nakao et al., 2007). When compared to coxibs, grapiprant is as effective at inhibiting inflammatory edema, pro-inflammatory biomarker production, bone destruction, synovial inflammation, and leukocyte infiltration in murine adjuvant-induced arthritis models (Okumura, et al., 2010). Additionally, grapiprant reduces pain in naturally occurring canine OA when compared to placebos. However, grapiprant has been associated with mild GI side effects including vomiting and diarrhea (Rausch-Derra, et al., 2016). Additionally in a long-term study, grapiprant induced mild changes in hematologic and urinary variables that the authors concluded were not associated with negative clinical outcomes (Rausch-Derra et al., 2015).

6.3.2 Anti-PGE_2 Antibodies.

PGE_2-targeting antibodies inhibit PGE_2 activity and decrease PGE_2-EP4 receptor signaling *in vitro*. *In vivo,* PGE_2 targeting antibodies reduce severity of arthritis in mouse RA models, but are not as potent as anti-TNFα antibodies. However, dual treatment with anti-PGE_2 and anti-TNFα antibodies show increased anti-inflammatory ability when compared to anti-TNFα

therapy alone (Norberg et al., 2013). Currently, a patent has been filed for PGE_2-binding antibody therapeutics (Gu et al., 2014), but no products are commercially available.

6.3.3 15-PGDH Activation.

15-PG hydrogenase (15-PGDH) oxidizes PGE_2 to produce the inactive metabolite, 15-keto PGE_2. Adenovirus-mediated delivery and activation of the 15-PGDH gene in mouse models leads to decreased systemic PGE_2 and cytokine production (Eruslanov et al., 2009). While in development, no 15-PGDH-activating therapeutics are available at this time.

6.4 PGEs as Anti-Inflammatories.

In addition to its role as a pro-inflammatory mediator, PGE_2 has also been shown to exert anti-inflammatory effects in numerous cell systems. Specifically, agonists of EP2 and EP4 have been shown to dampen many leukocyte effector functions (Hirata & Narumiya, 2012; Sugimoto & Narumiya, 2007). Agonists of EP2 and EP4 activate adenylate cyclases to increase intracellular cAMP production. Elevated intracellular cAMP activates multiple downstream targets, such as PKA, that inhibit leukocyte adhesion, respiratory burst, and pro-inflammatory cytokine production (Cheng et al., 2008; Chilcoat et al., 2002; Wen et al., 2010). Thus, EP2 and EP4-targeting PGE analogs, and PGE_2 itself, could serve as anti-inflammatories in both horses and humans.

6.4.1 PGE Analogs: Misoprostol.

Misoprostol is an EP2, EP3, and EP4 receptor agonist that has been shown to increase intracellular cAMP levels (Ahluwalia et al., 2014; Blikslager, 2012; Smallwood & Malawista, 1995). Misoprostol inhibits many neutrophil effector functions, including adhesion, chemotaxis, lysosomal enzyme release, respiratory burst, and pro-inflammatory cytokine production (Farmer et al., 1991; Gobejishvili et al., 2015; Kocher et al.,1997; Talpain et al., 1995). In murine inflammation models, misoprostol has been shown to decrease edema and leukocyte tissue influx (Moraes et al., 2007; Rossetti et al., 1995; Smith et al., 1996; Yamada et al., 1991), exert protective effects in cerebral ischemia (Li et al., 2008), and inhibit neutrophil influx and oxidative burst in cerebral hemorrhage (Wu et al., 2015). Excitingly, our lab demonstrated that misoprostol similarly exerts anti-inflammatory effects on equine leukocytes *in vitro*. In our model, misoprostol inhibits equine neutrophil respiratory burst, adhesion, and chemotaxis in response to a number of different stimulants (see Chapter III). Additionally, misoprostol inhibits LPS-stimulated equine leukocyte TNFα, IL-6, and CXCL8 production at the mRNA and protein levels (see Chapter IV).

Misoprostol has also been shown to inhibit effector functions of adaptive immune cells. This includes inhibition of human T cell proliferation, IFNγ production, and phagocytosis, as well as increased T cell production of the anti-inflammatory cytokine IL-10 (Waiser et al., 2003). Misoprostol is also able to discriminate between T cell subsets, inhibiting functions of the more pro-inflammatory Th1 cells, while stimulating Th2 cells to produce anti-inflammatory cytokines. Additionally, misoprostol can stimulate T helper cells to take on more Th2-like functions (Gold et al., 1994).

Specifically in OA cartilage, misoprostol has been shown to exert a protective effect on cartilage tissues. In a study by Dingle, *et. al*., application of misoprostol reversed the inhibitory effect of IL-1β on cartilage GAG synthesis. Additionally, misoprostol mitigated the negative effects of NSAIDs on GAG synthesis by increasing cartilage anabolic activity (Dingle, 1993). These effects could be explained by the potential regulatory role of PGEs on IL-1β synthesis and articular cartilage, as groups have shown that PGE_2 exerts both anabolic and catabolic effects on chondrocytes (Attur et al., 2008; Tung et al., 2002a).

Taken together with our recent data (see Chapters III, IV, and V), misoprostol shows promise as an inhibitor of leukocyte-mediated inflammation in horses and remains to be evaluated in horses *in vivo.* Misoprostol is FDA-approved to treat NSAID-induced gastropathy in humans, and is also used off-label in horses as a gastroprotectant, but has yet to be evaluated clinically as an anti-inflammatory.

6.4.2 Modulation of PGE_2 Transport

Prostaglandin transporter (PGT) is responsible for transporting extracellular prostaglandins into cells where they are subsequently enzymatically inactivated. Inhibition of PGT increases systemic PGE_2 levels and dampens many leukocyte functions and inflammatory processes. Additionally, studies have demonstrated that increased systemic PGE_2 can decrease COX-2 and EP receptor expression, leading to desensitized PGE_2 responses (Norberg et al., 2013; Schuster, 2002). While not commercially available, these drugs are currently in development.

7. Horses as Translational Models of Inflammatory Orthopedic Disease

The most reliable translational models are those that closely match the disease process of interest in humans, especially with concern to naturally occurring disease (Gregory et al., 2012). Horses are one of the only species that naturally develops inflammatory orthopedic diseases at rates similar to humans. Horses are exposed to many of the same environmental factors, athletic pressures, and wear-and-tear injuries as their human counterparts. Furthermore, diseases such as OA and septic arthritis share similar etiologies, pathophysiology, and biological mechanisms between species. Additionally, the equine joint more similarly mimics that of humans when compared to other animal models, including similar size, anatomy of tissues, and manner of load bearing. For all of these reasons, horses are an ideal translational model for studying orthopedic disease and injury. Recognition of the utility of equine models has led to multiple translational studies using horses to assess the pathogenesis, pathophysiology, and treatment of human orthopedic disorders. In this section, we will give a brief overview of equine models developed for inflammatory orthopedic translational studies and their ability to recapitulate the human condition.

Articular cartilage injury is common in humans and horses, and horses are an excellent translational model of cartilage defects and repair. Multiple equine joints are anatomically equivalent to those in humans, including the equine femoropatellar and femorotibial joints (stifle) that are similar to the human knee (McIlwraith et al., 2011). Within the stifle, thickness of the subchondral bone and multiple layers of the articular cartilage, including the non-calcified and calcified layers, are very comparable to that of

humans (Frisbie et al., 2006). Equine models that have been developed to study disorders of the articular cartilage include induction of cartilage defects to observe cartilage healing, (Convery et al., 1972) and generation of subchondral bone microfractures to evaluate their effect on articular cartilage remodeling (Frisbie et al., 1999). Following induction of injury, horses can be subjected to similar rehabilitation and controlled exercise regimens as humans to assess their effect on return to performance. Additionally, several reparative strategies involving mesenchymal stromal cells (MSCs) have been evaluated in equine joint and tendon injury and show potential translational benefit to humans. Therapeutic use of equine MSCs reduces reinjury rates and improves structural organization in healing tissues, and thus could be of substantial benefit to human athletes. Furthermore, a recent study comparing the characteristics of human and equine MSCs showed many similarities in cell surface expression, proliferation, and multi-lineage differentiation potential of these cells between species (Hillmann et al., 2016). Overall, the effects of these various cartilage repair models are easier to assess in horses when compared to smaller animals. This is due to the size of equine joints, which produce a greater amount of tissue and fluid for biochemical analysis, and are also better suited to clinical imaging modalities to measure a number of outcome parameters (McIlwraith et al., 2011).

Horses are also excellent translational models of developmental orthopedic disease such as OCD. As previously discussed, most human osteochondrosis lesions are not observed until clinical signs begin, and therefore only late-stages of the disease have been studied in people. In horses, resection of vessels within the epiphyseal cartilage mimics the pathophysiology of naturally occurring osteochondrosis in horses and humans, and thus

indicates that inadequate vascular supply is a major etiologic factor for osteochondrosis development in both species (Olstad et al., 2013). Similarly, it has been shown that horses naturally develop osteochondrosis in similar predilection sites as humans, and that end-stage disease between these species shares many similarities (McCoy et al., 2013). Together, this indicates that horses are excellent means of studying osteochondrosis and OCD in humans.

Horses have also been extensively used as translational models of OA. Etiologies of naturally occurring OA are similar between horses and humans, including acute trauma, altered biomechanical loading, and wear-and-tear ageing processes. Experimental models have been developed that utilize either surgical or IL-1β/LPS-induced OA and display similar pathologic changes as those seen in naturally occurring OA in humans (Frisbie et al., 2002; Ross et al., 2012). Furthermore, multiple studies have used naturally occurring OA in horses to measure synovial biomarkers as potential therapeutic targets (Kamm et al., 2010; Ley et al., 2009). In addition to the similarities of OA pathophysiology between species discussed in this review (subsection 5.1.1), the lucrative nature of the equine racing industry has made a push for novel OA joint therapies with the intention of translating their use to human athletes (Gregory et al., 2012). These include application of hyaluronic acid in synovial joints (Auer et al.,1980), gene therapy for expression of IL-1R antagonists (Frisbie et al., 2002), and chondrocyte implantation to repair articular cartilage defects (Frisbie et al., 2009).

Horses have also been studied as translational models of many other inflammatory diseases. Equine leukocytes mount a powerful immune response to pro-inflammatory mediators that is similar to that observed in human leukocytes. Additionally, equine and human orthopedic tissues produce similar inflammatory responses to pro-inflammatory

stimulants in multiple disease states (for a review, see Section 5.2). LPS-stimulation of equine and human neutrophils results in similar quantities and profiles of pro-inflammatory cytokine production including IL-8, IL-1β, and TNFα (Hirsch et al., 2012). Furthermore, equine and human neutrophils possess similar protease enzymes, including elastases, with similar degradative activity (Dubin et al., 1994). Additionally, modulation of inflammatory responses using anti-inflammatory therapeutics displays similar effects in both species (Hirsch et al., 2012). Our recent studies indicate that equine leukocytes are an excellent translational model for studying PGE_2 targeting, specifically through inhibition of mPGES-1 (Chapter II) or manipulation of EP receptor signaling (Chapters III, IV, and V). In the case of mPGES-1, multiple putative human mPGES-1 inhibitors show no cross-reactivity to rodent mPGES-1 due to differences in protein structure (Xu et al., 2008). However, these inhibitors were effective against equine mPGES-1. The similarity between human and equine mPGES-1 protein, as well as the tendency of equine athletes to naturally develop similar inflammatory orthopedic diseases, indicates that horses represent a strong translational model of studying mPGES-1 targeting in inflammatory orthopedic disorders.

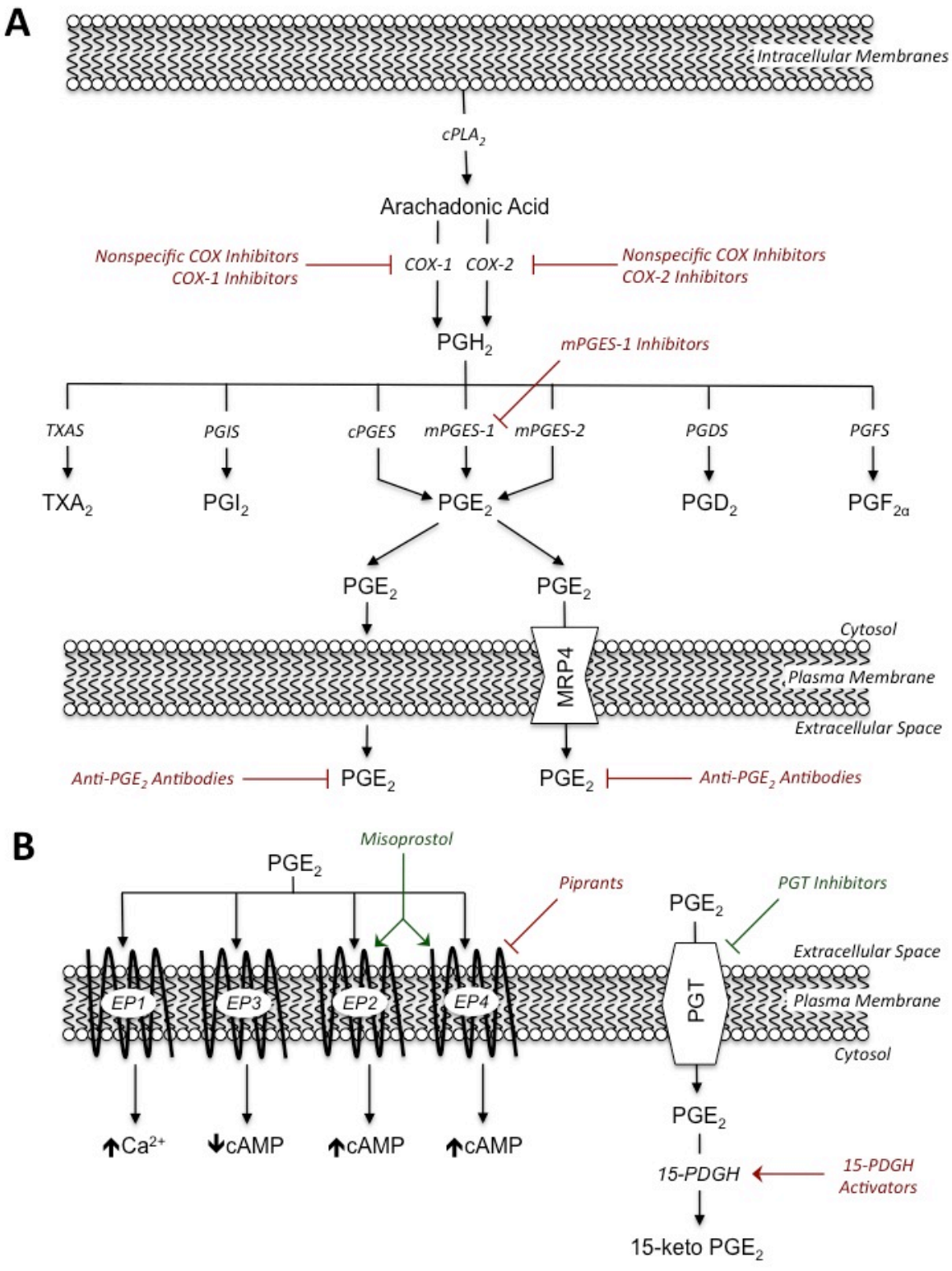
Additionally, genetic inflammatory disorders have also been studied in horses, including Glanzmann's Thromasthenia (GT) and severe combined immunodeficiency with radiation sensitivity (RS-SCID). GT is characterized by abnormal platelet aggregation and adhesion, and has been observed in both humans and horses. SCID is characterized by an inability to mount an antigen-specific immune response. In humans, RS-SCID is due to a defect in a specific component of DNA repair cellular machinery termed Artemis, which is needed to create functional proteins on T and B cells following recombination of various

segments of T cell and immunoglobulin receptors (Bauer et al., 2009). In horses, specifically Arabians, a defect in the DNA repair mechanism DNA-PK makes their cells more vulnerable and less able to repair following radiation damage (Perryman, 2004). In either case, the immunodeficiency seen in both species is similar, and thus horses can serve as a model for human genetic adaptive immune deficiency.

4. Conclusions

This review summarizes the potent role that PGE_2 plays in the pathogenesis and pathophysiology of equine and human inflammatory orthopedic diseases. While PGE_2 plays multiple homeostatic roles in both species, PGE_2 is also a critical modulator of cartilage and bone metabolism, edema formation, angiogenesis, and a potent orchestrator of the immune response in multiple orthopedic disorders. Therapeutic PGE_2-targeting strategies that have been implemented in humans and horses, particularly COX inhibitors, show similar anti-inflammatory benefits and adverse side effects including GI, renal, and cardiovascular complications. Thus, novel mechanisms of safely and effectively inhibiting PGE_2 are critically needed in both species. The similar roles of PGE_2 in disease etiology and progression in humans and horses supports the use of equine athletes as a model to study PGE_2 targeting strategies in inflammatory orthopedic disease that would benefit horses and humans alike.

Figure 1. PGE_2 synthesis and targeting strategies. (A) PGE_2 synthesis and secretion. Activated cytosolic phospholipase 2 ($cPLA_2$) cleaves arachadonic acid from cellular membranes. Arachadonic acid is oxidized by constitutively expressed cyclooxygenase-1 (COX-1) or inducible COX-2 to form the unstable prostaglandin intermediate, prostaglandin H_2 (PGH_2). PGH_2 serves as a substrate for multiple prostaglandin synthase enzymes including thromboxane synthase (TXAS), prostacyclin synthase (PGI2), PGD synthase (PGDS), PGF synthase (PGFS), and three different isoforms of PGE synthase (PGES). Cytosolic PGES (cPGES) and microsomal PGES-2 (mPGES-2) are constitutively expressed and produce basal levels of PGE_2. mPGES-1 is an inducible synthase and produces increased levels of inflammatory PGE_2. PGE_2 exits the cell via passive diffusion and active transport by the multidrug resistance protein 4 (MRP4). **(B) PGE_2 signaling and degradation.** PGE_2 binds to four different E-prostanoid (EP) receptors. Binding to EP1 leads to an increase in intracellular calcium. EP3 receptor activation leads to decreased adenylate cyclase activity and subsequently decreases intracellular cyclic AMP (cAMP) concentrations. PGE_2 interaction with EP2 and EP4 activates adenylate cyclase to increase intracellular cAMP concentrations. PGE_2 can be actively transported into cells via prostaglandin transporter (PGT), where it is converted to the inactive metabolite 15-keto PGE_2 by 15-prostaglandin dehydrogenase (15-PDGH). In both **(A)** and **(B)**, therapeutics that inhibit PGE_2 synthesis are shown in red, and therapeutics that increase PGE_2 levels (or are PGE analogs) are shown in green. Arrows drawn from these therapeutics indicate inhibition (blunted arrows) or stimulation (pointed arrows) of given components of the PGE_2 synthesis and signaling cascade.


A
Intracellular Membranes
cPLA2
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Nonspecific COX Inhibitors
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PGH2
mPGES-1 Inhibitors
TXAS
PGIS
cPGES
mPGES-1
mPGES-2
PGDS
PGFS
TXA2
PGI2
PGE2
PGD2
PGF2α
PGE2
PGE2
Cytosol
MRP4
Plasma Membrane
Extracellular Space
Anti-PGE2 Antibodies
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Misoprostol
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Piprants
PGE2
PGT Inhibitors
Extracellular Space
EP1
EP3
EP2
EP4
PGT
Plasma Membrane
Cytosol
↑Ca2+
↓cAMP
↑cAMP
↑cAMP
PGE2
15-PDGH
15-PDGH Activators
15-keto PGE2

Chapter II

Inhibition of microsomal prostaglandin E-synthase-1 (mPGES-1) selectively suppresses PGE_2 in an *in vitro* equine inflammation model

Emily G. Martin[1], Samuel L. Jones[1,2]

[1]Department of Clinical Sciences and [2]Comparative Medicine Institute, College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27606 USA

1. Abstract

Specific inhibition of leukocyte prostaglandin E_2 (PGE_2) production effectively limits inflammation in many disease models. Selective PGE_2 targeting elicits fewer clinical side effects compared to cyclooxygenase (COX) inhibitors, and is safely achievable through inhibition of the terminal enzyme downstream of COX in the inducible PGE_2 synthesis cascade: microsomal PGE synthase-1 (mPGES-1). To date, mPGES-1 has not been evaluated as an anti-inflammatory target in horses, which are excellent translational models of naturally-occurring inflammatory orthopedic disease in humans. Therefore, we tested the hypothesis that mPGES-1 is induced by lipopolysaccharide (LPS) in an *in vitro* equine inflammation model. Furthermore, we predicted that mPGES-1 inhibition in equine leukocytes would selectively reduce extracellular concentrations of PGE_2, but not other prostanoids in our model. To test this hypothesis, equine leukocyte-rich plasma (LRP) was primed and stimulated with GM-CSF and LPS, respectively, in the presence or absence of an mPGES-1 inhibitor (MF63), a COX-2 inhibitor (NS-398), or a nonselective COX inhibitor (indomethacin). Interestingly, LPS induced mPGES-1 mRNA, but not protein levels as measured by qPCR and western blot, respectively. In contrast, COX-2 mRNA and protein were coordinately induced by LPS. Excitingly, treatment of LPS-stimulated leukocytes with indomethacin and NS-398 significantly inhibited extracellular concentrations of multiple prostanoids (PGE_2, TXA_2 and PGI_2), while selective mPGES-1 inhibition suppressed only PGE_2. We conclude that mPGES-1 is a key regulator of PGE_2 and is a PGE_2-selective anti-

inflammatory target in equine leukocytes. Additional studies utilizing this translational model would benefit investigators of mPGES-1/PGE_2-selective therapeutics.

2. Introduction

Leukocytes such as neutrophils and monocytes play a pivotal role in the pathophysiology of many inflammatory orthopedic and musculoskeletal diseases in humans and horses (Deligne et al., 2015; DiCarlo & Kahn, 2011; Katz & Bailey, 2012; la RebiÃ re de Pouyade et al., 2011; Lieberthal et al., 2015; Prisk & Huard, 2003; Wright et al., 2010). Neutrophils infiltrate tissues and secrete toxic products that significantly contribute to the pathogenesis of rheumatoid arthritis (Wright et al., 2014), septic arthritis (DiCarlo & Kahn, 2011), and equine laminitis (la RebiÃ re de Pouyade & Serteyn, 2011). Monocytes, macrophages, and neutrophils are also implicated in the pathology of musculoskeletal injury and osteoarthritis-associated synovitis (Deligne et al., 2015; Prisk & Huard, 2003; Sellam & Berenbaum, 2010). An inability to control inflammatory cells in these diseases can lead to overwhelming and irreversible tissue injury.

Prostaglandins (PGs) are lipid messenger molecules that are critical mediators of leukocyte responses during inflammation. PGs are produced at low levels in most tissues under homeostatic conditions. However in disease states, the PG profile changes dramatically to orchestrate inflammatory responses. Of these PGs, prostaglandin E_2 (PGE_2) is one of the most potent mediators of inflammation. PGE_2 is significantly elevated in the joints of horses with naturally occurring or experimentally-induced joint disease and has been shown to play a role in muscle injury and osteoarthritis (Bertone et al., 2001; Frisbie et al., 2008; Hardy et al., 2002; Prisk & Huard, 2003). PGE_2 produced in damaged tissue recruits blood leukocytes to areas of tissue injury and induces vascular changes to aid these cells in crossing the

endothelium (Kalinski, 2011; Ricciotti & FitzGerald, 2011). Within the tissues, leukocytes are activated by lipid mediators, cytokines, and products of tissue breakdown, culminating in the secretion of inflammatory mediators and toxic products. This includes additional PGE_2 and other cytokines that amplify the inflammatory response, as well as reactive oxygen species and proteases for the destruction of microbial invaders (Kruger et al., 2015; Wong et al., 2012). While this physiologic process is essential for defending the body from bacterial infection, overabundant or dysregulated leukocyte responses injure normal tissue and contribute to inflammatory disease (Anderson et al., 1991; Varani & Ward, 1994; Wong et al., 2012).

Non-steroidal anti-inflammatory drugs (NSAIDS) effectively inhibit PGE_2 formation via targeting cyclooxygenase (COX) enzymes (Vane & Botting, 1996). COX-1 and COX-2 are two different COX isoforms that are key upstream enzymes in the synthesis of all prostanoids, a group that include the PGs, thromboxanes, and prostacyclins. COX-1 is constitutively expressed and governs prostanoid synthesis necessary for homeostatic functions including platelet aggregation, gastric mucosal protection, and renal electrolyte homeostasis. COX-2 is an inducible enzyme that is involved in the production of prostanoids under inflammatory conditions (Morita, 2002). COX enzymes convert arachadonic acid to the PG intermediate, PGH_2. PGH_2 is acted upon by a variety of terminal synthases to produce different prostanoids including PGE_2, $PGF_{2\alpha}$, PGD_2, thromboxane A_2 (TXA_2), and prostacyclin (PGI_2). Non-selective NSAIDs target both COX enzymes, leading to decreased constitutive and inducible prostanoid production. While effective anti-inflammatories, non-selective blockade of all prostanoids can lead to adverse effects in humans and horses

including renal and gastrointestinal toxicity (Harirforoosh et al., 2013; Marshall & Blikslager, 2011; Moses & Bertone, 2002). COX-2 selective inhibitors (coxibs) selectively inhibit induced prostanoid synthesis mediated by COX-2. However, dangerous cardiovascular side effects including myocardial infarction have been observed (Mukherjee et al., 2001). Thus, more specific and consequently safer therapeutics aimed at selectively inhibiting induced PGE_2 production would be beneficial in both horses and humans.

Potential PGE_2-specific therapeutic targets are the prostaglandin E-synthases (PGES) that act downstream of COX in the PGE biosynthetic pathway. Three isoforms of PGES exist that differentially regulate basal and induced PGE_2 production. Cytosolic PGES (cPGES) is constitutively expressed and couples with COX-1 to mediate basal PGE_2 synthesis (Tanioka et al., 2000)**.** Microsomal PGES-2 (mPGES-2) is also constitutively expressed and couples with either COX enzyme (Murakami et al., 2003). In contrast, microsomal PGES-1 (mPGES-1) is generally inducible and preferentially couples with COX-2 for increased PGE_2 production in inflammatory states (Murakami et al., 2000). mPGES-1 is essential for leukocyte influx into inflamed tissues and development of pain in rodent inflammatory models (Engblom et al., 2003; Hara et al., 2010; Kamei et al., 2004; Trebino et al., 2003; Uematsu et al., 2002). Additionally, mPGES-1 is significantly increased in both naturally occurring and experimental human inflammatory orthopedic diseases (Kojima et al., 2004; Li et al., 2005; Masuko-Hongo et al., 2004; Shimpo et al., 2009). Naturally occurring inflammatory orthopedic conditions are important problems in equine athletes, and horses are an excellent model for many human inflammatory orthopedic diseases and injuries.

We propose that mPGES-1 is a potential target for PGE_2-selective anti-inflammatory therapies in horses. However, mPGES-1 has not yet been investigated as an anti-inflammatory target in this species. Importantly, structural differences between humans and rodent mPGES-1 lead to a lack of activity of some human mPGES-1 functional inhibitors in rodent species, specifically phenanthrene imidazole derivatives such as MF63 (Xu et al., 2008), and it has yet to be determined if these inhibitors are effective in horses. Additionally, the kinetics and specific roles of PGES enzymes under inflammatory conditions are not fully characterized in equine models that include multiple types of blood leukocytes that are present in natural ratios and a similar native environment. These mixed cell models better mimic *in vivo* conditions in which leukocytes interact with one another to play a key role in systemic and tissue inflammation**.** Therefore, our goal was to further define the PGE_2 synthetic pathway by exploring PGE synthases in a mixed population of primary equine leukocytes. We sought to characterize mPGES-1 and cPGES mRNA and protein kinetics, as well as the role of mPGES-1 in regulating extracellular concentrations of PGE_2 in an *in vitro* equine model of inflammation. We tested the hypothesis that mPGES-1 mRNA and protein are upregulated in equine leukocytes by pro-inflammatory stimuli and are essential for increasing extracellular PGE_2 concentrations in our system. We predicted that inhibition of mPGES-1 would selectively reduce PGE_2, but not TXA_2 or PGI_2 levels in our model when compared to non-selective or COX-2-selective inhibitors.

3. Materials and Methods

Reagents

Experimental reagents were obtained from the following: 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).

Equine Donors and Leukocyte Isolation

All procedures were approved by the Institutional Animal Care and Use Committee at North Carolina State University (NCSU). All horses were fed and housed in similar conditions at the Teaching Animal Unit at NCSU 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) with some modification. Briefly, 30-60cc of equine whole blood was collected into heparinized syringes via jugular venipuncture of male and female horses ages 5-25 years. Whole blood was allowed to settle at room temperature for 1 hour. The leukocyte-rich plasma (LRP) supernatant layer was collected, and leukocytes within this layer were used for the following experiments.

Equine Leukocyte Priming and Stimulation

Equine leukocytes in LRP were first primed for 30 minutes at 37°C using GM-CSF at 1ng/mL, the concentration found to be optimal for cell priming in our experiments (data not shown), or the vehicle control (PBS). Following priming, cells were stimulated with 100ng/mL LPS or vehicle control (PBS) at 37°C for the indicated time periods before downstream analysis. Cell viability was evaluated via trypan blue exclusion and was routinely >95% prior to and following stimulation.

RNA Isolation and First-strand cDNA Synthesis

All RNA isolation materials were obtained from Qiagen (Valencia, CA, USA). Equine leukocytes in 1ml 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 Qiagen's RNase-free DNase set: one on-column digestion prior to elution of RNA, followed by a second DNase digestion once RNA was eluted. 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 (Invitrogen, Thermo Fischer Scientific, Grand Island, NY, USA) with random hexamers (50ng/ul) per manufacturer's protocol.

Real-Time PCR

Real-time PCR was performed using a MyIQ Single-Color Real-Time PCR Detection System from Biorad (Hercules, CA, USA). PCR reactions were prepared with 10ng cDNA, Taqman Gene Expression Master Mix (Applied Biosystems, Thermo Fischer Scientific, Grand Island, NY, USA), gene-specific Taqman primers and probes (Invitrogen, Thermo Fischer Scientific, Grand Island, NY, USA), and RNase/DNase-free water up to a final reaction volume of 25µl. Taqman primers and probes were obtained from Invitrogen's proprietary database of pre-designed Taqman Gene Expression Assays for equine mPGES-1 (Assay ID Ec04321097), COX-2 (Assay ID Ec03467558), COX-1 (Assay ID Ec03469511), and housekeeping genes. Primers and probes for equine cPGES were designed using Invitrogen's proprietary Custom Taqman Assay Design Tool. For all assays, Invitrogen identifies the NCBI reference sequence of the transcript used for primer and probe design, the 25 nucleotide binding location of the probe, and the amplicon length (Canales et al., 2006; Shi et al., 2006). 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 also 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 minutes, once;

95°C for 10 minutes, once; 95°C for 15 seconds, followed by 60°C for 1 minute (with data-collection and real-time enabled), 40 times.

Data analysis was performed using the ΔΔCt method using β_2 Microglobulin as the housekeeping gene for normalization. The housekeeping gene was selected by evaluating expression stability of three common housekeeping genes (β_2 Microglobulin, GAPDH, and β-Actin) using protocols established by Radonic *et al.* ΔΔCt values for all three genes were calculated as previously described (Radonic et al., 2004) with values closest to 0 indicating the least amount of change in gene expression. Of the three genes, β_2 Microglobulin was found to be the most stably expressed in equine leukocytes in our system (data not shown), and was therefore used as the housekeeping gene in all real-time PCR experiments (Assay ID Ec03468699).

Cell Lysis

Equine leukocytes in 1ml aliquots of LRP were primed and stimulated as described. Cells were then lysed using 2X concentrated radioimmunoprecipitation assay (RIPA) lysis buffer (0.2% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate, 2% NP-40, 10mM sodium pyrophosphate and 100mM sodium fluoride) containing protease inhibitors (1mM PMSF, and 1 EDTA-free protease inhibitor cocktail tablet (Roche, Mannheim, Germany) per 5ml buffer). Cells were placed on ice with agitation for 20 minutes, sonicated briefly, and centrifuged. Supernatants containing protein were stored at -80°C until further analysis.

Western Blot

Protein concentrations in lysates were determined by bicinchoninic acid (BCA) Assay (Pierce, Thermo Fischer Scientific, Grand Island, NY, USA). Equal concentrations of protein ranging from 85-100µg were loaded onto precast 4-12% bis-tris gels (Invitrogen) for electrophoresis. Protein was transferred to Immobilon-P PVDF Membranes (EMD Millipore, Billerica, MA, USA) and blocked for 1 hour 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), cPGES (1:2000), COX-2 (1:500), COX-1 (1:500), and β-actin (1:5000). Next, membranes were incubated in appropriate HRP-conjugated secondary antibodies in 5% skim milk for 2 hours at room temperature prior to development using Biorad clarity western ECL substrate. Membranes were visualized using the Biorad ChemiDoc MP system, and Biorad Image Lab software was used for image normalization and quantification.

Inhibitor Studies

Equine leukocytes in 100µl aliquots of LRP were primed for 30 minutes with 1ng/ml GM-CSF. Following priming, 100ng/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 (did not exert a significant effect (data not shown)). Cells were incubated for 18 hours at 37°C, centrifuged, and supernatant was collected and stored at -80C. Cell viability was evaluated via trypan blue exclusion and was routinely >95% following incubation with inhibitors.

ELISA

Plasma PGE_2, a stable metabolite of TXA_2 (TXB_2), and a stable metabolite of PGI_2 (6-keto-Prostaglandin $F_{1\alpha}$) 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.

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 (noted in figure legends). Log transformed data were back transformed for presentation, and thus all data are expressed as mean ± 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.

4. RESULTS

4.1 LPS stimulation of GM-CSF primed equine leukocytes increases mPGES-1, but not cPGES mRNA levels

Prostaglandin E Synthases (PGES) are differentially regulated in various cell types. mPGES-1 is constitutively expressed in a number of tissues where it is believed to play a role in normal cellular physiology, including the kidney, gastric mucosa, and spleen (Boulet et al., 2004; Samuelsson et al., 2007). cPGES has also been shown to be constitutively expressed in most tissues, excluding the brain (Tanioka et al., 2000). In other cell types, including peritoneal macrophages, synoviocytes, chondrocytes, and fibroblasts, mPGES-1 expression is markedly induced by pro-inflammatory mediators (Han & Smith, 2002; Kojima et al., 2004; Murakami et al., 2000; Samuelsson et al., 2007; Stichtenoth et al., 2001). A lack of substantial knowledge of PGE synthase regulation and kinetics in equine leukocytes led us to characterize the effect of pro-inflammatory mediator stimulation on mPGES-1 and cPGES mRNA levels using an *in vitro* equine model of inflammation. Equine leukocytes were primed with 1ng/ml GM-CSF followed by stimulation with 100ng/ml LPS, and mRNA was extracted for real-time PCR. Results demonstrate that mPGES-1 mRNA is not detectable in equine leukocytes prior to stimulation (data not shown), but is markedly increased by GM-CSF/LPS treatment. The peak increase in mPGES-1 mRNA was approximately 15-fold when compared to time-matched controls (PBS/PBS) after 2 hours of stimulation, and slowly declined over the following 18 hours (Figure1A). In contrast, cPGES mRNA levels remained unchanged over an 18-hour period, regardless of treatment. These kinetics closely

mimic those of COX enzymes in our system. COX-2 mRNA was increased by GM-CSF/LPS treatment and peaked after 6 hours of stimulation, whereas COX-1 mRNA levels remained unchanged regardless of treatment (Figure 1A).

LPS alone significantly increased mPGES-1 and COX-2 mRNA levels after 2 hours of stimulation. While GM-CSF priming alone had little effect, GM-CSF priming augmented the LPS-stimulated increase in mPGES-1 and COX-2 mRNA levels. The GM-CSF priming effect on COX-2, but not on mPGES-1 mRNA was significant when compared to LPS stimulation alone. (Figure1B).

4.2 mPGES-1 and cPGES proteins are constitutive in GM-CSF primed equine leukocytes and are not increased by LPS treatment

PGES proteins are necessary for PGE_2 production in other cell systems (Kamei et al., 2004; Nakatani et al., 2007), thus we next sought to characterize the effect of GM-CSF/LPS treatment on mPGES-1 and cPGES protein content in equine leukocytes. 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 GM-CSF/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 hours of stimulation (Figure 2A and 2B). In contrast, COX-2 protein levels were low at baseline and significantly increased following 6 hours of GM-CSF/LPS treatment when compared to controls (PBS/PBS) and GM-CSF treatment alone. These levels remained significantly elevated over the 18-hour incubation

period (Figure 2A and 2C). cPGES and COX-1 protein were constitutive, as both proteins were present at baseline and remained unchanged under all treatment conditions over 18 hours (Figure 2A).

4.3 LPS stimulation of GM-CSF primed equine leukocytes significantly increases extracellular PGE_2

To test the hypothesis that mPGES-1 is essential for increasing extracellular PGE_2 concentrations in equine leukocyte cell supernatants, we first sought to evaluate the effect of GM-CSF priming and LPS stimulation on PGE_2 levels in our *in vitro* inflammation model. 100µl of freshly isolated equine leukocytes were primed with 1ng/mL GM-CSF followed by stimulation with 100ng/mL LPS. Supernatants were collected and analyzed for PGE_2 via ELISA. Prior to GM-CSF/LPS treatment, PGE_2 was present in the plasma at 265.833 ± 59.633 pg/mL (data not shown), which is similar to previously reported data in equine whole blood (Brideau et al., 2001). GM-CSF priming alone (GM-CSF/PBS) did not significantly increase PGE_2 concentrations at any of the time points evaluated. LPS stimulation in the absence of priming (PBS/LPS) did increase PGE_2 levels, but this effect was not significant beyond 6 hours of stimulation. However, GM-CSF priming prior to LPS stimulation led to a significant increase in extracellular PGE_2 concentrations by 6 hours when compared to time matched controls (PBS/PBS) and cells treated with 1ng/mL GM-CSF only (GM-CSF/PBS), and these levels continued to increase and were significantly elevated throughout the 24-hour period of assessment (Figure 3).

4.4 Simultaneous treatment with LPS and the mPGES-1 inhibitor, MF63, selectively decreases extracellular PGE_2 in GM-CSF primed equine leukocytes

An important benefit of selective mPGES-1 inhibition is the ability to block induced PGE_2 production without decreasing synthesis of other beneficial prostanoids. Therefore, we evaluated the ability of a known mPGES-1 functional inhibitor, MF63 (Xu et al., 2008), to selectively decrease PGE_2 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 1ng/mL GM-CSF for 30 minutes, followed by simultaneous application of the aforementioned inhibitors and 100ng/mL LPS over an 18-hour period. We believe this method more closely mimics 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 PGE_2, 6-keto prostaglandin $F_{1\alpha}$ (a stable metabolite of PGI_2), and TXB_2 (a stable metabolite of TXA_2) via ELISA. We found that MF63, indomethacin, and NS-398 all significantly decreased extracellular PGE_2 concentrations in our model (IC_{50} = 0.1147uM, 0.0159uM, and 0.0528uM, respectively (Figure 4). Maximal concentrations of indomethacin and NS-398 led to more complete inhibition of PGE_2 to 5.24% and 4.66% of controls, respectively, when compared to MF63-mediated PGE_2 inhibition to 12.5% of controls (Figure 5). MF63 was a selective inhibitor of PGE_2 in our model, as concentrations of 0.1uM MF63 and above significantly decreased PGE_2, but not 6-keto prostaglandin $F_{1\alpha}$ or TXB_2 extracellular levels (Figure 5A). In contrast,

indomethacin and NS-398 significantly inhibited all three prostanoids evaluated at concentrations at and above 1 and 0.1uM, respectively (Figure 5B-C).

Shunting of the PGH_2 precursor to other PG synthases has been reported in some model systems following mPGES-1 inhibition or deletion. While application of MF63 led to increased 6-keto prostaglandin $F_{1\alpha}$ and TXB_2 levels to a maximum of 118.63% and 118.50% of controls, respectively, this trend was not statistically significant (Figure 5A). Thus, we conclude that no significant shunting to other prostanoids was found in our system.

5. Discussion

The aim of this study was to investigate the role of mPGES-1 in induced PGE_2 synthesis in an equine inflammation model, and to characterize this enzyme as a potential novel anti-inflammatory therapeutic target in horses. To our knowledge, this study is the first to define mRNA and protein kinetics of two PGES enzymes in a mixed population of equine leukocytes under inflammatory conditions. Interestingly, we found that GM-CSF/LPS treatment rapidly increased mPGES-1 mRNA levels, but did not lead to an increase in mPGES-1 protein (Figure1 and 2). In contrast, COX-2 mRNA and protein coordinately increased following GM-CSF/LPS treatment, while cPGES and COX-1 were constitutive at both the mRNA and protein level (Figure1 and 2). Our study also revealed that mPGES-1 is essential for induced PGE_2 synthesis in our equine inflammation model, as inhibition of mPGES-1 with a known mPGES-1 functional inhibitor (MF63) led to a significant decrease in PGE_2 levels. As this was determined via evaluation of PGE concentrations in cell

supernatants, this decrease could be due to both inhibited PGE synthesis and inhibited PGE cellular secretion, as occurs with certain NSAIDs (Reid et al., 2003). However, evidence of MF63 potently inhibiting mPGES-1 enzymatic activity in cell-free assays (IC_{50} = 1.3nM) (Xu et al., 2008) leads us to conclude that MF63 is suppressing extracellular PGE_2 levels through inhibition of PGE_2 synthesis in our *in vitro* system.

MF63 significantly inhibited PGE_2 without effecting levels of other essential prostanoids (Figure 4A). This data is consistent with previous reports in which mPGES-1 inhibition selectively suppressed PGE_2 *in vitro* in human whole blood assays and *in vivo* in rodent models of inflammation (Côté et al., 2007; Giroux et al., 2009; Leclerc et al., 2013; Xu et al., 2008). *In vivo* studies also demonstrate that mPGES-1 inhibition induces dose-dependent analgesia, decreased hyperalgesia, decreased signs of acute and chronic inflammation, and increased gastrointestinal tolerability with fewer gastric mucosal erosions compared to NSAID use (Xu et al., 2008). This data is supported in mPGES-1 knockout animals, which display decreased pain, inflammation, and fever (Engblom et al., 2003; Kamei et al., 2004; Trebino et al., 2003) and a more favorable cardiovascular profile than COX-2 knockout animals (Cheng et al., 2006). To our knowledge, this is the first evidence that selective mPGES-1 inhibitors developed for human use are also selective for equine mPGES-1. Taken together, our data suggests that mPGES-1 is an excellent candidate for investigation as an effective, PGE_2-specific, anti-inflammatory therapeutic target in horses with fewer adverse effects than NSAIDs. Additionally, as horses and humans develop many of the same orthopedic disorders, this study indicates that equine leukocytes are an

exemplary model for studying the role of mPGES-1 and mPGES-1 inhibitors as therapeutics in naturally-occurring inflammatory orthopedic diseases in humans.

We have demonstrated that mPGES-1 mRNA is increased by proinflammatory stimulation in a mixed population of equine blood leukocytes, consistent with data in isolated equine monocytes and leukocytes from other species (Silva et al., 2008; St-Onge et al., 2007; Uematsu et al., 2002). Additionally, we found that cPGES mRNA is constitutively present in equine leukocytes, similar to other cell and tissue types (Tanioka et al., 2000). To our knowledge, this study is the first to evaluate cPGES in blood leukocytes of any species. Interestingly, mPGES-1 mRNA levels peaked at 2 hours, while the enzyme immediately upstream in the PGE_2 synthetic pathway, COX-2, peaked at a slightly later time point (Figure 1A). This information supports previous work in other cell types reporting differential induction kinetics of mPGES-1 and COX-2 and suggests independent regulation in equine leukocytes as well (Stichtenoth et al., 2001). Differential regulation of these enzymes could aid in fine-tuning cellular control of PGE_2 production in response to pro-inflammatory stimuli.

Interestingly, mPGES-1 protein was constitutively present at baseline and following LPS stimulation (Figure 2A-B). A discrepancy in mPGES-1 mRNA and protein levels has been observed in a number of cell types including pancreatic islet cells (Parazzoli et al., 2012) and human neutrophils (St-Onge et al., 2007). Neutrophils make up a significant portion of the equine leukocyte population and provide one of the first lines of defense against infection *in vivo*. Neutrophils must respond quickly to injurious stimuli, and thus might maintain a stable, ready-made source of mPGES-1 protein for rapid PGE_2 production

in early immune responses. The presence of mPGES-1 protein but not mRNA prior to LPS stimulation, as well as a lack of coordinate mPGES-1 mRNA and protein induction following LPS stimulation, could be a consequence of multiple mechanisms. One potential explanation is that mPGES-1 protein is highly stable and not completely transcriptionally-dependent in equine leukocytes; transcription dictates only a fraction of protein levels found in mammalian cells, as post-transcriptional mRNA modifications and translational regulation are also critical, if not more important determinants of cellular protein levels (Vogel & Marcotte, 2012). Additionally, it is possible that mPGES-1 protein turnover is accelerated with increased intracellular enzymatic activity, and increased mPGES-1 mRNA transcription and subsequent translation function to quickly replenish mPGES-1 protein to maintain rapid PGE_2 production. Multiple intracellular enzymes, including COX, produce reactive oxygen species (ROS) during enzymatic activity (Morgan & Liu, 2010). ROS lead to oxidant-damage of multiple intracellular proteins that are then ubiquitinated and degraded by immunoproteasomes (Seifert et al., 2010), resulting in increased protein turnover in inflammatory states. We hypothesize that either or both of these mechanisms could be involved in mPGES-1 protein regulation in equine leukocytes.

In contrast to neutrophils, LPS has been shown to increase mPGES-1 protein in isolated human monocytes, a cell type that contributes significantly to PGE_2 production in leukocyte models (Mosca et al., 2007). While we did not observe mPGES-1 protein induction in our model, these differences may be explained by the high ratio of neutrophils to monocytes in equine whole blood. On average, approximately 43-60% of cells (2,300-8,600 cells/µl) in equine whole blood are neutrophils, whereas only 0-7% (or 0-1,000 cells/µl) are

monocytes (Smith et al., 2015). Thus, our western blotting technique might not be sensitive enough to detect minute increases in mPGES-1 protein contributed by the small monocyte population. Additionally, it is possible that both monocytes and neutrophils serve as a ready-made source of mPGES-1 protein in the horse.

COX-2 mRNA and protein levels were coordinately inducible (Figures 1 and 2), in agreement with previous studies in blood leukocytes (Fasano, 1998; Maloney et al., 1998). In conjunction with constitutive mPGES-1 protein, induction of COX-2 protein coordinately with PGE_2 production in our system suggests that COX-2, and not mPGES-1, is the critical driver of inflammation-induced PGE_2 production in equine leukocytes. This theory has also been suggested in human umbilical vein endothelial cells (Båge et al., 2011). Despite the prominent role of COX-2 in orchestrating PGE_2 production, we have demonstrated that mPGES-1 is equally essential to increasing extracellular PGE_2 concentrations in equine leukocytes, as both selective mPGES-1 inhibition and COX inhibition led to significantly decreased levels of PGE_2 in our model.

LPS stimulation of primed equine leukocytes significantly increased PGE_2 levels in our *in vitro* inflammatory model (Figure 3). Furthermore, a non-selective COX inhibitor (indomethacin), a COX-2 selective inhibitor (NS-398), and an mPGES-1 specific inhibitor (MF63) all significantly decreased extracellular PGE_2 concentrations (Figure 5). Importantly, compared to MF63, indomethacin and NS-398 led to more complete inhibition of PGE_2, with maximal doses abolishing approximately 95% of PGE_2 compared to controls (IC_{50} = 0.0159 and 0.0528uM, respectively) (Figures 4B-C, 5B-C). The maximal dose of MF63 inhibited 87.5% of PGE_2 compared to controls (IC_{50} = 0.1147uM, Figure 4A, 5A), and we hypothesize

that the remaining 12.5% of PGE_2 was synthesized via cPGES (Tanioka et al., 2000) and mPGES-2 (Murakami et al., 2003) coupling to both COX enzymes. Preservation of basal PGE_2 levels is crucial to maintaining renal function and gastroprotection in a clinical setting. We have demonstrated that this is more readily achievable with selective mPGES-1 inhibition compared to COX inhibitors, as COX-1 and COX-2 remain available for coupling to constitutive PGE synthases to mediate homeostatic PGE_2 production. COX-2 specific inhibitors suppress COX-2-derived PGH_2 formation and eliminate all PGE_2 production mediated through mPGES-1 or mPGES-2. Dual COX-1 and 2 inhibitors inhibit all PGH_2 formation, and thus inhibit both inducible and basal PGE_2 synthesis through all PGE synthases. Thus, our data supports that selective mPGES-1 inhibition significantly decreases the elevation of extracellular PGE_2 concentrations following GM-CSF/LPS treatment, while maintaining more substantial and presumably advantageous basal levels of PGE_2 in equine leukocytes compared to COX inhibitors.

Indomethacin and NS-398 significantly inhibited multiple prostanoids, including PGE_2, TXA_2, and PGI_2, while MF63 specifically inhibited PGE_2 (5). Non-specific prostanoid inhibition is associated with unwanted and potentially lethal side effects in horses (Cook & Blikslager, 2014; Marshall & Blikslager, 2011) as inhibition of both basal (COX-1-mediated) and induced (COX-2-mediated) prostanoid levels can cause gastrointestinal inflammation, ulceration and bleeding, renal dysfunction, and disturbances in cardiovascular homeostasis (Sharma & Jawad, 2005). While coxibs display improved gastrointestinal safety, they are associated with a risk of dangerous adverse cardiovascular events in humans**,** possibly as a result of differential effects on the prothrombotic and antithrombotic

prostanoids, TXA_2 and PGI_2 (Grosser, 2005; Sharma & Jawad, 2005). Consistent with data in other species (Koeberle et al., 2010; Mbalaviele et al., 2010; Xu et al., 2008), our data supports that mPGES-1 inhibition is selective for PGE_2 in horses, significantly reducing extracellular PGE_2 concentrations without altering TXA_2 and PGI_2 levels (Figure 4). This PGE_2 selectivity, coupled with maintenance of basal PGE_2 levels (Figure 4A), indicates that therapeutic mPGES-1 inhibition would have similar efficacy and a safer gastrointestinal, renal, and cardiovascular profile compared to traditional and COX-2 selective NSAIDs in horses.

A possible consequence of mPGES-1 inhibition is redirection of the PGH_2 precursor to other prostanoid synthases, leading to potentially dangerous outcomes. Previous *in vivo* and *in vitro* models have had mixed results. In some studies, mPGES-1 inhibition or deletion leads to a shunting of PGH_2 to other prostanoid synthases and increased production of TXA_2, PGI_2, or PGF_{2a} (Kapoor et al., 2006; Leclerc et al., 2013; Xu et al., 2008). This was not observed in our system, as neither PGI_2 nor TXA_2 were significantly increased over baseline. This is in agreement with Bruno 2010 *et al,* who did not observe shunting to other prostanoids in human whole blood following mPGES-1 inhibition. This author postulates that instead, PGH_2 might quickly diffuse or be transported out of blood cells to be degraded and not converted to other end-point prostanoids. While our data is promising, studies will need to be conducted *in vivo* in horses to evaluate potential prostanoid shunting and adverse effects.

We have demonstrated that equine leukocytes are an excellent model for human mPGES-1 inhibitor studies. MF63 has been reported to have IC_{50} values of 1.3uM (Côté et

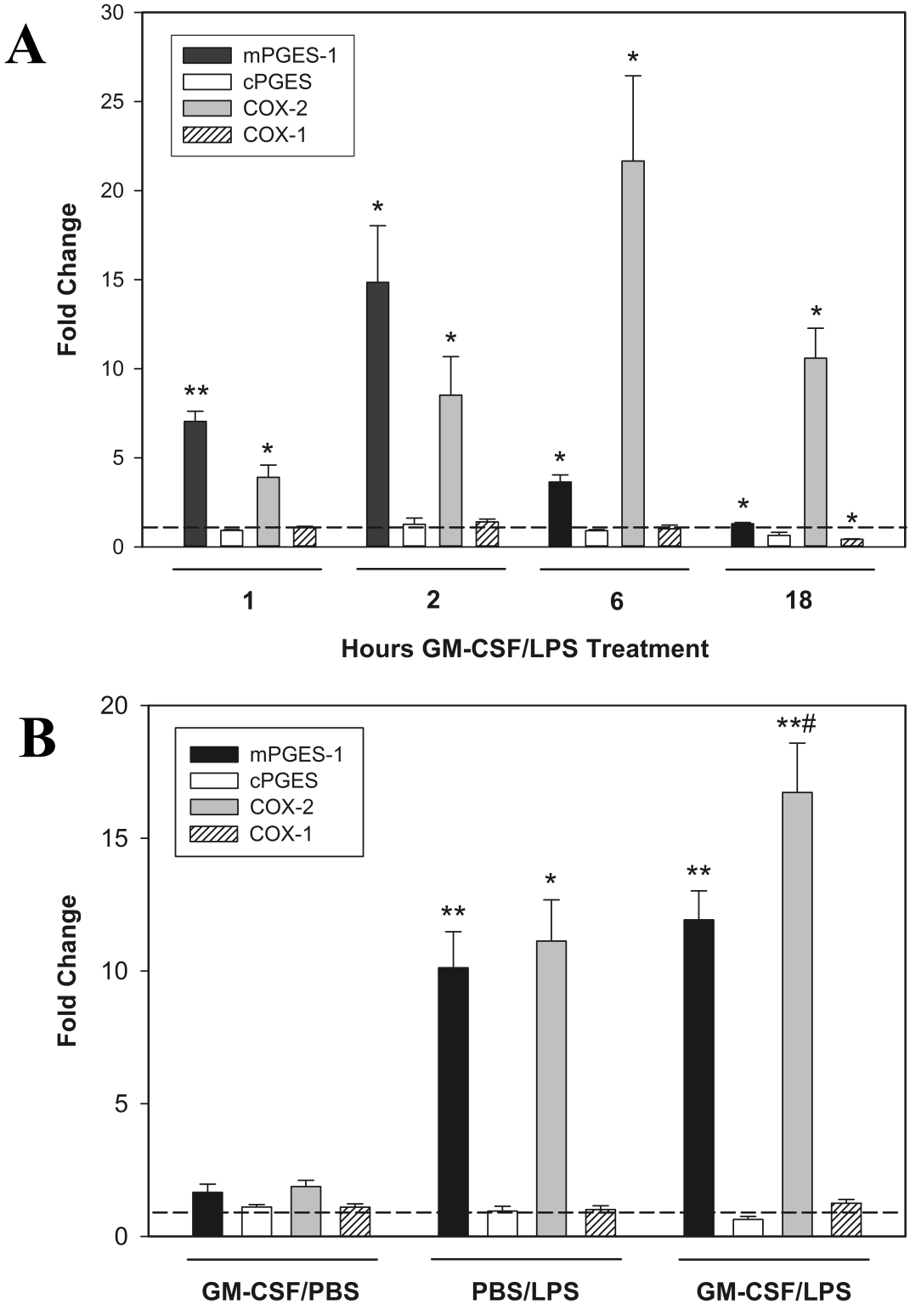
al., 2007) and 0.8uM (Xu et al., 2008) for LPS-stimulated PGE_2 in two different human whole blood assays, which are similar to the IC_{50} value obtained in equine leukocyte-rich plasma of 0.1147uM (Figure 4A). Additionally, previous studies report a lack of activity of phenanthrene imidazole derivatives, such as MF63, against mouse and rat mPGES-1 (Xu et al., 2008), suggesting that equine leukocytes are a better-suited translational model for studying these mPGES-1 inhibitors. Importantly, equine athletes and humans develop similar orthopedic diseases and injuries, and thus horses are an excellent model for assessing the importance of mPGES-1 inhibition in naturally-occurring inflammatory orthopedic disease in humans.

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 dangerous adverse effects of NSAIDS. Critically, this data also establishes that horses are an exemplary model for human mPGES-1 studies and could be especially useful for evaluating mPGES-1 as therapeutic target in naturally-occurring human inflammatory orthopedic diseases. Future studies using this system will seek to uncover the specific mechanisms regulating mPGES-1 induction in equine leukocytes in order to further elucidate all available mechanisms for targeting this enzyme. Additionally, *in vivo* approaches will be utilized to evaluate the efficacy of mPGES-1 inhibition in equine inflammatory disease.

6. Figures

Figure 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 (1ng/mL) or control for 30 minutes, followed by stimulation with LPS (100ng/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 follows: priming agent/stimulating agent. **(A)** mRNA kinetics in GM-CSF/LPS treated leukocytes over 18 hours. Data are expressed as mean fold change ± SEM over time-matched controls (PBS/PBS), denoted by dotted line equal to 1, and represents three different donor horses. $^{**}p < 0.001$ and $^{*}p < 0.05$ via two-tailed t-test compared to time-matched PBS/PBS. **(B)** mRNA levels in leukocytes primed and stimulated for 2 hours with indicated treatments. Data are expressed as in (A) and represent three different donor horses. $^{**}p < 0.001$ and $^{*}p < 0.05$ compared to enzyme-matched control (PBS/PBS) and GM-CSF/PBS levels, and $\#p < 0.05$ compared to enzyme-matched PBS/LPS levels via One Way ANOVA.


A
mPGES-1
cPGES
COX-2
COX-1
Fold Change
30
25
20
15
10
5
0
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*
*
*
*
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*
1
2
6
18
Hours GM-CSF/LPS Treatment
B
mPGES-1
cPGES
COX-2
COX-1
Fold Change
20
15
10
5
0
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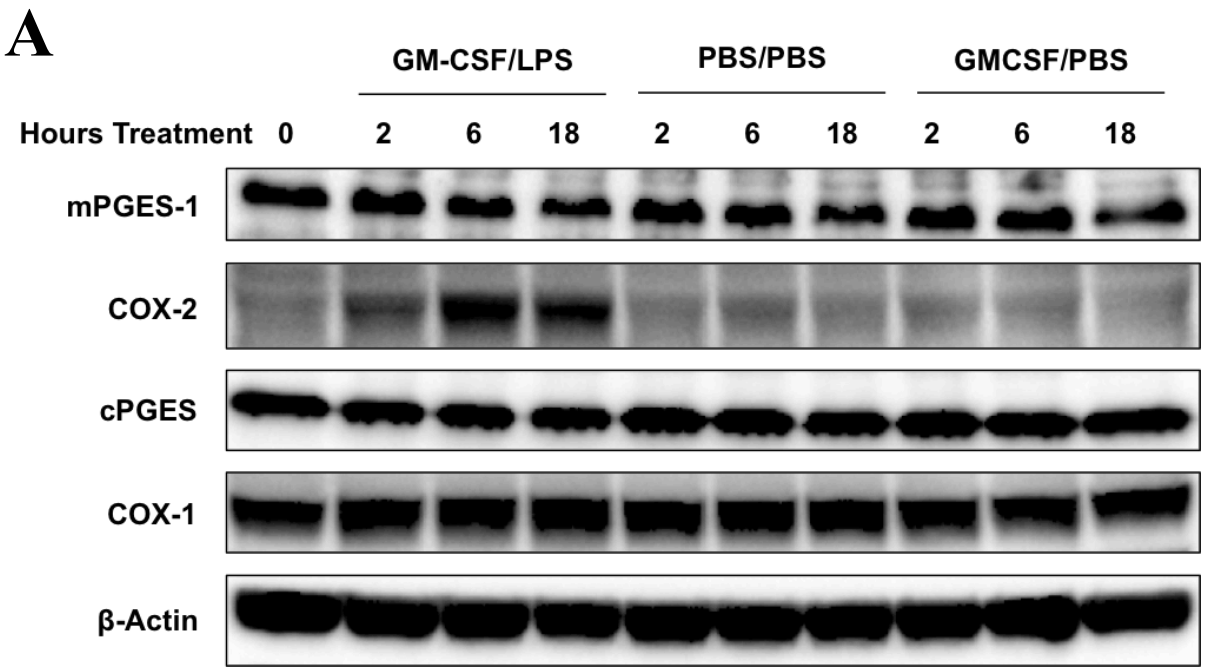
*
**
**#
GM-CSF/PBS
PBS/LPS
GM-CSF/LPS

Figure 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 (1ng/mL) or control for 30 minutes, followed by stimulation with LPS (100ng/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 follows: priming agent/stimulating agent. **(A)** Representative western blots and **(B-C)** quantification of mPGES-1 and COX-2 protein content from three independent experiments from different donor horses. 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.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GM-CSF/LPS
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COX-2
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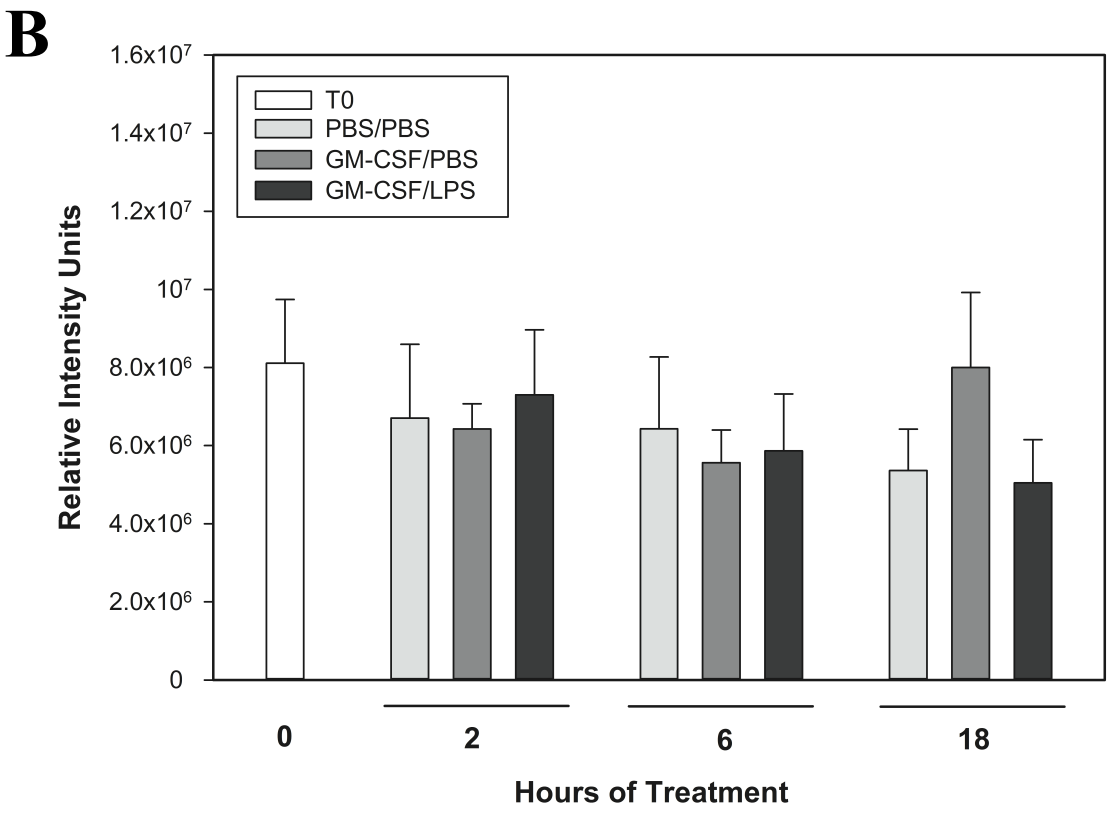
COX-1
β-Actin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GM-CSF/PBS
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Relative Intensity Units
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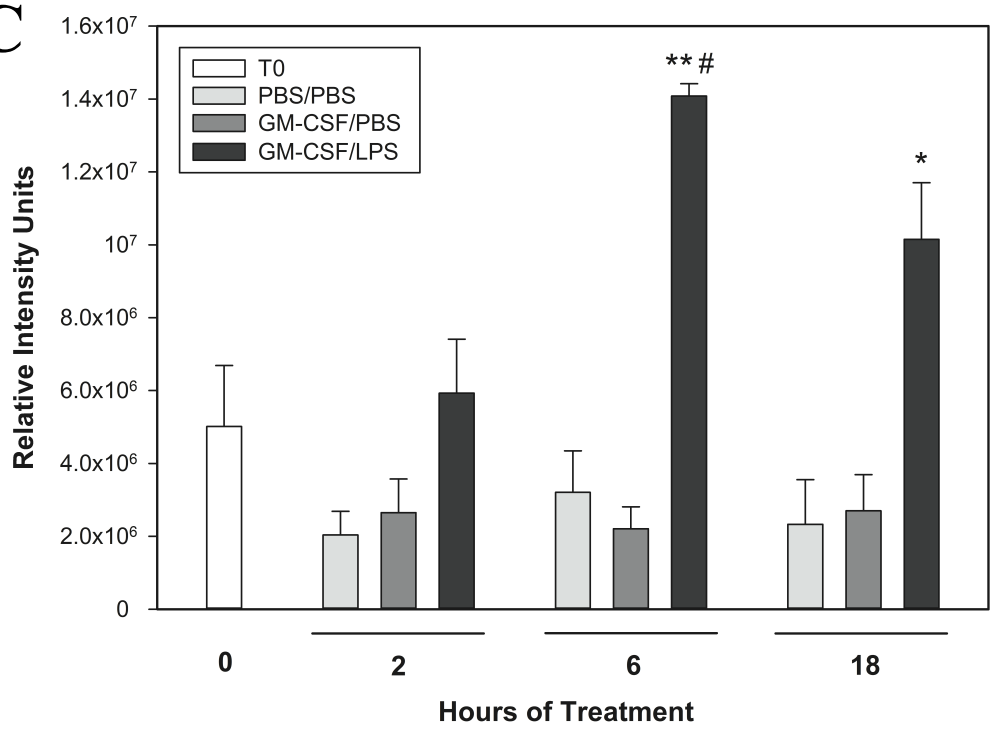
2
6
18
Hours of Treatment

C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Hours of Treatment

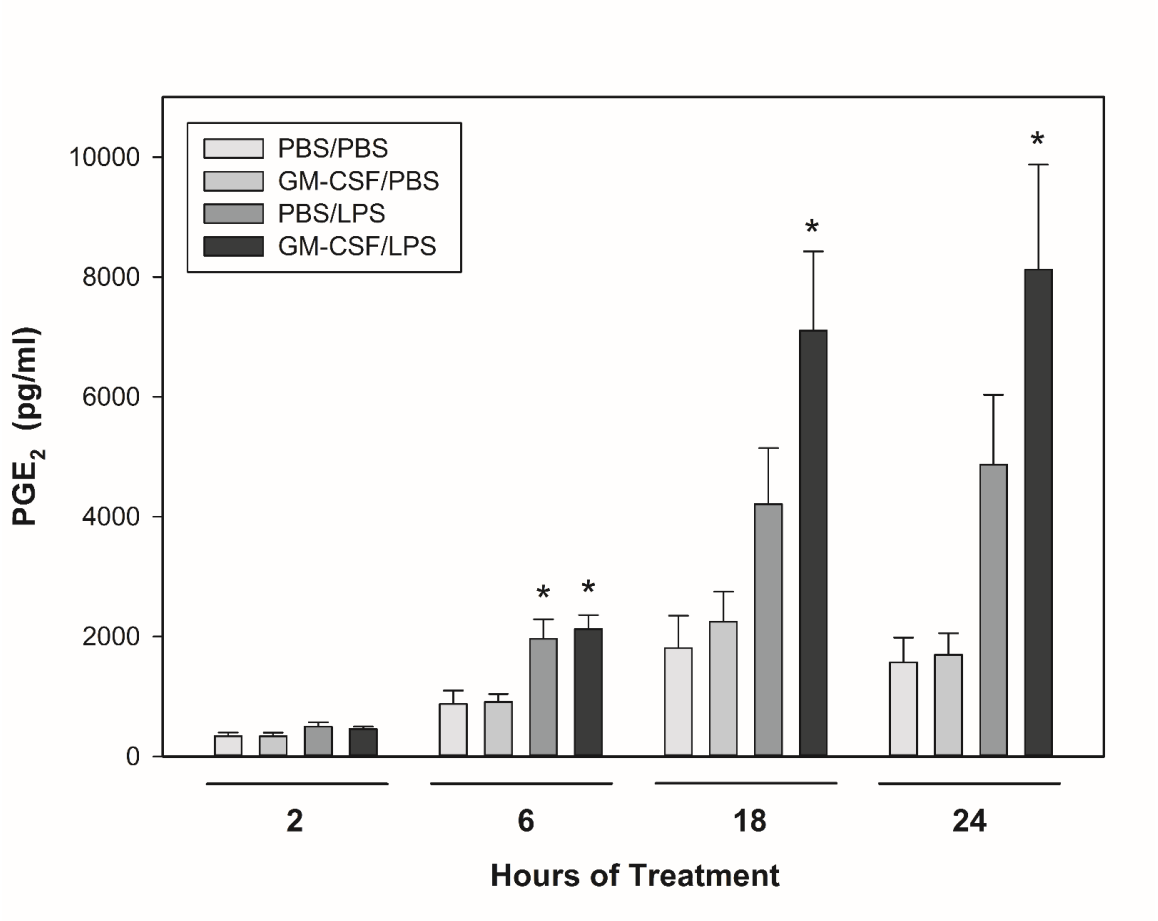
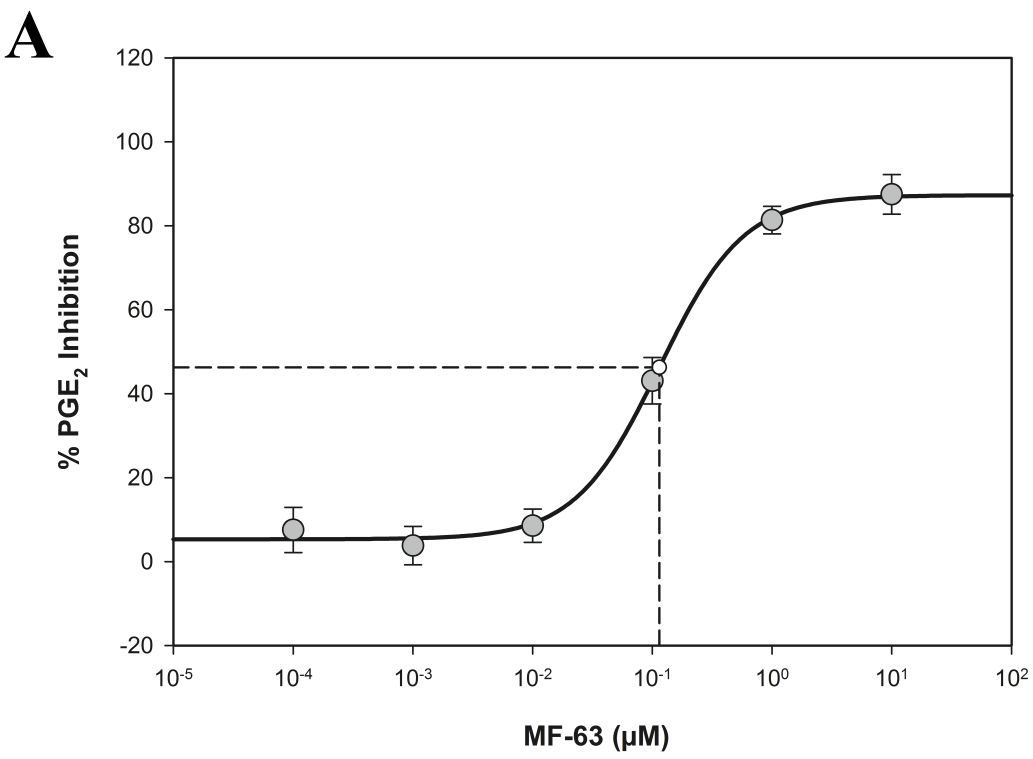


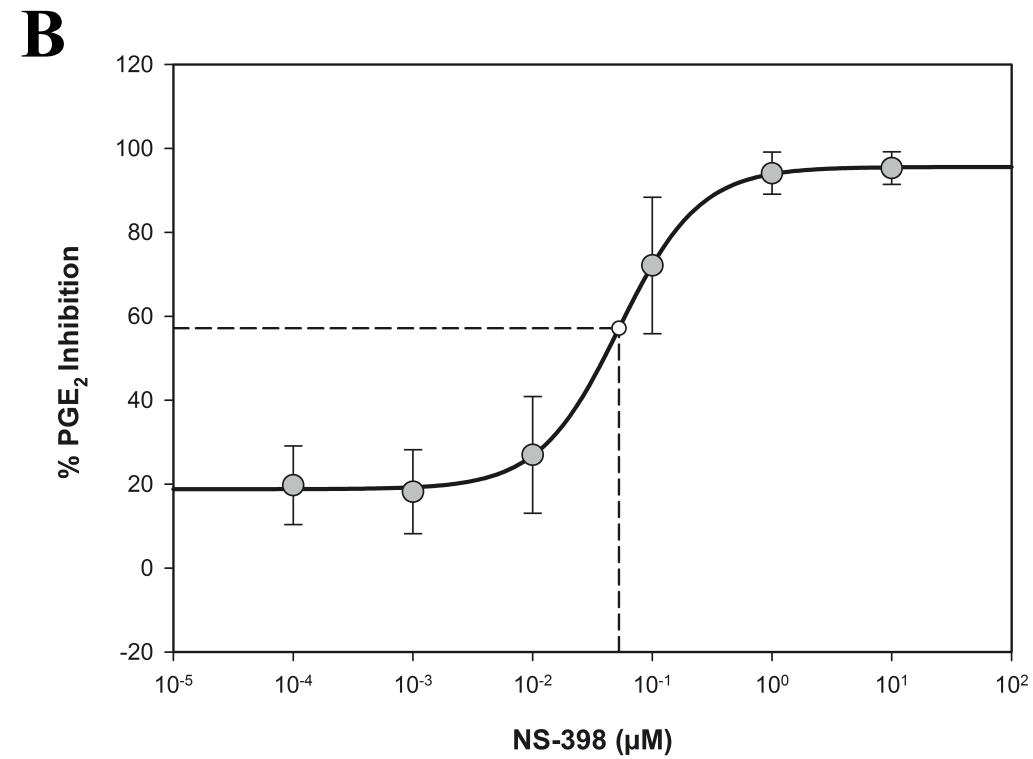
Figure 3: LPS stimulation significantly increases PGE_2 levels in GM-CSF primed equine leukocyte cell supernatants.

Equine leukocytes were primed with GM-CSF (1ng/mL) or control for 30 minutes, followed by stimulation with LPS (100ng/mL) or control for indicated times. PGE_2 levels were assessed via ELISA. Treatment designations are structured as follows: priming agent/stimulating agent. Values represent mean PGE_2 production ± SEM in pg/ml of six independent experiments from different donor horses. * $p<0.05$ compared to time-matched PBS/PBS and GM-CSF/PBS samples, via One-Way ANOVA. Data at 24 hours were log transformed for analysis, but back transformed for data presentation.

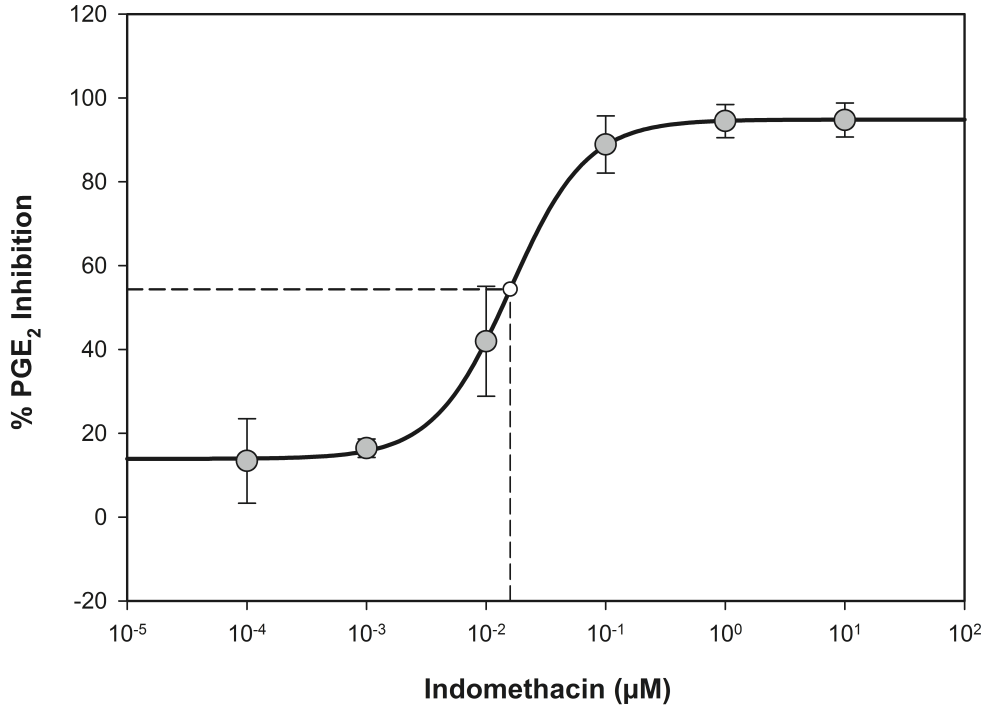
Figure 4: MF63, NS-398, and indomethacin decrease PGE_2 in equine leukocyte cell supernatants.

Equine leukocytes were primed with GM-CSF (1ng/ml), followed by stimulation with LPS (100ng/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 hours. PGE_2 production was assessed via ELISA**.** Data represent % inhibition of PGE_2 production ± SEM compared to cells stimulated in the presence of the vehicle for each inhibitor (0.04% DMSO), plotted against $\log_{10}$ inhibitor concentration. IC_{50} values were calculated for MF63 (0.1147uM), NS-398 (0.0528uM), and indomethacin (0.0159uM). Experiments represent three different donor horses.


A
120
100
80
60
40
20
0
-20
% PGE2 Inhibition
10-5
10-4
10-3
10-2
10-1
100
101
102
MF-63 (µM)


B
120
100
80
60
40
20
0
-20
% PGE2 Inhibition
10-5
10-4
10-3
10-2
10-1
100
101
102
NS-398 (µM)

C


120
100
80
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40
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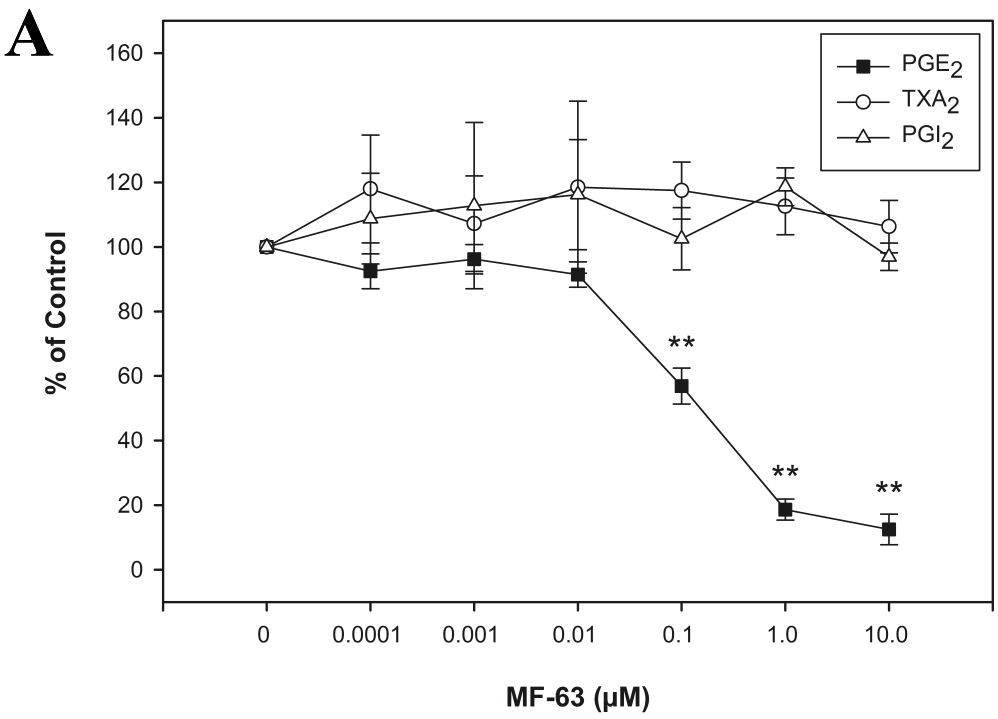
0
-20
% PGE2 Inhibition
10-5
10-4
10-3
10-2
10-1
100
101
102
Indomethacin (μM)

Figure 5: Inhibition of mPGES-1 selectively decreases PGE_2, but not TXA_2 or PGI_2 in LPS-stimulated, GM-CSF primed equine leukocyte cell supernatants.

Equine leukocytes were primed with GM-CSF (1ng/ml), followed by stimulation with LPS (100ng/ml) in the presence of (A) a selective mPGES-1 inhibitor (MF63), (B) a selective COX-2 inhibitor (NS-398), (C) a nonselective COX inhibitor (indomethacin), or vehicle control (0.04% DMSO) for 18 hours. PGE_2, TXB_2 (stable metabolite of TXA_2), and 6-keto-Prostaglandin $F_{1\alpha}$ (stable metabolite of PGI_2) 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). $^{**}p<0.001$ and $^{*}p<0.05$ PGE_2 secretion, $^{\#\#}p<0.001$ and $^{\#}p<0.05$ TXA_2 secretion, and $^{\dagger\dagger}p<0.001$ and $^{\dagger}p<0.05$ PGI_2 secretion compared to prostanoid-matched control, via One-Way ANOVA. In (C), PGI_2 and TXA_2 values were log transformed for analysis and back transformed for data


A
PGE2
TXA2
PGI2
% of Control
160
140
120
100
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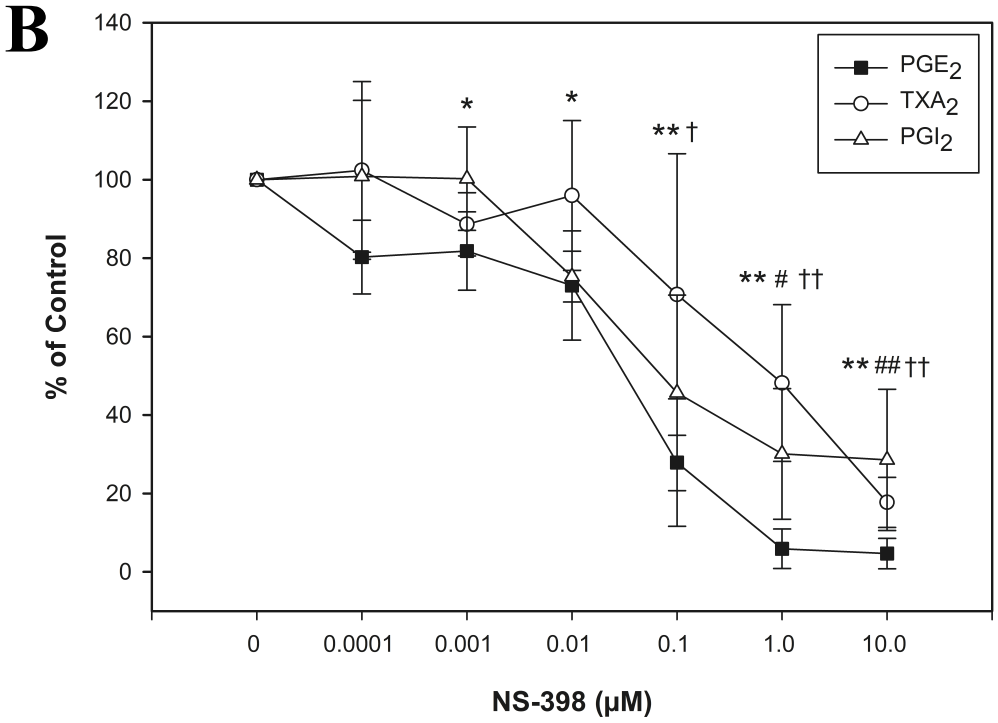
10.0
MF-63 (μM)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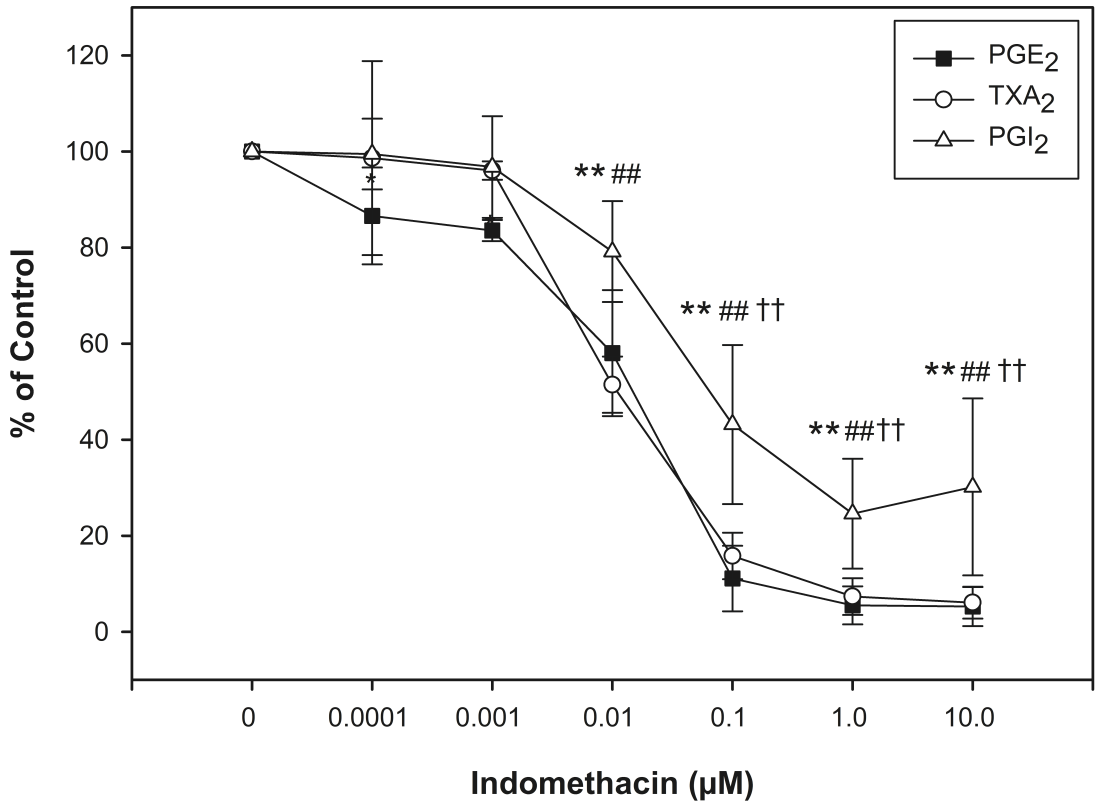
NS-398 (μM)

C


PGE2
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Chapter III

The PGE_1 analog misoprostol inhibits equine neutrophil adhesion, chemotaxis, and respiratory burst in an *in vitro* model of inflammation

Emily G. Martin[1], Rebecca L. Till[1], Samuel L. Jones[1,2]

1Department of Clinical Sciences and, 2Comparatie Medicine Institute, College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27607 USA

1. Abstract

The consequences of neutrophilic inflammation are a double-edged sword; neutrophils are absolutely necessary for clearance of microbial pathogens but can also inadvertently elicit tissue injury. In equine inflammatory disease, tightly controlled neutrophil functions such as endothelial adhesion, intraluminal crawling, interstitial tissue migration, and reactive oxygen species (ROS) production, can become dysregulated, excessive, and exacerbate disease. Anti-inflammatory therapeutics that are currently available are either ineffective or elicit dangerous adverse effects. Therefore, novel mechanisms of inhibiting neutrophil function are needed. One potential neutrophil-targeting strategy is the PGE_1 analog misoprostol. Misoprostol is a gastroprotectant that induces formation of the secondary messenger molecule cyclic AMP (cAMP), which has been shown to exert many anti-inflammatory effects on neutrophils. Misoprostol is currently used in horses to treat NSAID-induced gastrointestinal injury, however its effects on equine neutrophils have not been determined. We hypothesized that pretreatment of equine neutrophils with misoprostol would inhibit neutrophil adhesion, chemotaxis, and ROS production in response to multiple pro-inflammatory stimulants. Results of this study indicate that misoprostol inhibits equine neutrophil adhesion induced by LTB_4, but not immune complexes (IC) or PMA. In addition, misoprostol inhibited equine neutrophil chemotaxis towards LTB_4, CXCL8, and PAF in an *in vitro* chemotaxis plate assay. Furthermore, misoprostol also inhibited LPS-, IC-, and PMA-induced ROS production, as measured via luminol-enhanced chemiluminescence. This data

indicates that misoprostol is a potent regulator of neutrophil effector functions *in vitro* and inhibits multiple mechanisms of neutrophil-mediated tissue injury.

2. Introduction

Neutrophils provide a first line of defense against bacterial pathogens in both humans and horses. Upon infection, neutrophils move from the vasculature into areas of tissue infection by undergoing intricate mechanisms of recruitment and activation. These steps include endothelial adhesion, crawling along the vascular lumen, extravasation, interstitial tissue migration, and culminates in release bactericidal products such as reactive oxygen species (ROS) and antibacterial proteins (Borregaard, 2010; Kolaczkowska & Kubes, 2013; Pick et al., 2013; Sadik et al., 2011). While these steps are necessary to defend the host against pathogens, disregulated or overabundant neutrophil responses occur in many disease states and elicit substantial tissue injury (Mittal et al., 2014; Wong et al., 2012)**.** Neutrophils have been implicated in the pathogenesis of many devastating disorders in horses, including laminitis (la RebiÃ re de Pouyade & Serteyn, 2011), heaves (Bullone & Lavoie, 2015; Leclere et al., 2011), and gastrointestinal ischemia-reperfusion injury (Moore et al., 1995). Therefore, there is a significant need for neutrophil-targeted therapeutics in equine medicine.

Currently, therapies designed to inhibit neutrophilic inflammation in humans and animals are limited (Schofield et al., 2013). Thus, it is imperative that novel mechanisms of neutrophil inhibition are investigated. One potential neutrophil-targeting strategy is elevation of intracellular cyclic AMP (cAMP). cAMP is a ubiquitously produced second messenger molecule that is generated through G-protein coupled receptor (GPCR) signaling in neutrophils. Ligand binding to GPCRs leads to activation of intracellular adenylate cyclase (AC), which catalyzes the cyclization of AMP to form cAMP. cAMP activates two different

intracellular pathways, protein kinase A (PKA) and exchange proteins directly activated by cAMP (Epac), that mediate neutrophil adhesion (Bloemen et al., 1997; Chilcoat et al., 2002), chemotaxis (Harvath et al., 1991), and production of ROS (Bengis-Garber & Gruener, 1996; Chilcoat et al., 2002; Wang et al., 2003). Interestingly, the two pathways activated by cAMP have been shown to induce contradictory cellular functions: PKA elicits predominantly inhibitory effects within neutrophils, while Epac signaling is associated with cellular stimulation (Cheng et al., 2008). Recently, it has been suggested that PKA is the preferred pathway acted upon by cAMP within neutrophils, and thus agents that elevate cAMP hold great promise as inhibitors of neutrophil function in inflammatory disease (Scott et al., 2016).

Naturally-occurring cAMP elevating agents include E-type prostaglandins (PGEs) such as PGE_1 and PGE_2. Binding of PGEs to E prostanoid (EP) 2 and EP4 receptors increases intracellular cAMP and attenuates multiple neutrophil functions *in vitro* (Armstrong, 1995; Hirata & Narumiya, 2012; Hong, 1996; Sugimoto & Narumiya, 2007; Talpain et al., 1995; Wise, 1996). However, clinical use of prostaglandins is limited due to instability, poor absorption, and inadequate oral availability. PGE analogs have been developed that demonstrate better stability and absorption, including the PGE_1 analog misoprostol (Smallwood & Malawista, 1995). Misoprostol is an EP2, EP3, and EP4 receptor agonist that increases intracellular cAMP and is FDA-approved to treat NSAID-induced ulceration in humans due to its protective effects in the gastrointestinal (GI) tract (Ahluwalia et al., 2014; Blikslager, 2012). In horses, misoprostol has been shown to decrease gastric acid secretion, increase recovery of ischemia injured equine jejunum, and is currently used to treat NSAID-induced colitis and ulceration (Marshall & Blikslager, 2011; Sangiah et al.,1989;

Tomlinson & Blikslager, 2005). While these benefits alone justify the use of misoprostol in NSAID-treated patients, misoprostol could provide additional anti-inflammatory benefits in horses suffering from neutrophil-mediated disease. The anti-inflammatory properties of misoprostol have not been studied in equine neutrophils, and therefore our goal was to evaluate misoprostol as a novel anti-inflammatory therapeutic in horses. We hypothesized that the PGE_1 analog misoprostol would inhibit equine neutrophil effector functions *in vitro*. This study is the first to demonstrate that pretreatment with misoprostol leads to concentration-dependent inhibition of equine neutrophil adhesion, chemotaxis, and ROS production in response to multiple pro-inflammatory stimulants.

3. Materials and Methods

Experimental Reagents

Lipopolysaccharide (LPS) from E. coli 055:B5, phorbol 12-myristate 13-acetate (PMA), CXCL8, dibutyryl cyclic-AMP (db-cAMP), wortmannin, staurosporine, bovine serum albumin (BSA), and anti-BSA antibody were from Sigma Aldrich (St. Louis, MO); heat-inactivated fetal bovine serum (FBS) was from Gemini-Bioproducts (West Sacramento, CA); misoprostol, LTB_4, and PAF were from Cayman Chemical (Ann Arbor, MI); equine recombinant granulocyte-monocyte colony-stimulating factor (GM-CSF) was from Kingfisher Biotech (Saint Paul, MN); and hanks balanced salt solution (HBSS) was from Thermo Fischer Scientific (Grand Island, NY).

Equine Donors and Neutrophil Isolation

All experiments were approved by the Institutional Animal Care and Use Committee at North Carolina State University (NCSU). Horses included in this study were part of the NCSU Teaching Animal Unit herd, 5-15 years of age, and of mixed breed and gender. All horses were housed under similar conditions and did not receive any medications for the duration of the study.

Neutrophils were isolated from equine whole blood by density-gradient centrifugation as previously described (Sheats et al., 2014). Briefly, 30-60 cc of heparinized equine whole blood was collected via jugular venipuncture. Whole blood was placed into sterile conical tubes for 1 hour at room temperature to allow erythrocytes to settle out of suspension. The leukocyte-rich plasma layer (supernatant) was layered onto Ficoll-Paque Plus (GE Healthcare, Sweden) at a 2:1 ratio. Cells were centrifuged and erythrocyte contamination was removed from the neutrophil pellet via a 1-minute hypotonic lysis procedure.

Misoprostol Pretreatment

Neutrophils were pretreated with various concentrations of misoprostol, db-cAMP, wortmannin, staurosporine, or vehicle for each inhibitor, for 30 minutes at 37°C. Cell viability was evaluated before and after pretreatment using trypan blue exclusion and was routinely >95%.

Neutrophil Adhesion

Equine neutrophil adhesion methods have been optimized in our lab previously (Sheats et al., 2014). Neutrophils were resuspended to a concentration of $1x10^7$ cells per ml in HBSS. 2μg/mL of the fluorescent dye calcein am (Anaspec, Fremont, CA) was added to cells and incubated in the dark at room temperature for 30 minutes. Following calcein labeling, cells were resuspended in HBSS supplemented with 1mM Ca^{2+}, 1mM Mg^{2+}, and 2% fetal bovine serum.

For immune complex (IC)-induced adhesion, Immulon2HB plates (Thermo Fischer Scientific) were coated with 10 μg BSA overnight at 4º C and then incubated at 37º C for 2 hours with 5 μg of anti-BSA antibody. $1x10^5$ cells were plated per well and incubated for 30 minutes at 37º C. Wells that were not coated with anti-BSA antibodies served as unstimulated controls. For LTB_4- and PMA-induced adhesion, plates were coated overnight with 5% FBS at 4º C. $1x10^5$ cells were plated in each well and allowed to rest at 37º C for 10 minutes before addition of 10 ng/ml PMA (or $1x10^{-5}$ % DMSO vehicle) or 10nM LTB_4 (or $3x10^{-3}$ % ethanol vehicle). Cells were incubated at 37º C for 30 minutes with PMA or 75 seconds with LTB_4.

Following stimulation in all treatments, fluorescence in each well was read on an fMax plate reader (485nm excitation, 530nm emission) then washed 2 (LTB_4) or 3 (IC and PMA) times before final read. The number of plate washes performed was determined as the first wash where fewer than 10% of unstimulated neutrophils were adherent to the plate. Percent adhesion was calculated as the difference between the initial and final fluorescence readings in each well.

Neutrophil Chemotaxis

Equine neutrophil chemotaxis methods have been optimized in our lab previously (Cook et al., 2009b; Sheats et al., 2014). Neutrophils were labeled with calcein am and resuspended in media as described above for adhesion experiments. Neuroprobe Disposable ChemoTx Systems (Neuroprobe, Gaithersburg, MD) with 3 micron pore size and polycarbonate track-etch (PCTE) filters were used for chemotaxis experiments. Cell media containing chemoattractant or vehicle was added to the bottom chamber of each well. The following chemoattractants were utilized: 10nM LTB_4, 10nM PAF, 100ng/mL CXCL8, and vehicle for each chemoattractant ($3x10^{-3}$ % ethanol for LTB_4 and PAF, HBSS for CXCL8). Additionally, 100% migration control wells were prepared by adding $1x10^4$ calcein-labeled cells to the bottom chamber of replicate wells. Porous filters were placed over the bottom chambers so that contact between filter and chemoattractant or control media was established. $1x10^4$ misoprostol-pretreated, calcein-labeled neutrophils were then added in triplicate to the top portion of each well filter and were incubated for 1 hour at 37°C to allow cells to chemotax into bottom chambers (Sheats et al., 2014). Following incubation, non-migrated cells were scraped from the top of the filters and EDTA was added for 10 minutes at room temperature. EDTA was then scraped away and fluorescence of the bottom well was taken using an fMax plate reader as described above. Percent cell migration was determined by percent fluorescence of wells in each treatment group compared to the 100% migration control wells.

Neutrophil Reactive Oxygen Species Production

Production of reactive oxygen species (ROS) was measured using luminol-enhanced chemiluminescence, and has been optimized previously for equine neutrophils (Benbarek et al., 1996). Cells were plated on sterile, white, 96-well high-binding plates (Sigma), which were coated with 5% FBS (for LPS- and PMA-mediated respiratory burst) or 5ug/well IC (for IC- mediated respiratory burst) as described above for adhesion experiments. Neutrophils were then stimulated using three different experimental protocols: (1) priming for 30 minutes with 1ng/mL GM-CSF, followed by stimulation with 100ng/mL LPS (or PBS vehicle); (2) 100ng/mL PMA (or 0.01% DMSO vehicle); or (3) 5ug/well immobilized IC (or no IC unstimulated control). 1mM luminol was added to each well, and luminesce was measured using a Fluoroskan Ascent FL Microplate Fluorometer and Luminometer (Thermo Scientific).

As this assay had not yet been attempted in our lab, preliminary experiments were conducted prior to data collection to determine the optimal number of neutrophils to be used for each stimulant. A standard curve was created by plotting neutrophil cell numbers, versus raw luminescence values produced by increasing quantities of neutrophils treated with each stimulant. From this curve, neutrophil quantities that fell on the linear portion of the curve were selected for analysis (data not shown). In accordance with this preliminary data, neutrophils were resuspended to achieve a final concentration of $3x10^5$ cells/well (IC), $2x10^5$ cells per well (LPS), or $1x10^5$ cells/well (PMA) in HBSS supplemented with 10μM Ca^{2+}, 10μM Mg^{2+}, and 2% FBS.

A series of kinetics studies were completed to determine the time of maximal significant ROS production in response to each stimulant, and were determined to be 35 minutes for LPS, 40 minutes for PMA, and 55 minutes for IC. The effects of misoprostol pretreatment on stimulated ROS production were then evaluated at those time points. ROS production in misoprostol-pretreated cells was determined as a percentage of stimulated cells that were pretreated with the misoprostol vehicle (PBS in experimental protocol (1), or HBSS in experimental protocols (2) and (3), above). ROS production from one horse was considered a significant outlier via the ESD (extreme studentized deviate) method and was excluded from analysis ($\alpha = 0.05$).

Statistical Analysis

Data were analyzed using SigmaPlot (Systat Software, San Jose, CA). All data were normally distributed (Shapiro-Wilk test) and are presented as mean ± SEM. Significant differences between treatments were determined via One-Way Repeated Measures Analysis of Variance (One-Way RM ANOVA) with Holm-Sidak multiple comparisons *post hoc* testing, or via two-tailed t-test, where appropriate. A p value <0.05 was considered statistically significant.

4. Results

4.1 Misoprostol pretreatment inhibits LTB_4-, but not PMA- or immune complex-induced equine neutrophil adhesion

To enter areas of tissue inflammation, circulating peripheral blood neutrophils must first arrest to endothelial cells neighboring injured tissues. This requires transient chemoattractant-induced adhesion, followed by firmer adhesion of activated neutrophils. Both adhesion events are mediated by β2 integrins (Langereis, 2013). Increased intracellular cAMP has been shown to inhibit β2 integrin-dependent adherence of equine neutrophils (Bloemen et al., 1997; Chilcoat et al., 2002), and thus we hypothesized that the cAMP-elevating agent misoprostol would decrease equine neutrophil adhesion in an *in vitro* assay. Furthermore, we hypothesized that the cell-permeant cAMP analog, db-cAMP, would serve as a model for increased intracellular cAMP and a positive control for inhibition of adhesion in this study. In addition, because integrin binding ability, strength, and duration differ in response to particular adhesion stimulus, we wanted to evaluate the effect of misoprostol on adhesion stimulated by the chemoattractant LTB_4, the FcγR agonist immune complexes (IC), and a direct stimulator of PKC (phorbol 12-myristate 13-acetate, or PMA).

Our results show that 53.5% of cells stimulated with LTB_4 adhered to FBS-coated plates. In contrast, pretreatment with 300μM misoprostol significantly inhibited LTB_4-stimulated adhesion to only 39.8%. Furthermore, in agreement with previous reports (Bloemen et al., 1997), only 22.2% of LTB_4-stimulated neutrophils pretreated with the cell-permeant cAMP analog db-cAMP were adherent in our assay (Figure 1A). This finding is

consistent with conclusions proposed by Smallwood *et al*., who suggested that misoprostol inhibits human neutrophil functions through elevation of intracellular cAMP (Smallwood & Malawista, 1995). The PI3K-inhibitor wortmannin was unable to inhibit LTB_4-stimulated equine neutrophil adhesion in this assay (data not shown), in agreement with findings by Jones *et. al.* that chemoattractant-stimulated adhesion is PI3K-independent in human neutrophils (Jones et al., 1998). Thus, our results extend these findings and indicate that chemoattractant-stimulated adhesion is also PI3K-independent in equine neutrophils. In contrast, the PKC inhibitor staurosporine significantly inhibited LTB_4-induced adhesion to 33.4%, supporting a role for PCK in chemoattractant-induced β2 integrin adhesion in equine neutrophils (Figure 1A).

Consistent with previous results from our lab (Sheats et al., 2014), IC stimulated 71.3% of equine neutrophils to firmly adhere. However in contrast to previous findings (Chilcoat et al., 2002), pretreatment of cells with misoprostol and db-cAMP had no significant effect on IC-induced adhesion (Figure 1B). Pretreatment with the PI3K inhibitor wortmannin significantly inhibited IC-induced adhesion in this assay to 12.1%, in accordance with findings by Jones *et. al.* that FcγR-mediated β2 integrin adhesion is PI3K-dependent in human neutrophils (Jones et al., 1998).

PMA stimulated 67.5% of equine neutrophils to adhere to FBS-coated plates, which is similar to previous findings (Sheats et al., 2014). db-cAMP had no significant effect on PMA-mediated neutrophil adhesion, however misoprostol pretreatment significantly enhanced PMA-induced adhesion to a maximum of 87.5%, supporting previous reports (Chilcoat et al., 2002). Neutrophil pretreatment with the PKC inhibitor staurosporine was

utilized as a positive control for inhibition in this assay and significantly inhibited PMA-induced equine neutrophil adhesion to 48.0% (Figure 1C).

4.2 Misoprostol pretreatment inhibits equine neutrophil migration towards LTB_4, CXCL8, and PAF

Following adhesion to the vascular endothelium, neutrophils must migrate along chemoattractant gradients to reach sites of tissue injury or infection. This includes intraluminal crawling along the vascular endothelium, as well as interstitial tissue migration to target sites (Kolaczkowska & Kubes, 2013; R. Pick et al., 2013). Human neutrophil migration is enhanced by 1uM PGE_1 pretreatment in response to fMLP, but inhibited at higher PGE_1 concentrations (Farmer et al., 1991). The effect of the PGE_1 analog misoprostol on neutrophil chemotaxis *in vitro* has not yet been evaluated. Because fMLP is a weak chemoattractant for equine neutrophils (Sedgwick et al., 1987), we utilized CXCL8, LTB_4, and PAF to investigate the effects of misoprostol pretreatment on equine neutrophil chemotaxis.

Concentrations of chemoattractants utilized for migration experiments were chosen based on previous work in our lab (Cook et al., 2009b; Eckert et al., 2009; Sheats et al., 2014). LTB_4 and CXCL8 were the most potent chemoattractants, and stimulated directed migration of 73.6% and 70.8% of equine neutrophils, respectively (Figure 2A-B). PAF was slightly less potent, as only 58.0% of neutrophils migrated towards PAF (Figure 2C). Similar to previously reported data, 1uM misoprostol enhanced neutrophil chemotaxis towards LTB_4 and CXCL8 but did not achieve significance. Higher concentrations of misoprostol

pretreatment inhibited neutrophil chemotaxis in response to LTB_4 and CXCL8 and significantly inhibited chemotaxis at a concentration of 300μM (Figure 2A-B). Misoprostol pretreatment did not enhance PAF-induced chemotaxis, and instead led to concentration-dependent inhibition of neutrophil migration at 100μM misoprostol and above (Figure 2C). In addition, db-cAMP significantly inhibited PAF-mediated neutrophil chemotaxis, while LTB_4- and CXCL8-induced chemotaxis was unaffected (Figure 2). The PI3K inhibitor wortmannin was used as a positive control for inhibition of chemotaxis for all 3 chemoattractants, as PI3K is known to be required for neutrophil migration. Interestingly, 300μM misoprostol more potently inhibited neutrophil migration towards all chemoattractants when compared to db-cAMP. While this could be attributed to different inhibitor concentrations, this potentially implicates an additional cAMP-independent mechanism in inhibition of equine neutrophil chemotaxis.

4.3 Neutrophil production of reactive oxygen species (ROS) is increased by PMA and immune complexes in unprimed cells, and by LPS in GM-CSF primed cells

Once at the site of infection, neutrophils release ROS such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) to kill microbial invaders. Neutrophils are capable of releasing ROS intracellularly within phagosomes containing engulfed microbes, as well as into the surrounding tissues to kill nearby pathogens. Release of ROS into surrounding tissues can cause significant tissue injury in many neutrophil-mediated diseases. Therefore, to detect both intra- and extra-cellular neutrophil ROS production, we utilized the highly-sensitive luminol-enhanced chemiluminescence method (Dahlgren & Karlsson, 1999).

Equine neutrophils stimulated with 5ug of immobilized IC produced a robust ROS response that peaked following 60 minutes of stimulation. This was followed by a steep decline in ROS production over the following 60 minutes (Figure 3A). Because of high horse-to-horse variability, ROS production at this 60-minute point was not considered significantly increased over unstimulated cells. However, the next highest point of ROS production, observed following 55 minutes of IC stimulation, was considered significantly different than controls (Figure 3A).

Stimulation with 100ng/mL PMA also resulted in a robust ROS response in equine neutrophils that was significantly greater than vehicle treated cells by 10 minutes of stimulation. ROS production continued to increase up to 40 minutes post stimulation, at which point a plateau of ROS production was observed (Figure 3B).

Equine neutrophils stimulated with 100ng/mL LPS mounted a small increase in ROS production that was not significantly different than unstimulated controls. In contrast, priming cells with 1ng/mL GM-CSF for 30 minutes prior to LPS stimulation led to a significant increase in ROS production which peaked following 35 minutes of LPS treatment. ROS production then steadily declined over the subsequent 1.5 hours. GM-CSF priming alone did not have a significant effect on ROS production (Figure 3C).

Of note is that LPS treatment of primed equine neutrophils resulted in a less potent ROS response than PMA and IC. Peak luminescence values in these cells were one order of magnitude lower than those produced by IC and PMA treatment, indicating that LPS is a weaker stimulant of equine neutrophil ROS production, even following neutrophil priming (Figure 3).

4.4 Misoprostol pretreatment significantly inhibits LPS-, immune complex-, and PMA-induced equine neutrophil reactive oxygen species production by equine neutrophils

Misoprostol and other cAMP-elevating agents have been demonstrated to inhibit human neutrophil ROS production (Ottonello et al., 1995; Talpain et al., 1995). Therefore, we hypothesized that misoprostol pretreatment would inhibit ROS production by equine neutrophils as well, which has yet to be investigated.

Pretreatment with concentrations ≤1μM misoprostol had no effect on IC-stimulated ROS production. However, a concentration-dependent decrease in ROS was observed when cells were pretreated with misoprostol concentrations at and above 10μM. This effect became statistically significant at 200μM and 300μM misoprostol pretreatment (to 49.4% and 42.9% of control, respectively). Similarly, pretreatment with db-cAMP led to a concentration-dependent decrease in IC-stimulated ROS production at concentrations at and above 750μM (Figure 4A). This supports the theory that misoprostol inhibits ROS production through elevation of cAMP (Ottonello et al., 1995; Smallwood & Malawista, 1995).

Conversely, PMA-stimulated equine neutrophil ROS production was enhanced in response to misoprostol pretreatment in a concentration-dependent manner and was significantly elevated at a concentration of 100μM misoprostol (Figure 4B). This pattern was similar to that observed in PMA-induced adhesion experiments (Figure 1). Multiple doses of db-cAMP had no effect on PMA-stimulated ROS production, which is similar to previously published data (Bengis-Garber & Gruener, 1996). Surprisingly, 300uM misoprostol pretreatment significantly inhibited PMA-mediated neutrophil ROS production (Figure 4B). This indicates that the inhibitory effect of 300μM misoprostol might be attributed to cAMP-

independent mechanisms. The PKC inhibitor staurosporine was used as a positive control for our ability to inhibit PMA-stimulated respiratory burst in equine neutrophils in this assay (Figure 4B).

Misoprostol pretreatment was a potent inhibitor of LPS-stimulated ROS production in primed equine neutrophils. This effect was concentration-dependent, and was observed even at the lowest concentration of misoprostol evaluated (0.001μM). Similarly, db-cAMP pretreatment had a concentration-dependent effect on ROS production as well, which was significantly decreased at all concentrations of db-cAMP measured (Figure 4C).

5. Discussion

The aim of this study was to investigate the effect of misoprostol on equine neutrophil functions including adhesion, chemotaxis, and reactive oxygen species (ROS) production. Surprisingly, we found that misoprostol pretreatment had no effect on IC-induced adhesion, and in fact increased adhesion of neutrophils treated with PMA (Figure 1B-C). In contrast, misoprostol inhibited transient LTB_4-stimulated adhesion of equine neutrophils in our assay (Figure 1A). Furthermore, we demonstrated that misoprostol pretreatment decreased equine neutrophil chemotaxis towards LTB_4, CXCL8, and PAF in a concentration-dependent manner (Figure 2), as well as the production of ROS in response to LPS, IC, and PMA (Figure 4). To our knowledge, this study is the first to establish the anti-inflammatory effects of misoprostol on equine neutrophil functions.

Overabundant and disregulated neutrophil responses elicit substantial tissue damage in many equine diseases including laminitis (la RebiÃ re de Pouyade & Serteyn, 2011), heaves (Bullone & Lavoie, 2015; Leclere et al., 2011), and gastrointestinal ischemia-reperfusion injury (Moore et al., 1995). Previous efforts to develop neutrophil-targeting therapeutics have fallen short clinically (Arslan et al., 2008; Baran et al., 2001; Faxon et al., 2002; Flaherty et al., 1994; Mertens et al., 2006; Richard et al., 1988; Schofield et al., 2013), thus the inhibitory effect of misoprostol on equine neutrophil functions is exciting. Misoprostol has been shown to exert protective effects *in vivo,* such as inhibition of neutrophil tissue influx and oxidative damage in mouse models of cerebral ischemia-reperfusion injury and hemorrhage (Li et al., 2008; Wu et al., 2015). Additionally, misoprostol has been shown to reduce edema and leukocyte infiltration in murine models of paw, air pouch, airway, and colonic inflammation (Moraes et al., 2007; Rossetti et al., 1995; Smith et al., 1996; Yamada et al., 1991). Taken together with data from the current study, misoprostol is a promising therapeutic for neutrophil-mediated disease in horses.

EP2 and EP4 receptor agonists increase intercellular cAMP, which is the presumed mechanism through which misoprostol exerts inhibitory effects in neutrophils (Armstrong, 1995; Smallwood & Malawista, 1995; Talpain et al., 1995). Previous reports demonstrate a direct link between elevation of intracellular cAMP and inhibition of equine neutrophil functions, and thus db-cAMP was used to model this phenomenon in our study (Brooks et al., 2011; Chilcoat et al., 2002; Sun et al., 2007). Similarities between the effects of misoprostol and db-cAMP in our system suggest that increased cAMP is the predominant mechanism through which misoprostol inhibits most, but not all equine neutrophil cellular functions

assayed. However, as cAMP levels were not directly measured in response to misoprostol pretreatment, a direct link could not be made.

The first step in neutrophil tissue recruitment is adhesion of circulating blood neutrophils to the vascular endothelium. During this process, interaction of neutrophil G protein-coupled receptors (GPCRs) with endothelial-bound chemoattractants, such as LTB_4, leads to "inside-out" signaling within the neutrophil (Mócsai et al., 2015). Inside-out signaling initiates transient neutrophil adhesion by increasing the ligand-binding affinity and clustering of β2 integrins on the neutrophil cell surface. Engagement of β2 integrins by endothelial cell adhesion molecules (CAMs) results in "outside-in" signaling that leads to increased expression of cell surface β2 integrins, increased β2 integrin affinity for endothelial CAMs, and ultimately induces more stable adhesion (Abram & Lowell, 2009; Kolaczkowska & Kubes, 2013). Experimental inhibition of neutrophil and endothelial CAMs significantly decreases neutrophil tissue influx and reduces tissue damage in IRI models (Mayadas et al., 1993; Wilson et al., 1993; Aversano et al., 1995; Lefer et al., 1994; Palazzo et al., 1998). However, these experimental methods have not been clinically translatable. Thus, there is a critical need for more effective therapeutics that inhibit neutrophil adhesion.

In this study, misoprostol pretreatment inhibited LTB_4- and IC-induced equine neutrophil adhesion in a concentration-dependent manner, but was only considered statistically significant in LTB_4 stimulated cells (Figure 1A-B). We hypothesize that divergent signaling pathways induced by these endogenous stimulants leads to differing effects of misoprostol on transient versus firm neutrophil adhesion. In human neutrophils, GPCR-stimulated adhesion is phosphatidylinositol-3 kinase (PI3K)-independent, while

FcγR-mediated adhesion is PI3K-dependent. However, it is hypothesized that these pathways converge on activation of GTPases that are necessary for cellular adhesion (Jones et al., 1998; Laudanna et al., 1996; Nobes et al., 1995). PKA has been shown to directly inhibit the Rho GTPase, RhoA, which is critical to β2-integrin mediated neutrophil adhesion (Chilcoat et al., 2002; Lang et al., 1996; Laudanna et al., 1996; 1997). However, additional distinct GTPases play a role in adhesion stimulated by different mediators. LTB_4-GPCR signaling stimulates Rho GTPases by activation of guanine nucleotide exchange factors (GEFs) including P-Rex1 and CALDAG-GEF1, which in turn activate Rho GTPase, Rac, and Ras GTPase, Rap1, respectively (Ma et al., 1998; Welch et al., 2002). It has been shown that cAMP-activated PKA can inhibit GPCR-induced activation of P-Rex1 and CALDAG-GEF1, thus inhibiting activation of GTPases necessary for cellular adhesion (Mayeenuddin & Garrison, 2006, Subramanian et al., 2013). Conversely, IC-FcγR-PI3K signaling activates GTPases preferentially by stimulating the GEF Vav (Futosi et al., 2013; Utomo et al., 2006). It has been reported that in contrast to P-Rex1 and CALDAG-GEF1, cAMP-activated PKA increases Vav activity in certain cell types, such as endothelial cells (Birukova et al., 2010), and thus could enhance FcγR-induced neutrophil adhesion. Thus, we hypothesize that PKA's inhibitory effect on RhoA activity could be agonized in GPCR-stimulated cells by PKA-induced inhibition of P-Rex1 and CALDAG-GEF1, or antagonized in IC-stimulated cells by PKA-stimulated Vav. This could explain the weaker inhibitory effects of misoprostol on IC- versus LTB_4-stimulated equine neutrophil adhesion.

An alternative explanation for the different effects of misoprostol on transient and firm neutrophil adhesion pertains to cAMP-activated Epac. Epac and PKA elicit divergent

functions within neutrophils to maintain fine-tuned control of intracellular cAMP signaling. While cAMP-activated PKA generally inhibits neutrophil functions, Epac signaling has been shown to enhance cellular adhesion (Cheng et al., 2008). This effect is mediated through the Ras GTPase Rap1 that is essential for Mac-1-mediated neutrophil and macrophage adhesion (Bergmeier et al., 2007; Lim et al., 2011). We hypothesize that increased intracellular cAMP could potentially activate both PKA and Epac in equine neutrophils, and these pathways could antagonize one another to modulate the effects of misoprostol on transient and firm adhesion.

In contrast to LTB_4 and IC, misoprostol pretreatment led to a dose-dependent increase in PMA-stimulated adhesion that is similar to previous reports from our lab (Chilcoat et al., 2002). PMA is a synthetic mimic of diacylglycerol (DAG), and thus mediates adhesion through a PI3K-independent mechanism. Cell-free assays have demonstrated that Rho GTPases can activate various PKC isoforms and thus may be upstream of PKC signaling (Slater et al., 2001). Therefore, inhibitory effects of cAMP-PKA on upstream Rho GTPases would not affect PMA-stimulated PKC activation. Furthermore, PKC can directly activate RhoA, potentially outcompeting inhibitory effects of PKA on RhoA activity and adhesion (Laudanna et al., 1997). Additionally, as PMA-induced adhesion was increased by misoprostol, it is possible that PKA-stimulated Vav could work synergistically with PMA to increase adhesion through a PI3K-dependent mechanism (Birukova et al., 2010).

Following adhesion, neutrophils crawl along the vascular endothelium and undergo directed interstitial tissue migration in response to chemoattractant gradients. Chemoattractants induce neutrophil migration by engaging GPCRs and activating many

common downstream signaling pathways, including mitogen-activated protein kinases (MAPK), phospholipase C (PLC), and PI3K. These pathways lead to activation of multiple GTPases that are essential for neutrophil chemotaxis, including Rho, Rac, and Cdc42. While many of these mechanisms are shared, each unique GPCR initiates different chemotactic responses, intensities, and migration patterns (Kim & Haynes, 2012). One example of these differences is the lipid-cytokine-chemokine cascade in neutrophil-mediated disease. Lipid chemoattractants predominantly initiate neutrophil chemotaxis into inflamed tissues, while chemokines such as CXCL8 amplify neutrophil chemotaxis in later stages (Sadik & Luster, 2012). Because of these differences, we evaluated the effect of misoprostol on multiple types of chemoattractants including the lipids LTB_4 and PAF, and the chemokine CXCL8.

Misoprostol pretreatment inhibited equine neutrophil chemotaxis towards CXCL8, LTB_4, and PAF (Figure 2). Misoprostol most potently inhibited chemotaxis towards PAF, which was demonstrated to be the weakest inducer of equine neutrophil migration (Figure 2C). LTB_4 and CXCL8 were more potent chemoattractants, and significant inhibition of chemotaxis was only produced at the highest concentration of misoprostol tested (300μM). Interestingly, 1-10μM misoprostol increased chemotaxis towards LTB_4 and CXCL8, however this was not statistically significant (Figure 2A-B). This data agrees with previous reports indicating that low levels of cAMP can stimulate neutrophil migration, while higher cAMP concentrations can inhibit it (Elferink & de Koster, 1993). This could be explained by the critical role of Rac in cellular migration and Rac regulation by PKA. PKA has been shown to activate Rac in migrating neutrophils, leading to lamellapodia formation and directed migration. However, hyper-activation of PKA has also been shown to inhibit

GTPase activation and thus inhibits chemotaxis (Howe et al., 2004). Increased intracellular cAMP, as seen at higher doses of misoprostol, could serve as a hyper-activation signal to inhibit Rac activation. In light of these reports, surprisingly, 1mM db-cAMP did not significantly inhibit LTB_4 or CXCL8-induced chemotaxis (Figure 2A-B). Previous studies have demonstrated that endogenous PGEs inhibit cell migration through EP2, which is liked to increased intracellular cAMP (Armstrong, 1995; Harvath et al., 1991; Smallwood & Malawista, 1995). cAMP-activated PKA inhibits multiple downstream targets, including p38 MAPK, which is necessary for equine neutrophil migration (Costantini et al., 2010; Eckert et al., 2009). However in addition to PKA, cAMP also acts on multiple additional targets to initiate a complex array of intracellular signaling. Studies have shown that cAMP signaling can have divergent effects in human neutrophils that depend on the cAMP-elevating agent and the chemoattractant concentration. One study found that LTB_4-induced chemotaxis was insensitive to the synthetic cAMP agent 8Br-cAMP, but was potently inhibited by PGE_1. Additionally, cAMP has been shown to have little effect on chemotaxis in the presence of optimal concentrations of LTB_4, however significantly inhibited chemotaxis towards threshold LTB_4 concentrations (defined as the lowest concentrations of LTB_4 that elicit a significant chemotactic response) (Harvath et al., 1991). Taken together with our findings, it is likely that the effects of cAMP on equine neutrophil chemotaxis are dependent on the specific cAMP-elevating agent and the chemoattractant concentration utilized.

There are multiple additional mechanisms that could cause misoprostol to have different effects on neutrophil chemoattractant responses. Our first hypothesis is that distribution of specific chemoattractant receptors and downstream PKAs, ACs, or

phosphodiesterases in equine neutrophils could lead to cAMP compartmentalization. This limits the availability of critical intracellular secondary messenger molecules and modulates their effects throughout the cell (Zaccolo et al., 2006). A second potential explanation for these differences could be an unidentified cAMP-independent mechanism. In one study, cAMP was not implicated in PGE_2-medaited inhibition of chemotaxis in human neutrophils, however the alternative pathway through which EP2 suppressed neutrophil chemotaxis was not found (Armstrong, 1995). Previous reports also demonstrate that PGE_2 can inhibit PI3K activity through a PKA-independent mechanism. However, this study was not able to fully elucidate the mechanism involved in this process and remains to be studied (Kolsch et al. 2008; Burelout et al., 2004).

Once within the tissues, neutrophils produce reactive oxygen species (ROS) to kill bacterial pathogens. However, abundant ROS production can also elicit substantial oxidative tissue injury. ROSs are produced by active NADPH oxidase complexes that are formed following activation, translocation, and assembly of multiple soluble components at intracellular and plasma cell membranes. Of these components, activation of p47phox and Rac GTPase are essential to NADPH complex formation and generation of superoxide in neutrophils (El-Benna et al., 2009; E. Pick, 2014). Rac and p47phox are activated through induction of the PI3K pathway following stimulation by pro-inflammatory mediators such as LPS and IC (Chen et al., 2003; Han et al., 1998; Yamamori et al., 2002). PI3K also phosphorylates and activates p38 MAPK, which activates additional members of the NADPH complex and has been shown to play a role in membrane translocation of activated Rac (Brown et al., 2004; Yamamori et al., 2002). The kinetics of LPS-induced p38 MAPK

phosphorylation in neutrophils mimic the kinetics of ROS production seen in our assay (Figure 3), with phosphorylation peaking at 30 minutes and sustained through 60 minutes of stimulation (Haselmayer et al., 2009). In our study, peak time and intensity of ROS production varied between stimulants, with more robust ROS production in response to PMA and IC when compared to LPS stimulation. The different peaks and intensities seen with each stimulant indicate that signaling leading to ROS production within equine neutrophils is heavily dependent on the inciting factor.

Because LPS alone was a weak inducer of equine neutrophil ROS production, we hypothesized that priming neutrophils with granulocyte-monocyte colony-stimulating factor (GM-CSF) prior to LPS stimulation would produce a more robust response. GM-CSF induces low levels of p47phox phosphorylation, but does not induce translocation to areas of NADPH complex formation. However, priming neutrophils with GM-CSF prior to exposure to more potent stimulants leads to more robust phosphorylation and translocation of p47phox and subsequent ROS formation (Dang et al., 1999). This theory was supported in equine neutrophils, as priming with GM-CSF significantly increased ROS production in response to LPS stimulation in our study (Figure 3C).

Misoprostol and db-cAMP inhibited IC and LPS-stimulated ROS production in a concentration-dependent manner (Figure 4 A, C). Both IC and LPS require activation of PI3K and increased intracellular phosphatidylinositol (3,4,5) trisphosphate (PIP_3) for ROS production (Haselmayer et al., 2009; Kulkarni et al., 2011). P13K activates multiple downstream targets that orchestrate NAPDH oxidase assembly, such as p38 MAPK (Brown et al., 2004; Yamamori et al., 2002). Additionally, increased intracellular PIP_3 is a dual

activator of P-Rex1 GEF that stimulates Rac GTPase activity that is also required for NADPH complex function. Phosphorylation of P-Rex1 via cAMP-induced PKA has been shown to inhibit P-Rex1 function and subsequent Rac activity (Mayeenuddin & Garrison, 2006). Furthermore, PKA also exerts inhibitory effects on other essential NADPH complex components and activators including p47phox and p38 MAPK (Bengis-Garber & Gruener, 1996; Costantini et al., 2010). This data suggests that misoprostol likely inhibits ROS production in IC and LPS-stimulated neutrophils through cAMP-activated PKA. In contrast, PMA stimulates ROS production through direct activation of PKC, which is a potent inducer of p38 MAPK activity and activation of NADPH complex components (Capsoni et al., 2012; Dekker et al., 2000). Therefore, this pathway is generally thought to be insensitive to cAMP-elevating agents that act on Rho GTPases upstream of PKC (Haselmayer et al., 2009; Ottonello et al., 1995; Slater et al., 2001). Interestingly, while db-cAMP had no effect on PMA-mediated ROS production, 100μM misoprostol significantly increased ROS levels. Conversely, 300μM misoprostol significantly decreased ROS production (Figure 4B), which has been documented previously (Penfield & Dale, 1985). We hypothesize that similar to PMA-stimulated adhesion, cAMP-induced PKA activity could stimulate Vav GEFs and downstream Rho GTPases to enhance formation of the NADPH complex in response to PMA. Alternatively, cAMP-activated Epacs could also stimulate Rap1, which has been suggested, but not proven to be involved in superoxide production (Pick, 2014). As these enhancing effects were only observed at concentrations up to 100μM misoprostol, it is possible that further amplified PKA activation by increasing levels of intracellular cAMP can outcompete PKC activation of p38 MAPK to inhibit complex formation.

Misoprostol is currently used to treat and prevent NSAID-induced GI injury in equine patients suffering from inflammatory disease (Blikslager, 2012; Marshall & Blikslager 2011). Replenishing PGE levels with misoprostol restores mucosal barrier function following ischemia-reperfusion injury in equine small intestine, potentially through a cAMP-dependent mechanism (Blikslager et al., 2000; Tomlinson & Blikslager, 2005). Taken together with our data, this indicates that misoprostol would be a safe addition to NSAID therapy to preserve GI health while also exerting anti-inflammatory effects. Excitingly, previous reports suggest that misoprostol may augment the anti-inflammatory benefits of NSAIDs (Kitsis et al., 1991; Moraes et al., 2007). Select NSAIDs have been shown to inhibit neutrophil adhesion (Diaz-Gonzalez et al., 1995), chemotaxis (Bertolotto et al., 2014), and respiratory burst (Umeki, 1990); thus, addition of misoprostol could lead to more complete inhibition of neutrophilic inflammation with fewer GI side effects compared to NSAID treatment alone.

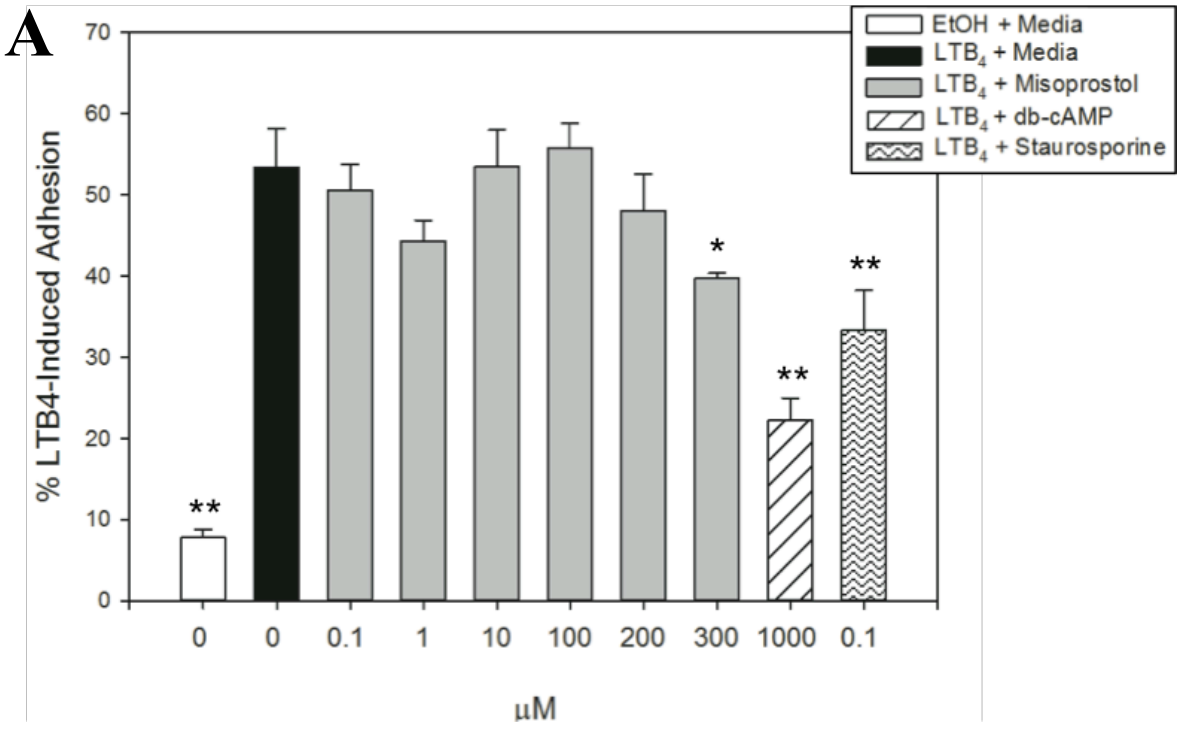
In conclusion, this study demonstrates for the first time that misoprostol exerts anti-inflammatory effects on equine neutrophil effector functions *in vitro.* Misoprostol pretreatment led to a concentration-dependent decrease in equine neutrophil LTB_4-stimulated adhesion, LTB_4, CXCL8-, and PAF-induced chemotaxis, and reactive oxygen species production in response to LPS, IC, and PMA stimulation. While many of these effects were observed at relatively high concentrations of misoprostol, lower doses of misoprostol may prove to be anti-inflammatory *in vivo* upon interacting with multiple cell types. In fact, it has been suggested that many of the protective effects provided by misoprostol *in vivo* following cerebral ischemia-reperfusion injury are mediated through EP2 and EP4 receptors on endothelial cells, which neutrophils closely interact with to elicit many of their effects (Li et

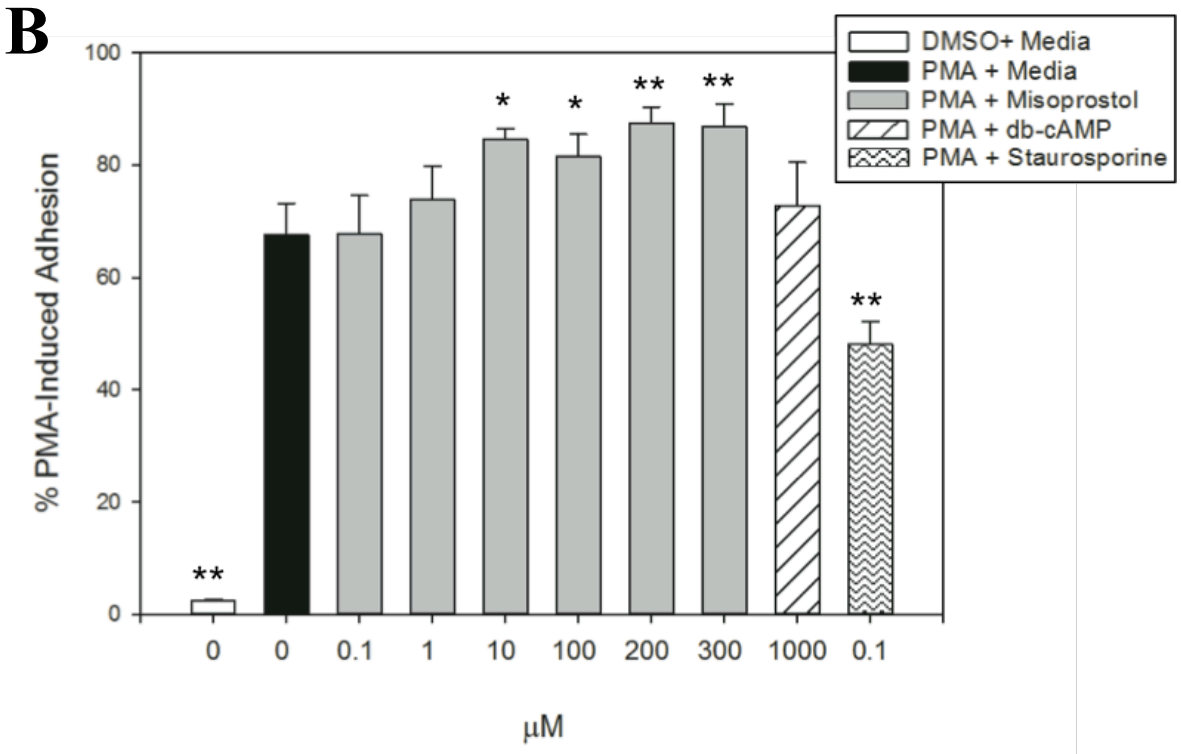
al., 2008). *In vivo* equine inflammatory models are needed to investigate this theory and are underway. Additionally, this study provides proof of principle that EP receptor agonists elicit anti-inflammatory effects on equine neutrophils and paves the way for evaluating other EP2 and/or EP4 receptor agonists as anti-inflammatory therapeutics is horses.

6. Figures

Figure 1: Misoprostol pretreatment inhibits LTB_4-, but not IC- or PMA-induced equine neutrophil adhesion.

Calcein-labeled equine neutrophils were pretreated with varying concentrations of misoprostol, db-cAMP, or the vehicle for each inhibitor (HBSS). Preincubation with known inhibitors of neutrophil adhesion - wortmannin (for IC) or staurosporine (for LTB_4 and PMA) - was utilized as a positive control for inhibition. Neutrophils were stimulated with the following stimulants or vehicles: **(A)** 10nM LTB_4 (or EtOH vehicle)**, (B)** 100ng/mL PMA (or DMSO vehicle), **(C)** 5ug immobilized IC (or 5% BSA vehicle). Cells were stimulated with LTB_4 for 75 seconds, or PMA and IC for 30 minutes. Initial fluorescence readings were taken prior to removal of non-adherent neutrophils via multiple washing steps including 2 washes for LTB_4, and 3 washes for IC and PMA. Percent adhesion was calculated as the final fluorescence reading versus the initial fluorescence reading in each well. Data are expressed as mean % adhesion ± SEM and represent 3 different horses. $**p<0.001$ and $*p<0.05$ indicate significant difference from stimulated cells pretreated with misoprostol vehicle, via One Way RM ANOVA.


A
% LTB4-Induced Adhesion
70
60
50
40
30
20
10
0
EtOH + Media
LTB4 + Media
LTB4 + Misoprostol
LTB4 + db-cAMP
LTB4 + Staurosporine
**
*
**
**
0
0
0.1
1
10
100
200
300
1000
0.1
µM


B
% PMA-Induced Adhesion
100
80
60
40
20
0
DMSO+ Media
PMA + Media
PMA + Misoprostol
PMA + db-cAMP
PMA + Staurosporine
**
*
*
**
**
**
0
0
0.1
1
10
100
200
300
1000
0.1
µM

C

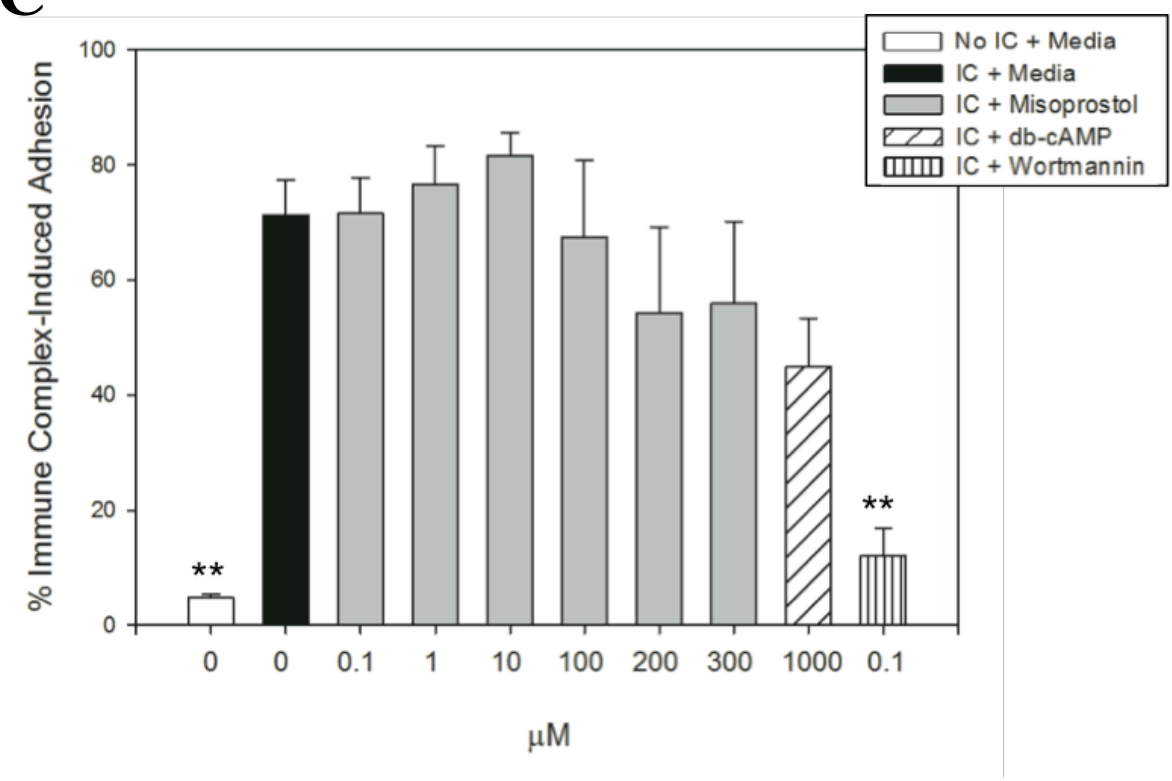
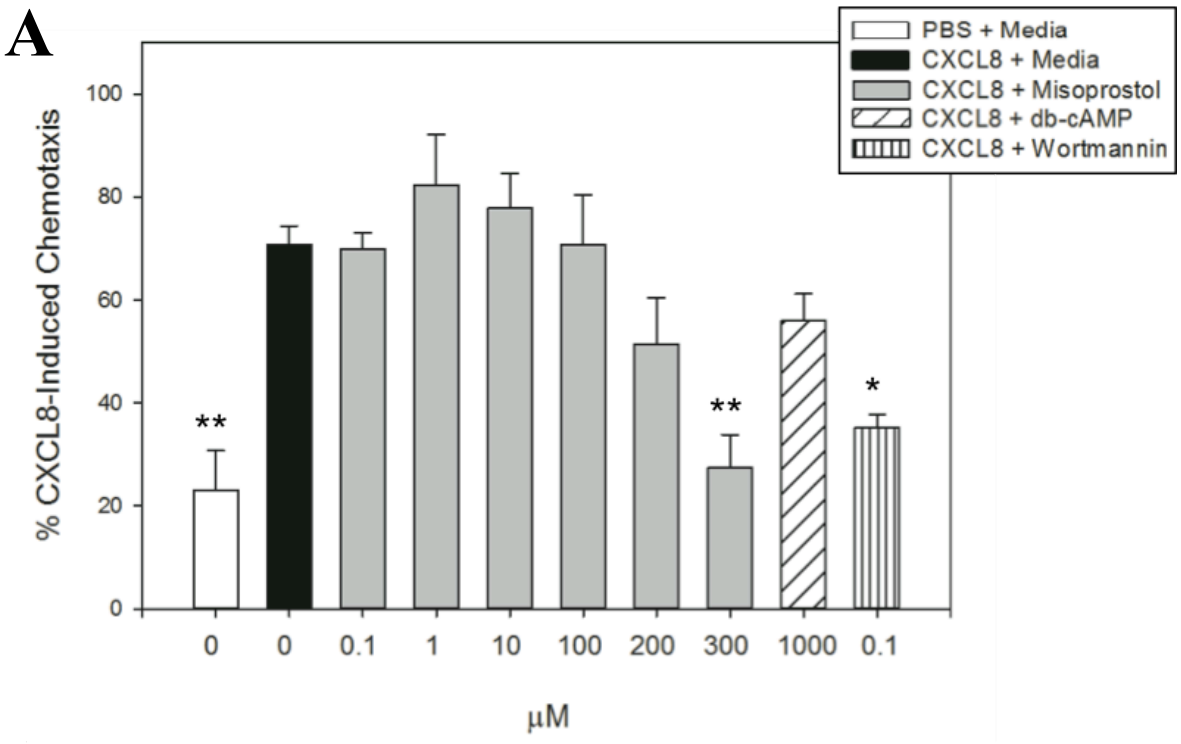
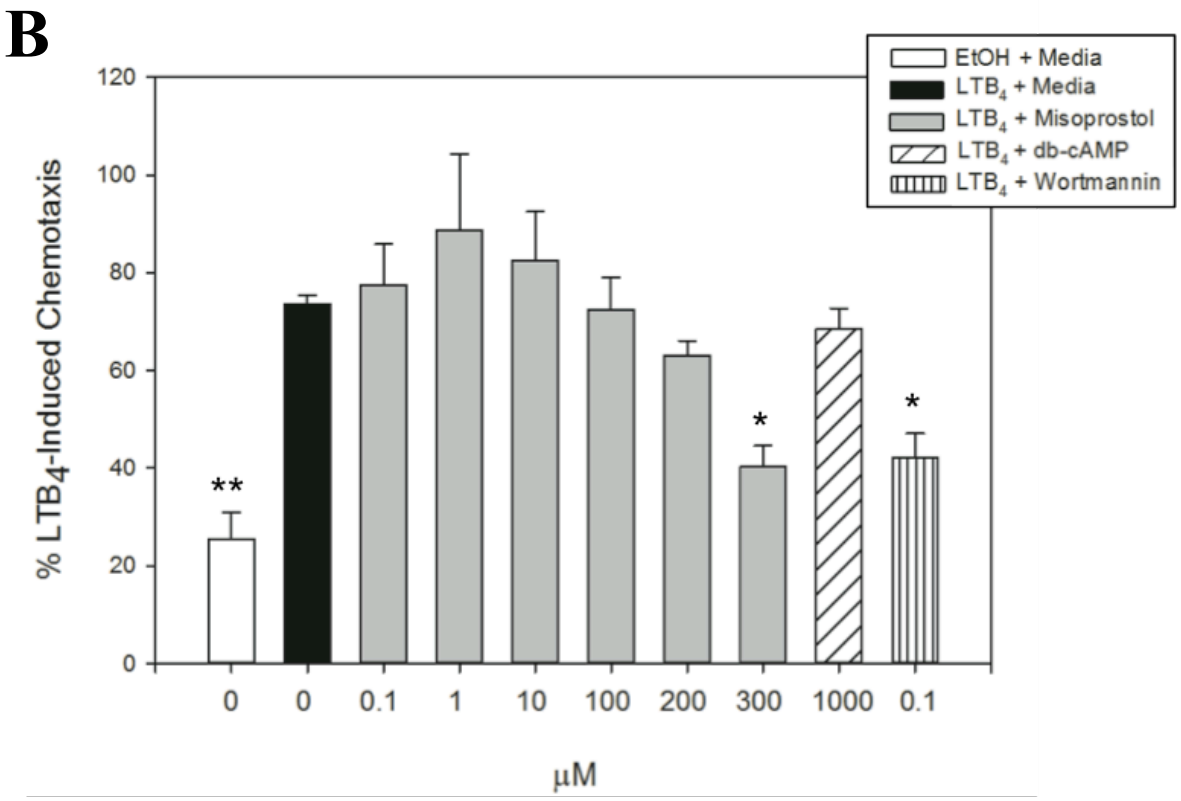

No IC + Media
IC + Media
IC + Misoprostol
IC + db-cAMP
IC + Wortmannin
% Immune Complex-Induced Adhesion
100
80
60
40
20
0
**
**
0
0
0.1
1
10
100
200
300
1000
0.1
µM

Figure 2: Misoprostol pretreatment inhibits CXCL8-, LTB_4-, and PAF-induced equine neutrophil chemotaxis.

Calcein-labeled equine neutrophils were pretreated with multiple concentrations of misoprostol, db-cAMP, or the vehicle for each inhibitor (HBSS). The PI3K inhibitor wortmannin was used as a positive control inhibition. Neutrophils were stimulated to chemotax for 1 hour towards the following chemoattractants or vehicles: **(A)** 100ng/mL CXCL8 (or HBSS vehicle), **(B)** 10nM LTB_4 (or EtOH vehicle), or **(C)** 10nM PAF (or EtOH vehicle). Percent chemotaxis was calculated as florescence in each bottom well following 1 hour of chemotaxis, versus 100% migration control wells. Data are expressed as mean % chemotaxis ± SEM and represent 3 different horses. $^{**}p<0.001$ and $^{*}p<0.05$ indicate significant difference from stimulated cells pretreated with the misoprostol vehicle, via One Way RM ANOVA.


A
PBS + Media
CXCL8 + Media
CXCL8 + Misoprostol
CXCL8 + db-cAMP
CXCL8 + Wortmannin
% CXCL8-Induced Chemotaxis
0
20
40
60
80
100
**
**
*
0
0
0.1
1
10
100
200
300
1000
0.1
µM


B
EtOH + Media
LTB4 + Media
LTB4 + Misoprostol
LTB4 + db-cAMP
LTB4 + Wortmannin
% LTB4-Induced Chemotaxis
0
20
40
60
80
100
120
**
*
*
0
0
0.1
1
10
100
200
300
1000
0.1
µM

C

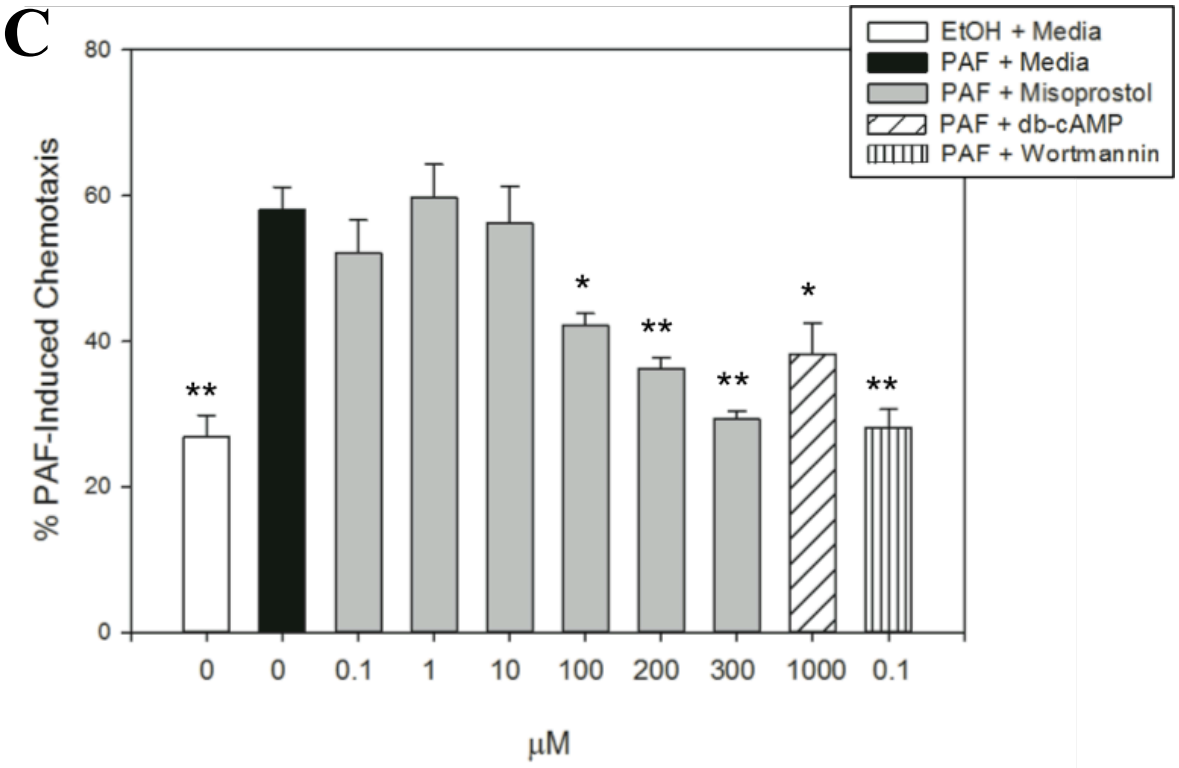
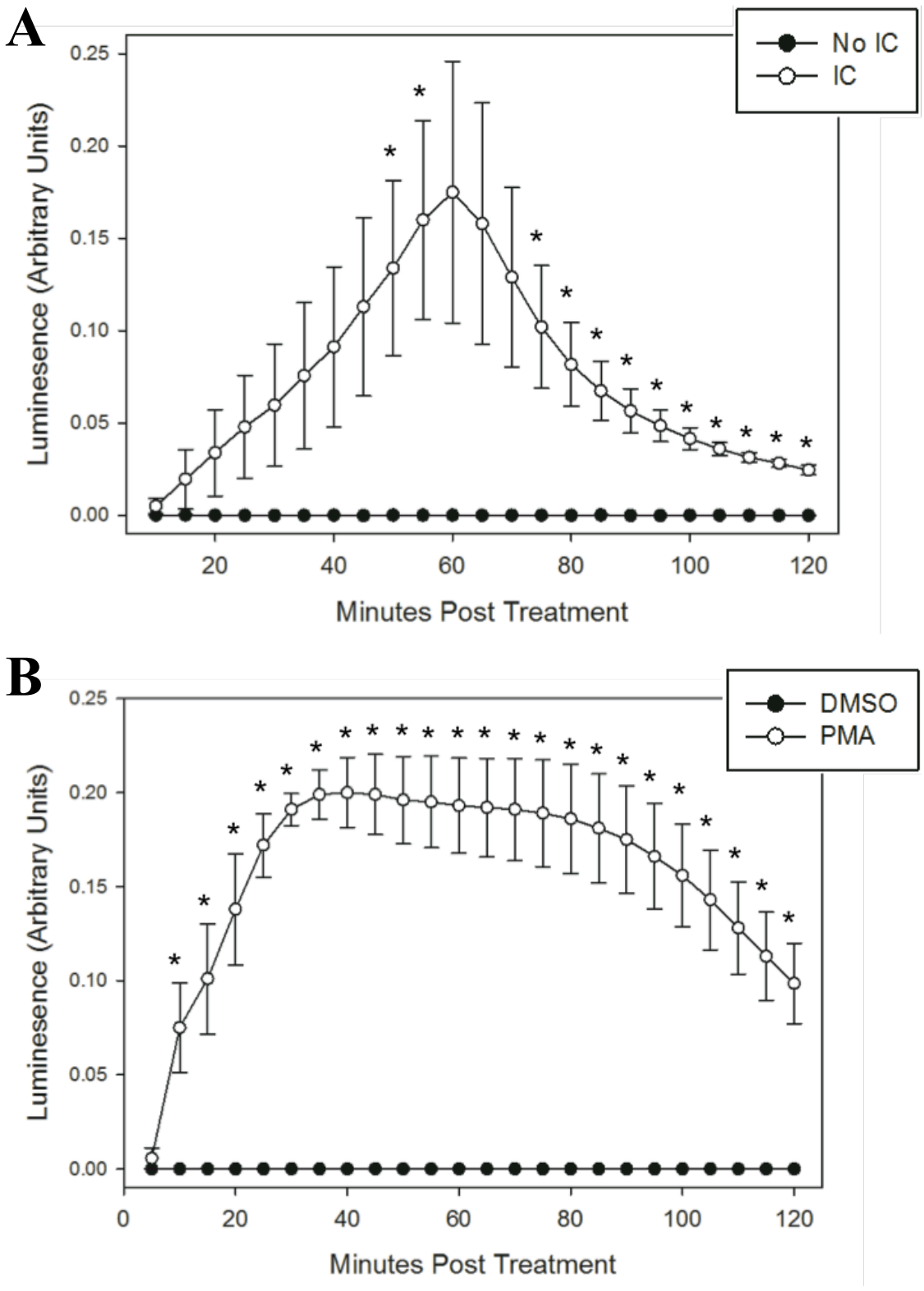

EtOH + Media
PAF + Media
PAF + Misoprostol
PAF + db-cAMP
PAF + Wortmannin
% PAF-Induced Chemotaxis
80
60
40
20
0
**
*
**
**
*
**
0
0
0.1
1
10
100
200
300
1000
0.1
µM

Figure 3: Kinetics of equine neutrophil ROS production stimulated by LPS, IC, and PMA.

Equine neutrophils were treated with the following stimulants or vehicles: **(A)** 1ng/mL GM-CSF priming for 30 minutes followed by stimulation with 100ng/mL LPS (or PBS vehicle), **(B)** 5ug immobilized IC (or 5% BSA vehicle), or **(C)** 100ng/mL PMA (or DMSO vehicle). Stimulated cells were incubated in the presence of 1mM luminol for 2 hours. Kinetic luminescence readings were taken every 5 minutes. Data are presented as mean ± SEM arbitrary units of luminescence and represent at least 3 different horses. $^{**}p<0.001$ and $^{*}p<0.05$ indicate significant difference from unstimulated vehicle treated cells via One Way RM ANOVA (LPS) or two-tailed t-test (IC and PMA).


A
No IC
IC
Luminesence (Arbitrary Units)
0.25
0.20
0.15
0.10
0.05
0.00
20
40
60
80
100
120
Minutes Post Treatment
B
DMSO
PMA
Luminesence (Arbitrary Units)
0.25
0.20
0.15
0.10
0.05
0.00
0
20
40
60
80
100
120
Minutes Post Treatment

C

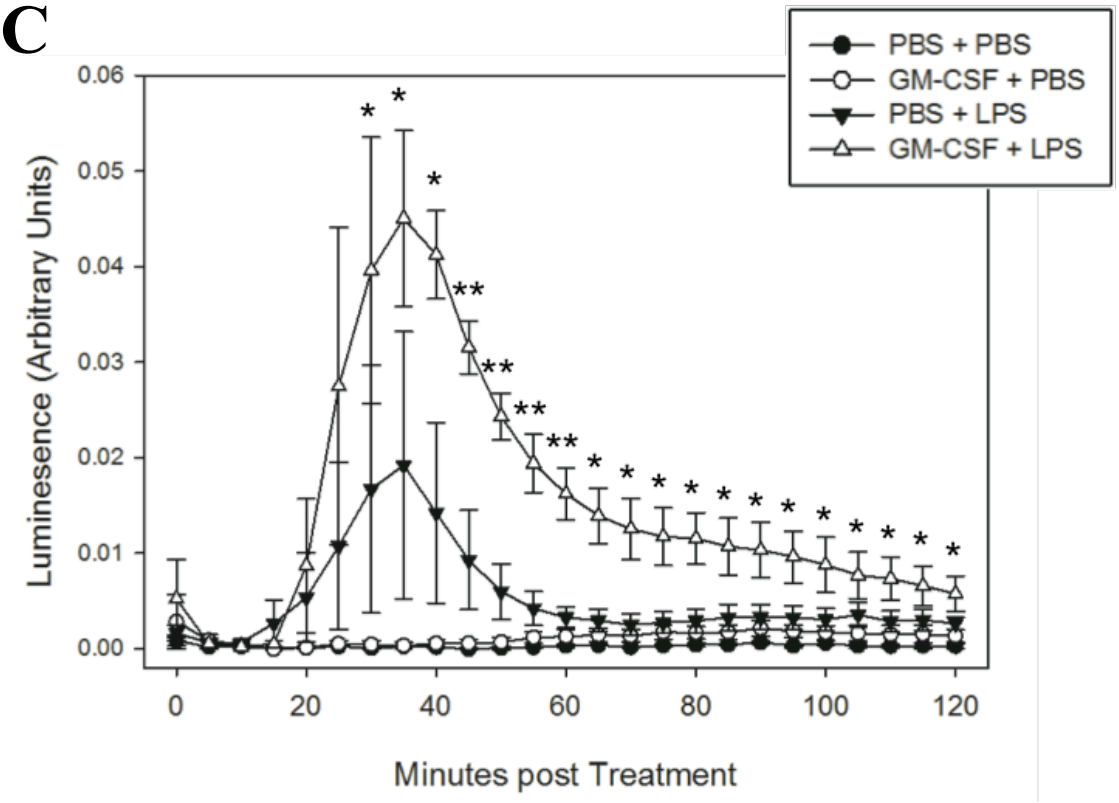
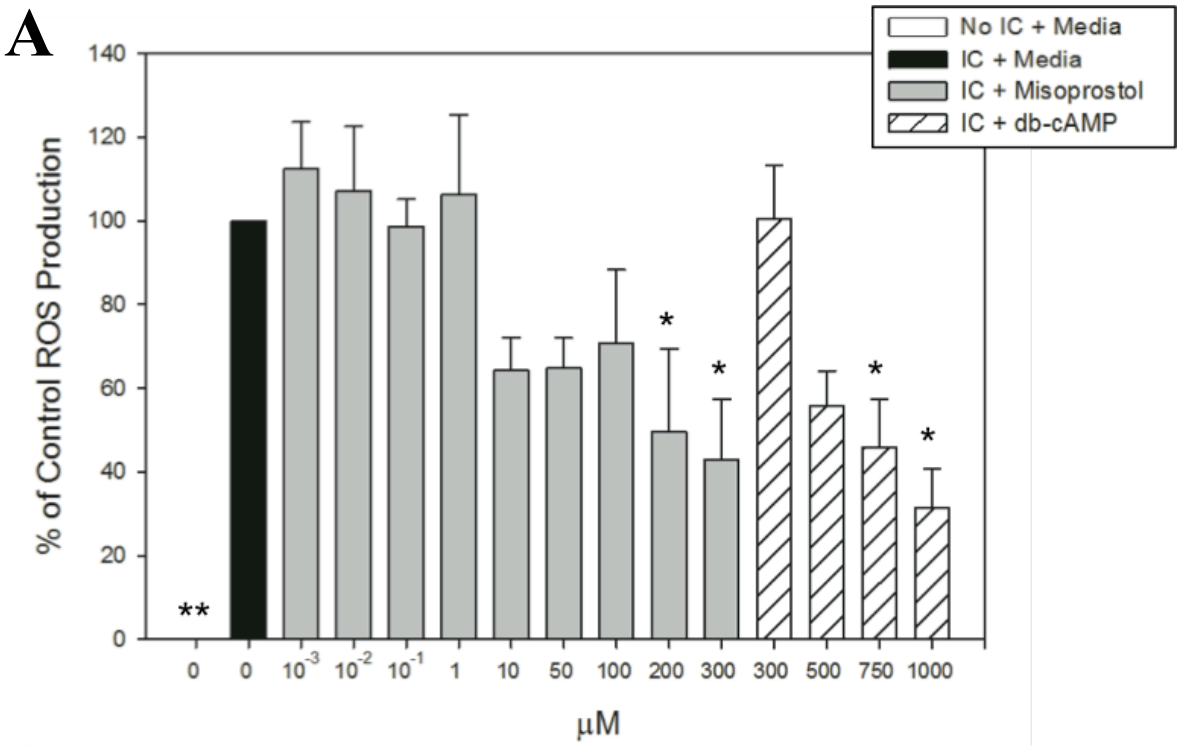
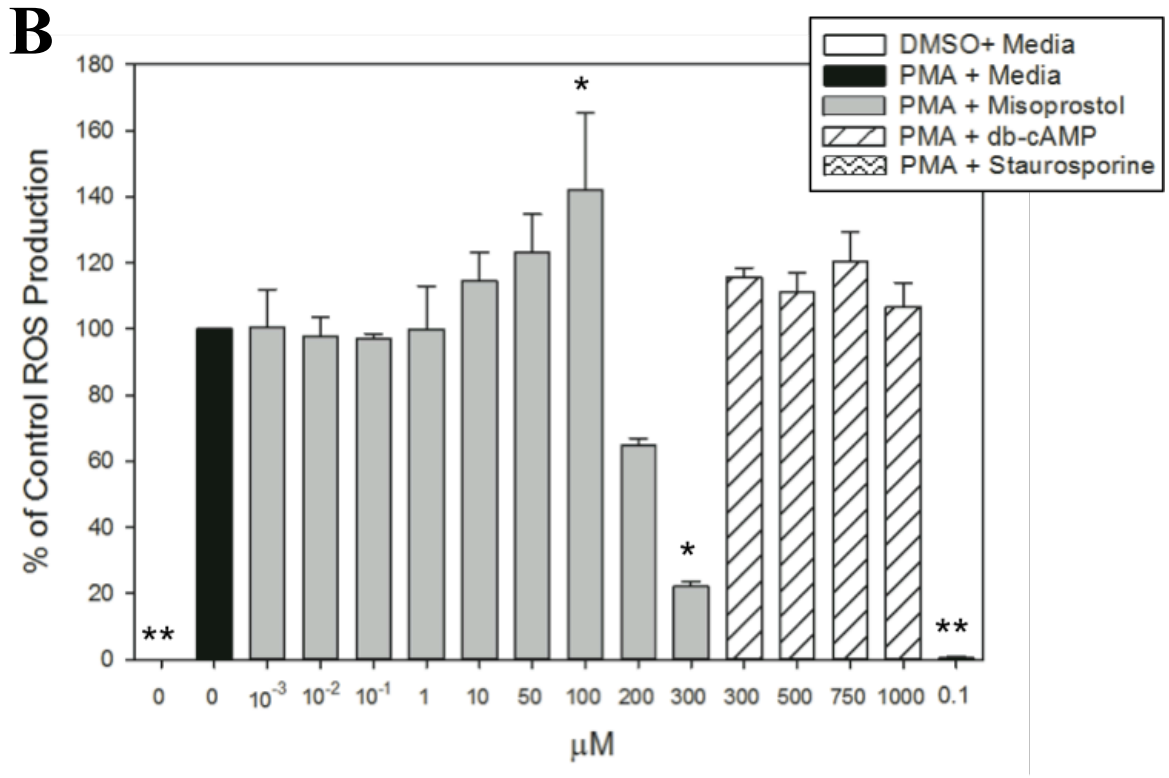
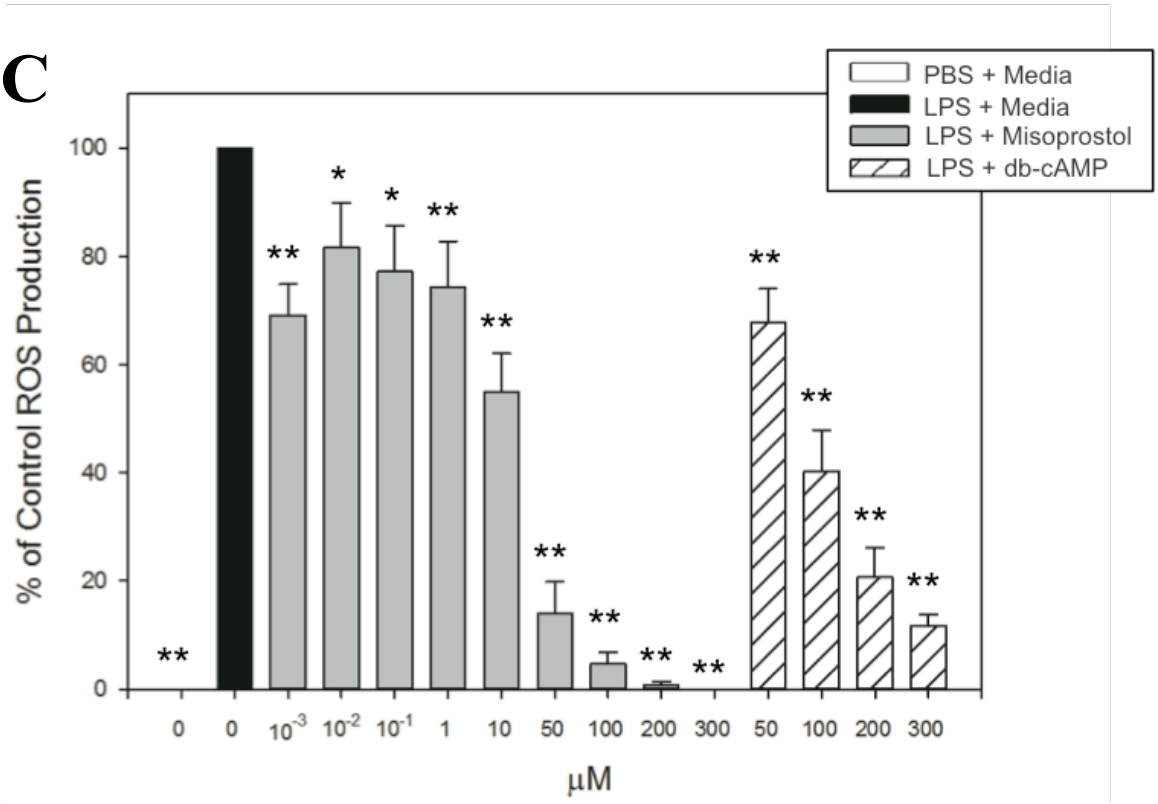

PBS + PBS
GM-CSF + PBS
PBS + LPS
GM-CSF + LPS
Luminesence (Arbitrary Units)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
0
20
40
60
80
100
120
Minutes post Treatment

Figure 4: Pretreatment with misoprostol inhibits LPS-, IC-, and PMA-stimulated equine neutrophil ROS production.

Equine neutrophils were pretreated with multiple concentrations of misoprostol, db-cAMP, or the vehicle for each inhibitor (HBSS (IC and PMA), or PBS (LPS)). The PKC inhibitor staurosporine was used as a positive control for inhibition of PMA-induced ROS production. Equine neutrophils were treated with **(A)** 1ng/mL GM-CSF priming for 30 minutes followed by stimulation with LPS (or PBS vehicle) for 35 minutes; **(B)** 5ug immobilized IC (or 5% FBS) for 55 minutes; or **(C)** 100ng/mL PMA (or DMSO vehicle) for 40 minutes, in the presence of 1mM luminol. Data are presented as mean % ROS production ± SEM compared to stimulated cells pretreated with the misoprostol vehicle, and represent at least 3 different horses. $^{**}p<0.001$ and $^{*}p<0.05$ indicate significant difference from stimulated cells pretreated with the misoprostol vehicle, via One Way RM ANOVA


A
No IC + Media
IC + Media
IC + Misoprostol
IC + db-cAMP
% of Control ROS Production
0
20
40
60
80
100
120
140
**
*
*
*
*
0
0
10-3
10-2
10-1
1
10
50
100
200
300
300
500
750
1000
µM


B
DMSO+ Media
PMA + Media
PMA + Misoprostol
PMA + db-cAMP
PMA + Staurosporine
% of Control ROS Production
0
20
40
60
80
100
120
140
160
180
**
*
*
**
0
0
10-3
10-2
10-1
1
10
50
100
200
300
300
500
750
1000
0.1
µM


C
PBS + Media
LPS + Media
LPS + Misoprostol
LPS + db-cAMP
% of Control ROS Production
100
80
60
40
20
0
0
0
10-3
10-2
10-1
1
10
50
100
200
300
50
100
200
300
μM

Chapter IV

The PGE_1 Analog Misoprostol Inhibits Pro-Inflammatory Cytokine mRNA and Protein Synthesis in Equine Leukocytes

Emily G. Martin[1], Kristen M. Messenger[2,3], Samuel L. Jones[1,3]

[1]Department of Clinical Sciences, [2]Department of Molecular Biomedical Sciences, and [3]Comparative Medicine Institute, College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27607 USA

1. Abstract

Proinflammatory cytokines including TNFα, IL-1β, IL-6, and IL-8 are potent mediators of immune responses in horses. While necessary for orchestration of protective leukocyte functions, disregulated cytokine signaling occurs in multiple disease states and is implicated in multiple leukocyte-induced equine pathologies. Safe and effective cytokine-targeting therapies have not been developed in horses, and thus novel mechanisms of inhibiting cytokine production are needed. One potential mechanism is elevation of intracellular cyclic AMP (cAMP), a secondary messenger molecule that modulates cytokine synthesis in leukocytes. Intracellular cAMP production can be induced by activation of E-prostanoid (EP) receptors 2 and 4 by the prostaglandin E (PGE_1) analog, misoprostol. Misoprostol is currently used as a gastroprotectant in horses, but has not been evaluated as a cytokine-targeting equine therapeutic. We hypothesized that misoprostol treatment before or after LPS stimulation would inhibit equine leukocyte production of the pro-inflammatory cytokines TNFα, IL-1β, IL-6, and IL-8 at the mRNA and protein level, as measured by RT-PCR and multiplex bead immunoassay, respectively. To test this hypothesis, freshly collected equine leukocyte-rich plasma was utilized to reflect the complex environment encountered by equine leukocytes *in vivo*. Treatment of leukocytes with misoprostol either before or after LPS stimulation inhibited TNFα, IL-1β, and IL-6 mRNA production, but did not affect IL-8 mRNA. At the protein level, misoprostol pre- and post-treatment inhibited LPS-induced TNFα and IL-6 production, but had no effect on IL-8. Interestingly, misoprostol pretreatment enhanced IL-1β protein synthesis following 6 hours of LPS stimulation, while post-treatment

with misoprostol inhibited IL-1β protein production when measured after 24 hours of LPS stimulation. These results indicate that misoprostol exerts anti-inflammatory effects on equine leukocytes when applied either before or after a proinflammatory stimulus. However these effects appear to be cytokine-specific, and can differentially mediate cytokine production at the mRNA and protein level.

2. Introduction

Pro-inflammatory cytokines are small protein mediators that initiate and propagate inflammation. These molecules are produced by many cell types in response to inflammatory insult and aid in clearance of microbial infection through orchestration of leukocyte functions. These functions include recruitment and activation of leukocytes at sites of tissue injury (Kolaczkowska & Kubes, 2013), upregulation of leukocyte adhesion molecules for leukocyte adhesion and migration (Radi et al., 2001), and amplification of pro-inflammatory mediator synthesis and secretion to augment the inflammatory cascade (Chaudhry et al., 2013). In the context of inflammatory disease, the fine-tuned balance of cytokine production and signaling often becomes disregulated and overabundant, resulting in destructive and catabolic leukocyte processes that elicit tissue injury and exacerbate disease. Disregulated cytokine production plays a role in multiple disease states in humans and horses, including osteoarthritis (Wojdasiewicz et al., 2014), sepsis (Chaudhry et al., 2013), colitis (Műzes, 2012), and equine laminitis (la RebiÃ re de Pouyade & Serteyn, 2011)**.** Of these cytokines, TNFα, IL-1β, IL-6, and IL-8 have been shown to play a pivotal role in the development of inflammatory disease**,** and thus have been a major target for therapeutic development (Chaudhry et al., 2013; Harada et al., 1994; Hunter & Jones, 2015; Ren & Torres, 2009; Sedger & McDermott, 2014). However, cytokine-targeting strategies have shown varied clinical efficacy and are associated with many deleterious side effects that limit their use in some patients (Calich et al., 2010; Song et al., 2002; Dinarello, 2003). Therefore, novel methods of safely and effectively inhibiting cytokine production are needed.

One potential mechanism of targeting cytokine production is elevation of intracellular cyclic AMP (cAMP). Increased cAMP inhibits many equine leukocyte functions, including chemotaxis (Brooks et al., 2011), adhesion, respiratory burst (Chilcoat et al., 2002), and production of pro-inflammatory cytokines (Sun et al., 2010). These effects are mediated primarily through activation of protein kinase A (PKA) in equine leukocytes (Chilcoat et al., 2002). In human leukocytes, it has been shown that cAMP-activated PKA phosphorylates cAMP response element binding protein (CREB), which inhibits transcriptional activity of NF-κβ that is essential for pro-inflammatory cytokine production (Wen et al., 2010). Thus, cAMP-elevating agents show great promise as anti-inflammatory therapeutics in equine and human leukocytes.

Multiple cAMP-elevating therapeutics have been investigated for anti-inflammatory potential in horses, including phosphodiesterase (PDE) inhibitors such as pentoxifylline, and β2 adrenergic agonists such as Clenbuterol. While pentoxifylline and Clenbuterol elicit anti-inflammatory effects in equine models (Barton & Moore, 1994; Barton et al., 1997a; Chilcoat et al., 2002; Cudmore et al., 2013; Laan et al., 2006; Venugopal et al., 2012), limitations to their use have been observed *in vivo.* One study demonstrated few clinically beneficial effects of pentoxifylline administration in an *in vivo* equine model of endotoxemia (Barton et al., 1997b). Additionally, it has been reported that Clenbuterol loses therapeutic efficacy for treatment of RAO in horses after 21 days and might even worsen the condition thereafter (Read et al., 2012). Furthermore, these therapies have been shown to elicit deleterious cardiovascular, neurologic, and musculoskeletal effects in humans and horses (Escolar et al.,

2012; Kearns & McKeever, 2009; Grimmer et al., 2016; Thompson et al., 2011). Thus, investigation of novel cAMP-elevating agents in horses is warranted.

This study proposes that the prostaglandin E_1 (PGE_1) analog misoprostol, a gastroprotectant and cAMP-elevating agent, is a promising anti-inflammatory therapy for use in horses. Misoprostol is FDA-approved to treat gastropathies associated with nonsteroidal anti-inflammatory drugs (NSAIDs) in humans, and is currently used for the same purpose in horses. Misoprostol is an E-prostanoid (EP) receptor 2, 3, and 4 agonist that has been demonstrated to elevate intracellular cAMP in leukocytes (Smallwood & Malawista, 1995). The anti-inflammatory effects of misoprostol on human leukocytes are mediated predominantly through the EP2 receptor, which is readily expressed on innate immune cells such as neutrophils (Armstrong, 1995; Meja et al., 1997; Talpain et al., 1995; Wheeldon & Vardey, 1993; Wise, 1996). Misoprostol has been demonstrated to modulate leukocyte pro-inflammatory cytokine production in multiple human and murine models (Gobejishvili et al., 2015; Haynes et al., 1992; Meja et al., 1997; Widomski et al., 1997). However, misoprostol has not yet been investigated as a cytokine-targeting anti-inflammatory therapy in horses. Thus, the goal of this study is to evaluate the effect of misoprostol on equine leukocyte pro-inflammatory cytokine production at the mRNA and protein level in response to lipopolysaccharide (LPS) stimulation. We hypothesized that misoprostol treatment of equine leukocytes before or after LPS stimulation would inhibit TNFα, IL-1β, IL-6, and IL-8 mRNA and protein production as measured by RT-PCR and multiplex bead immunoassay, respectively.

3. Materials and Methods

Equine Donors and Blood Collection

All horses utilized in this study were housed at the North Carolina State University (NCSU) Teaching Animal Unit. Horses were of mixed breed and gender, and included quarter horses, thoroughbreds, Tennessee walking horses, and breed crosses. Ages of horses ranged from 5-15 years. All horses were kept on pasture under similar conditions, and did not receive any anti-inflammatory medications while being used in this study. All procedures were approved by the NCSU Institutional Animal Care and Use Committee (IACUC).

Leucocyte-rich plasma was obtained as previously described (Eckert et al., 2007). Briefly, 30-60cc of heparinized equine whole blood was collected via jugular venipuncture. Blood sat for 1 hour at room temperature to allow erythrocytes to settle out of solution, and the supernatant leukocyte-rich plasma (LRP) layer was collected.

Misoprostol Treatment and LPS Stimulation

For pretreatment of leukocytes, misoprostol (Cayman Chemical, Ann Arbor, MI) or vehicle control (0.05% DMSO) was applied to LRP at various concentrations for 30 minutes at 37°C prior stimulation with lipopolysaccharide (LPS) from E. coli 055:B5 (Sigma Aldrich, St. Louis, MO). For post-treatment of leukocytes, misoprostol or vehicle control was applied to LRP either 30 or 60 minutes following LPS stimulation.

LRP was stimulated with LPS at a final concentration of 100ng/mL, or vehicle control for LPS (sterile PBS), for various time periods at 37°C. Cell viability was assessed

via trypan blue exclusion before and after misoprostol and LPS treatment and was routinely > 95%.

RNA Isolation and First-Strand cDNA Synthesis

All RNA isolation and DNAse materials were obtained from Qiagen (Valencia, CA). RNA was isolated using an RNeasy Mini Kit with QIAshredder column homogenation per manufacturer protocol. An RNase-free DNAse set was used to perform two DNase steps: one on-column DNase procedure, as well as one DNase procedure following elution of RNA. Following the second DNaase digestion, RNA was cleaned up using the RNeasy Mini Kit and quantified using a Nanodrop Spectrometer. Equal quantities of RNA from each sample were used for cDNA synthesis using the Superscript III First-Strand Synthesis System (Invitrogen, Thermo-Fischer Scientific, Grand Island, NY) per manufacturer's protocol with random hexamers (50ng/ul).

Real-Time PCR

Real-time PCR was carried out using a BioRad MyIQ Single Color Real-Time PCR Detection System (BioRad, Hercules, CA) using Taqman primers and probes (Invitrogen, Thermo-Fischer Scientific). Primers and probes were obtained from Invitrogen's proprietary equine-specific gene expression assay database and have been validated by the company. Invitrogen identifies the NCBI target sequence used to design the primers and probe, the 25 base pair region of probe binding, and predicted amplicon size (Canales et al., 2006; Shi et al., 2006). In preliminary experiments, all PCR products were run on a 2% agarose gel and

visualized using EZ Vision Three-DNA Dye (Amresco, Solon, OH) to verify specificity of the product. ID numbers of Taqman gene expression assays used are as follows: TNFα, Ec03467871; IL-6, Ec03468678; IL-1β, Ec04260296; IL-8, Ec-3468860 and β2M: Ec03468699 . PCR samples were run in triplicate, and each well contained equal quantities of cDNA (between 1-10ng), 1x Taqman primers and probes, 1x Taqman gene expression master mix, and RNase-free water up to 25ul. PCR cycle conditions were carried out per manufacturer's protocol as follows: 50°C for 2 minutes, once; 95°C for 10 minutes, once; 95°C for 15 seconds, followed by 60°C for 1 minute (with real-time data collection enabled), 40 times.

Outliers were removed from any triplicate with a standard deviation greater than 0.167, and the mean of the remaining two wells was used for analysis. Fold change in mRNA levels was determined using the ΔΔCt data analysis method, and β2M was chosen as the stably expressed housekeeping gene in preliminary experiments in equine leukocytes using procedures described by Radonic *et al.* (Radonic et al., 2004).

Multiplex Bead Immunoassay Sample Collection and Analysis

Prior to treatment, equine LRP was diluted 1:1 in RPMI media without phenol red and supplemented with 100U/mL penicillin and 100ug/mL streptomycin (Sigma Aldirch). Diluted LRP was then pre- or post-treated with misoprostol and stimulated with LPS as described above for 6 or 24 hours. Cell supernatant was collected and used for cytokine and chemokine analysis.

Simultaneous analysis of 23 different cytokines and chemokines was carried out using an equine-specific Milliplex® Map Magnetic Bead Panel (EMD Millipore, Billerica, MA) per manufacturer's protocol. Cytokines/chemokines included in the analysis were: interleukin-1α (IL-1α), IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 (p70), IL-13, IL-17A, IL-18, tumor necrosis factor-α (TNFα), interferon-γ (IFNγ), IFNγ-induced protein 10 (IP-10, or CXCL10), granulocyte-stimulating factor (G-CSF), granulocyte monocyte-stimulating factor (GM-CSF), growth-regulated protein (GRO, or CXCL1), monocyte chemotactic protein-1 (MCP-1, or CCL2), fibroblast growth factor-2 (FGF-2), Eotaxin (or CCL11), Fractalkine (or CX3CL1), and RANTES (or CCL5).

Briefly, supplied 96-well assay plates were washed using kit wash buffer before use. Background (assay buffer), standard, and control wells were loaded onto the plate and diluted 1:1 with serum matrix. The serum matrix for this kit was diluted 1:1 with RPMI media before use to mimic the sample environment. Next, 25ul of each sample and assay buffer were plated in triplicate in sample wells. Plates were covered and incubated on an orbital shaker overnight at 4°C in the presence of magnetic beads coated with fluorescently-labeled capture antibodies for each analyte. Beads were then washed and incubated with biotinylated detection antibodies, followed by addition of streptavidin-phycoerythrin. Beads were washed again before resuspension in drive fluid and sample analysis using a Luminex MagPix® instrument and Luminex xPONENT® software (Luminex, Austin, TX).

Data analysis was conducted using Milliplex Analyst® software (EMD Millipore). A minimum count of 50 beads per well was used for inclusion in analysis. Outliers were removed from any triplicate with a coefficient of variation (CV) >15%, and the mean of the

remaining duplicate samples was used for analysis. Values that fell below the lower limit of detection (LLOD) of the assay were assigned the lowest detectable concentration for that analyte, as determined by the Analyst® software. Cytokines/chemokines in which >50% of sample values fell below the LLOD were not analyzed. Mean fluorescence intensity (MFI) data using a 5-parameter logistic standard curve was used to calculate analyte concentration. One horse was removed from analysis due to high CV across all samples, and presence of multiple outlying analyte values at baseline and upon LPS stimulation at 6 and 24 hours (via extreme studentized derivative method, $\alpha=0.05$).

Statistical Analysis

Data was analyzing using SigmaPlot Version 12.0 (Systat Software, San Jose, CA). Data was assessed for normality via the Shapiro-Wilk test ($p<0.05$), and non-normal data were log transformed to achieve normality for analysis (noted in figure legends). Data that required log transformation included TNFα mRNA fold change at the 2 hour time point presented in figure 1A, TNFα pg/ml at 6hr presented in table 1, and Fractaline and IFNγ pg/ml at 24 hours presented in table 1. Data was back transformed for presentation. Data was analyzed via One Way RM ANOVA with Holm-Sidak multiple comparisons *post hoc* testing, or paired t-test where noted. Log transformation did not achieve normality or equality of variance for IL-1β protein values at 6 or 24 hour time points (table 1); thus raw median IL-1β values were analyzed using Friedman's RM ANOVA on Ranks with Student-Newman-Keuls multiple comparisons *post hoc* testing. For comparison, all data are presented as mean ± SEM.

4. Results

4.1 Kinetics of cytokine mRNA expression in LPS-stimulated equine leukocytes

Leukocyte production of pro-inflammatory cytokines occurs rapidly following LPS stimulation. To assess the effect of misoprostol on maximal LPS-stimulated cytokine mRNA production, we first determined the kinetics of cytokine mRNA expression in response to LPS in our equine LRP model. TNFα, IL-6, and IL-1β mRNA production peaked after 2 hours of stimulation with 100ng/mL LPS, and quickly declined by 6 hours of treatment (Figure 1A-C). In contrast, IL-8 displayed a biphasic pattern of mRNA production, peaking first at 2 hours before declining and peaking again at 18 hours (Figure 1D). This pattern is also observed in human whole blood following LPS stimulation (DeForge & Remick, 1991). Because all cytokines displayed a peak in production following 2 hours of LPS stimulation, this time point was chosen for initial analysis of the effects of misoprostol pre-treatment on LPS-induced cytokine mRNA levels in equine leukocytes.

4.2 Misoprostol pretreatment inhibits LPS-stimulated TNFα, IL-6, and IL-1β, but not IL-8 mRNA levels in equine leukocytes

Elevation of intracellular cAMP dampens multiple immune cell functions. However, different cAMP-elevating agents have been shown to have varied and even opposing effects on TNFα, IL-6, IL-1β, and IL-8 mRNA synthesis in distinct equine leukocyte populations (Barton & Moore, 1994; Laan et al., 2006; Sun et al., 2010). Additionally, cAMP can elicit different effects on cytokines at the mRNA and protein level, indicating a potential role for

cAMP in transcriptional and post-transcriptional regulation of some cytokines (D'Hellencourt et al., 1996; Verghese et al., 1995). The effects of misoprostol on proinflammatory cytokine mRNA production in leukocytes have not been thoroughly studied and have not been evaluated in horses. Thus, we began our investigation by evaluating the effect of misoprostol pre-treatment on pro-inflammatory cytokine mRNA expression in equine leukocytes.

Misoprostol pretreatment led to a concentration-dependent decrease in TNFα, IL-6, and IL-1β, but not IL-8 mRNA levels in equine leukocytes following 2 hours of LPS stimulation (Figure 2). TNFα mRNA was most potently inhibited by misoprostol at concentrations of 1μM and above (maximum inhibition to 10.3% of control; Figure 2A), which is in agreement with previous reports in human peripheral blood mononuclear cells (Gobejishvili et al., 2015). Comparatively, IL-6 and IL-1β mRNA levels were significantly decreased at and above 10uM misoprostol (maximum inhibition to 33.1% and 64.5% of control, respectively; Figure 2B-C), and thus were less sensitive to the effects of misoprostol. In contrast, IL-8 mRNA levels were unaffected by misoprostol pretreatment (Figure 2D). The effects of misoprostol on IL-6, IL-1β, and IL-8 mRNA expression have not yet been evaluated in leukocytes. Considering the opposing effects of other cAMP-elevating agents on IL-6, IL-1β, and IL-8 mRNA in other equine leukocyte models (Barton & Moore, 1994; Laan et al., 2006; Sun et al., 2010), our data suggests that misoprostol exerts a uniquely inhibitory effect on equine leukocyte IL-6 and IL-1β mRNA production.

4.3 Misoprostol treatment following LPS stimulation inhibits TNFα, IL-6, and IL-1β mRNA expression in equine leukocytes

In most clinical situations, veterinarians must apply anti-inflammatory therapy following an inflammatory insult. Thus, to assess the clinical relevance of misoprostol therapy on TNFα, IL-6, and IL-1β mRNA levels, we evaluated the effect of misoprostol when applied following LPS stimulation of equine leukocytes at both an early (2 hours) and late (6 hours) time point.

Application of misoprostol 30 and 60 minutes following LPS stimulation of equine leukocytes resulted in a statistically significant decrease in TNFα (to 29.5% and 33.7% of control) and IL-6 mRNA (to 73.3% and 57.8% of control) when assessed following 2 hours of LPS treatment (Figure 3A). Additionally, application of misoprostol 60 minutes following LPS stimulation significantly inhibited IL-1β mRNA production when evaluated at 2 hours (to 76.9% of control), however had no effect if applied at 30 minutes (Figure 3A). In contrast, when cells were stimulated with LPS for 6 hours, misoprostol treatment 30 and 60 minutes following LPS stimulation decreased IL-6 mRNA only (to 41.9% and 48.2% of control), whereas TNFα and IL-1β mRNA levels were not affected (Figure 3B). This indicates that misoprostol inhibits the initial equine leukocyte pro-inflammatory response to LPS, even after intracellular signaling has already been induced. However, these effects are varied after 6 hours of LPS stimulation.

4.4 Misoprostol significantly inhibits LPS-stimulated equine leukocyte secretion of TNFα, IL-6, IL-1β, and IP-10 in equine leukocytes

Reports have demonstrated that cAMP-elevating agents can elicit differential and even divergent effects on cytokines at the mRNA and protein level (D'Hellencourt et al., 1996; Verghese et al., 1995). Thus, we evaluated the effect of misoprostol treatment on cytokine secretion in LPS-stimulated equine leukocytes at both an early (6 hour) and late (24 hour) time point. For this study, a multiplex bead immunoassay was used to simultaneously evaluate the effect of LPS stimulation and misoprostol treatment on 23 different cytokines. As shown in Table 1, 100ng/mL LPS significantly increased secretion of almost all cytokines and chemokines evaluated at 6 hours, including TNFα, IL-6, IL-1β, IL-8, IP-10, IL-10, IL-4, IFN, IL-2, IL-18, IL-5, Fractaline, G-CSF, GRO, MCP-1, and Eotaxin. Of these analytes, only TNFα, IL-6, IL-1β, IL-8, and IL-10 remained significantly increased at 24 hours. Seven of the analytes (FGF-2, IL-13, IL-17A, IL-1α, GM-CSF, RANTES, and IL-12p70) were below the limit of detection for this assay and were not analyzed.

The results of misoprostol treatment on LPS-induced cytokine protein levels are shown in table 1. Misoprostol treatment both before and after LPS stimulation resulted in a statistically significant decrease in TNFα and IL-6 protein following 6 and 24 hours of LPS stimulation. IL-8 protein levels were unaffected by misoprostol treatment, similar to data obtained at the mRNA level (Figure 2D). Interestingly, misoprostol pretreatment led to a significant increase in IL-1β protein at 6 hours of LPS stimulation, while misoprostol post treatment had no effect at this time point. Furthermore, misoprostol treatment following LPS

stimulation led to a statistically significant decrease in IL-1β protein at 24 hours, at which time misoprostol pretreatment had no effect.

In addition to the above cytokines, misoprostol post-treatment significantly inhibited IP-10 protein production after 6 hours of LPS stimulation. No other cytokines or chemokines were significantly affected by misoprostol pre- or post-treatment. However, an insignificant decrease in IFNγ protein of greater than two-fold was noted in misoprostol pre- and post-treated leukocytes at 24 hours (Table 1).

Taken together, these results indicate that misoprostol pre- and post- treatment of LPS-stimulated equine leukocytes inhibits secretion of pro-inflammatory TNFα and IL-6 proteins, as well as exerts differential effects on IL-1β mRNA and cytokine secretion. Interestingly, the anti-inflammatory cytokine IL-10, which has been documented to be increased by misoprostol treatment in leukocytes (Gobejishvili et al., 2015) was unaffected.

5. Discussion

Dysregulated cytokine production and subsequent leukocyte responses have been implicated in the pathophysiology of multiple diseases that affect horses (Chaudhry et al., 2013; la RebiÃ re de Pouyade & Serteyn, 2011; Műzes, 2012; Visser & Pollitt, 2011; Wojdasiewicz et al., 2014). While increased intracellular cAMP has been demonstrated to inhibit cytokine synthesis in equine leukocytes, many cAMP-elevating therapies studied in horses are associated with limited clinical efficacy and adverse effects *in vivo* (Laan et al., 2006; Sun et al., 2010; Barton et al., 1997b; Escolar et al., 2012; Kearns & McKeever, 2009; Read et al.,

2012; Thompson et al., 2011). This study is the first to demonstrate that the cAMP-elevating PGE_1 analog, misoprostol, significantly decreases pro-inflammatory cytokine mRNA and protein levels in an *in vitro* model of equine inflammation. Misoprostol treatment prior to and following LPS stimulation decreased TNFα and IL-6 mRNA and protein levels in equine leukocytes, but had no effect on IL-8. Interestingly at early time points of analysis, misoprostol inhibited LPS-stimulated IL-1β mRNA synthesis but increased IL-1β protein levels. Conversely at later time points, misoprostol had no effect on IL-1β mRNA production but decreased IL-1β protein secretion (Figures 2-3, Table 1). This indicates that misoprostol is a potent modulator of equine leukocyte pro-inflammatory cytokine mRNA and protein synthesis, as well as suggests a potential role in cytokine regulation at the transcriptional and post-transcriptional level.

Misoprostol inhibition of TNFα mRNA and protein synthesis is documented in leukocytes from multiple species. Misoprostol decreases TNFα mRNA and protein synthesis in human and rodent leukocytes *in vitro* (Gobejishvili et al., 2015; Haynes et al., 1992; Meja et al., 1997), as well as in *ex vivo* human and *in vivo* rodent models of inflammation (Gobejishvili et al., 2015; Mahatma et al., 1991; Nakamura et al., 1992)**.** Furthermore, the cAMP analog db-cAMP has been shown to inhibit TNFα mRNA production *in vitro* in equine monocytes (Sun et al., 2010). In our study, misoprostol pre- and post- treatment inhibited equine leukocyte TNFα mRNA levels (Figures 2-3), and suppressed TNFα protein secretion (Table 1). These results are exciting, as TNFα is a critical mediator of inflammatory disease and has become an important target for therapy development. TNFα is

implicated in the pathophysiology of several equine inflammatory disorders, including dysregulated immune responses in endotoxemia and sepsis (Beutler et al., 2008; Morris et al., 1990), increased neutrophil infiltration in inflammatory airway disease (Richard et al., 2014), and enhancement of neutrophil reactive oxygen species production and tissue damage that plays a role in laminitis (Binder et al., 1999; Kwon, et al., 2013)**.** Therefore, our finding that misoprostol significantly inhibits TNFα mRNA and protein secretion in equine leukocytes is promising for treatment of equine inflammatory disease.

The mechanism by which misoprostol inhibits TNFα mRNA and protein production in equine leukocytes has not yet been elucidated, but we hypothesize that this is a cAMP-dependent mechanism. It has been shown that misoprostol elicits multiple anti-inflammatory effects in human leukocytes though elevation of intracellular cAMP (Armstrong, 1995; Gobejishvili et al., 2015; Smallwood & Malawista, 1995). Increased cAMP predominantly stimulates two intracellular pathways, including activation of protein kinase A (PKA), and/or exchange proteins directly activated by cAMP (Epacs). cAMP-activated PKA is generally associated with inhibitory effects in leukocytes, including decreased TNFα mRNA and protein synthesis (Gobejishvili et al., 2015). In contrast, Epac activation elicits pro-inflammatory effects mediated through the GTPase, Rap1, including stimulation of cellular adhesion and secretion (Cheng et al., 2008)**.** Recent reports suggest that cAMP preferentially activates the inhibitory PKA pathway in neutrophils, and that misoprostol inhibits TNFα mRNA expression in human leukocytes in a PKA-dependent manner. However functional Epacs are present in leukocytes and thus could also play a role in cytokine regulation (Scott et al., 2016). In other cell types, PKA and Epac can antagonize or agonize one another in

various processes, suggesting that cross-talk between these pathways could orchestrate cellular functions (Cheng et al., 2008). The Epac pathway has not been fully studied in leukocytes, however it is possible that both the Epac and PKA pathways maintain fine-tuned control of cAMP signaling to modulate equine leukocyte cytokine production.

The potential routes through which cAMP-activated PKA inhibits cytokine production have not been evaluated in equine leukocytes, however previous studies in other species implicate inhibition of transcription factor NF-κβ. NF-κβ is critical for LPS-stimulated toll-like receptor 4 (TLR4) signaling and upregulation of pro-inflammatory cytokine expression**.** In human leukocytes, cAMP-activated PKA inhibits NF-κβ transcriptional activity by activating CREB (cAMP response element binding protein) (Gobejishvili et al., 2015; Wen et al., 2010). CREB inhibits NF-κβ through competition for cofactors that are necessary for activity, and thus inhibits NF-κβ-mediated transcription of pro-inflammatory cytokine mRNA (Parry & Mackman, 1997; Wen et al., 2010). Interestingly, cAMP has also been demonstrated to inhibit elements of the AP-1 transcription factor complex through activation of Epac-Rap1 signaling, leading to reduced expression of many inflammatory genes. Additionally, Epac has been implicated in a second potential mechanism of cytokine inhibition involving activation of SOCS (suppressor of cytokine signaling) proteins. These proteins are upregulated by native PGEs and misoprostol through cAMP-activated Epac-Rap1 signaling (Cheon et al., 2006; Gasperini et al., 2002; Sands et al., 2006). SOCS1 has been demonstrated to inhibit TLR signaling by inducing ubiquitination and degradation of NF-κβ to prevent cytokine transcription (Ryo et al., 2003). Additionally, SOCS3 inhibits activation of TRAF6, which is a critical component of TLR signaling

(Frobøse et al., 2006). While these mechanisms have yet to be investigated in equine leukocytes, it is possible that multiple cAMP-activated pathways are responsible for misoprostol-mediated cytokine inhibition in horses.

In addition to the suppressive effects of misoprostol on TNFα expression in our model, misoprostol pre- and post- treatment also inhibited LPS-stimulated equine leukocyte IL-6 mRNA (Figures 2-3) and IL-6 protein synthesis (Table 1). These results somewhat contradict previous studies that report varied effects of cAMP-elevating agents on leukocyte IL-6 production. Earlier studies demonstrate that misoprostol and other cAMP-elevating agents can enhance IL-6 expression in innate immune cells (Haynes et al., 1992). This seemingly contradictory effect of cAMP has been attributed to the pleiotropic nature of IL-6, as it can exert both pro- and anti-inflammatory effects *in vivo***.** Interestingly, one study demonstrated that the phosphodiesterase (PDE) inhibitor and cAMP-elevating agent pentoxifylline has no effect on IL-6 mRNA or protein production in LPS-stimulated human monocytes, but inhibited IL-6 protein synthesis in human whole blood (D'Hellencourt et al., 1996)**.** Additional equine-specific studies have demonstrated that cAMP-elevating agents have no effect on IL-6 mRNA expression in equine alveolar macrophages (Laan et al., 2006), but decrease IL-6 activity in LPS-stimulated equine whole blood (Barton & Moore, 1994)**.** These studies suggest that increased intracellular cAMP has different effects on isolated cell types when compared to mixed cell populations. In whole blood or LRP, cross-talk between different cell types could serve to inhibit IL-6 expression in response to cAMP-elevating agents by decreasing TNFα and ROS production, or enhancing secretion of anti-inflammatory mediators such as IL-10. Taken together with our study, we hypothesize that

cAMP-elevating agents exert specific inhibitory effects on IL-6 expression in a mixed population of equine leukocytes when compared to a single cell type system.

The ability of misoprostol to inhibit TNFα and IL-6 mRNA and protein production when applied following LPS stimulation is exciting (Figure 3), as clinicians are most often faced with treating inflammation following inflammatory insult. Our data indicates that misoprostol is able to inhibit an active pro-inflammatory process that has initiated multiple intracellular signaling pathways. This demonstrates that misoprostol could be a practical choice of anti-inflammatory for clinicians in a setting in which patients are being treated for acute and possibly chronic inflammatory disorders.

The divergent effects of misoprostol on IL-1β mRNA and protein production in equine leukocytes suggest that misoprostol regulates IL-1β on a transcriptional and post-transcriptional level. Previous studies demonstrate that cAMP-elevating agents can decrease (Haynes et al., 1992; Laan et al., 2006), increase (Sung & Walters, 1991), or have no effect (Sun et al., 2010) on IL-1β mRNA and protein synthesis in human and equine monocytes, and exert opposing effects on IL-1β mRNA and protein production in human leukocyte models (D'Hellencourt et al., 1996; Verghese et al., 1995). At early time points in our study (i.e. 2 hours for mRNA, and 6 hours for protein assessment), pretreatment with misoprostol decreased LPS-stimulated IL-1β mRNA production (Figures 2C, 3A), but enhanced IL-1β protein secretion (Table 1). While we cannot definitely explain these divergent effects, we hypothesize that they are orchestrated by caspase-mediated IL-1β activation. In immune cells, IL-1β is synthesized as an inactive precursor (pro-IL-1β) and requires activation by the protease caspase-1 for secretion. Interestingly, caspase-1 is constitutively active in circulating

blood monocytes (Netea et al., 2009), and cAMP has been shown to induce caspase-1-dependent monocyte IL-1β synthesis. Furthermore, cAMP can directly stimulate caspase-1 activity in pulmonary microvascular endothelial cells (Larena et al., 2015; Renema et al., 2016). Thus, cAMP-stimulated caspase-1 activity could activate pro-IL-1β protein more rapidly in our assay leading to increased mature IL-1β protein secretion, even if IL-1β mRNA levels are decreased. *In vivo,* native PGEs might utilize this mechanism to regulate several processes in health and disease. IL-1β has been shown to play some homeostatic roles *in vivo* (Ren & Torres, 2009), and it is possible that decreased IL-1β mRNA levels coupled with increased IL-1β protein could serve to balance IL-1β synthesis to maintain homeostasis during the onset of inflammation in horses.

At later points of assessment (i.e. 6 hours for mRNA, and 24 hours for protein analysis), misoprostol had no significant effect on IL-1β mRNA production (Figure 3B), but decreased IL-1β protein secretion when applied following LPS stimulation (Table 1). It is possible that differences in effects on IL-1β between early and late time points are due to leukocyte differentiation over time in culture. 24 hours of LPS stimulation, in combination with the cytokine milieu present in cell supernatants, provides conditions needed for monocytes to begin differentiation into macrophages (Geissmann et al., 2010). Caspase-1 is constitutively inactive in macrophages, and thus requires two separate stimulatory events for mature IL-1β secretion: the first to upregulate IL-1β mRNA and pro-IL-1β protein synthesis, and the second to activate caspase-1 to process mature IL-1β. Rapid metabolism of misoprostol and downregulation of EP2 receptors following extended exposure to PGEs most

likely limits the effects of misoprostol on IL-1β mRNA synthesis at 24 hours in our model (Nishigaki et al., 1996). Thus, although IL-1β mRNA was not affected at this later time point, a lack of caspase-1 activity in these cells could decrease mature IL-1β protein secretion after 24 hours. *In vivo,* accumulated pro-IL-1β protein could potentially serve as substrate for rapid formation of active IL-1β upon caspase-1 activation once cells encounter a second inflammatory stimulus. The mechanisms regulating this differential effect remain to be determined in equine leukocytes.

The effects of misoprostol on IL-8 mRNA and protein synthesis have not been previously evaluated. Thus, we are the first to report that misoprostol has no effect on IL-8 mRNA or protein levels in leukocytes. IL-8 is a potent chemoattractant that recruits neutrophils to areas of tissue inflammation, upregulates neutrophil adhesion molecules, and induces neutrophil adhesion and enzyme release. Surprisingly, some cAMP-elevating agents increase IL-8 production in LPS-stimulated equine monocytes (Sun et al., 2010), but inhibit IL-8 mRNA synthesis in equine alveolar macrophages (Laan et al., 2006). Furthermore, similar to IL-6, one human study demonstrated that pentoxifylline has no effect on IL-8 mRNA or protein in human monocytes, but inhibits secretion of IL-8 protein in LPS-stimulated human whole blood (D'Hellencourt et al., 1996). These disparities suggest that cAMP-elevating agents have varied effects on IL-8 production in different types of leukocytes, as well as in isolated versus mixed leukocyte populations. As misoprostol had no effect on IL-8 production in equine LRP, while other cAMP-elevating agents decrease IL-8 in human whole blood, we hypothesize that this effect is specific to equine cells and to misoprostol. While the later has yet to be investigated, it is possible that misoprostol exerts

unidentified effects on equine leukocytes that do not affect IL-8. This could be a homeostatic function *in vivo*, as IL-8 has been hypothesized to play a reparative role in porcine gut models (Nguyen et al., 2014) and could be preserved by natural PGEs to maintain GI health and repair in horses.

The recent availability of a commercially available equine multiplex bead immunoassay that detects 23 different secreted cytokines and chemokines allowed us to investigate the effect of misoprostol pre- or post-treatment on a multitude of proteins secreted by equine leukocytes. In addition to the analytes previously discussed, misoprostol post-treatment significantly inhibited secretion of IP-10 (also known as CXCL10, or IFNγ-inducible protein 10) following 6 hours of LPS stimulation. IP-10 is rapidly induced following infection and is upregulated in LPS-stimulated innate immune cells to attract and stimulate effector T cells (Dufour et al., 2002). In addition to IP-10, we saw an insignificant 2-fold decrease in IFNγ secretion in misoprostol pre- and post-treated leukocytes following 24 hours of LPS stimulation. Significant inhibition of IFNγ protein secretion by misoprostol pretreatment has been demonstrated previously in human monocytes (Haynes et al., 1992). IFNγ is critical for defense against intracellular bacterial infections and some viruses, and is also a critical regulator of macrophage activation, MHC I and II antigen presentation, and effector T cell differentiation. Taken together, this indicates that misoprostol could aid in regulating the bridge between innate and adaptive immune responses *in vivo*.

Surprisingly, the anti-inflammatory IL-10 protein was not affected by misoprostol treatment. Previous studies have demonstrated that misoprostol treatment increases IL-10 protein production in an *ex vivo* human leukocyte model (Gobejishvili et al., 2015).

Additionally, other cAMP-elevating agents augment IL-10 protein secretion in LPS-stimulated human monocytes, suggesting that this is a cAMP-dependent mechanism. cAMP-dependent increases in IL-10 expression require PKA activity in human cells (Eigler et al., 1998), suggesting divergent roles for PKA in cytokine regulation. Previous work in equine monocytes describe that db-cAMP significantly augments LPS-stimulated equine monocyte IL-10 mRNA levels, however did not evaluate the effect on IL-10 protein (Sun et al., 2010). Because our study did not evaluate IL-10 mRNA, our data suggests that misoprostol and potentially other cAMP-elevating agents do not effect IL-10 protein secretion in equine leukocytes.

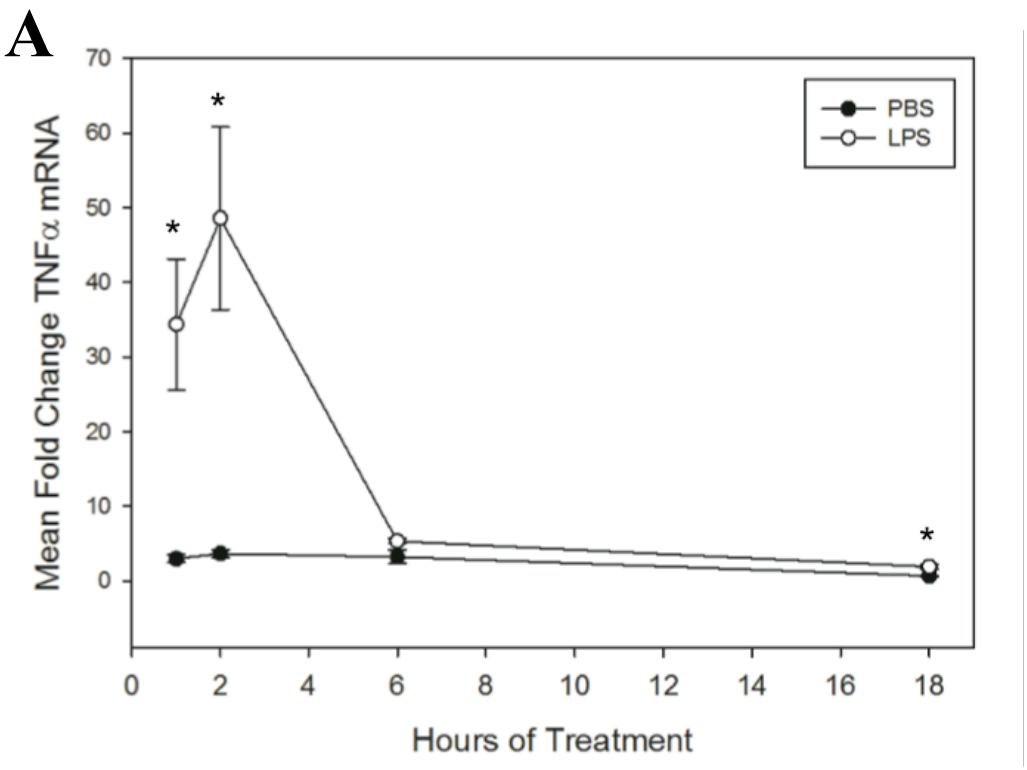
This study is the first to confirm that misoprostol regulates equine leukocyte cytokine production at the mRNA and protein levels. Significant inhibition of TNFα, IL-6, and IL-1β mRNA and protein at multiple points in the inflammatory cascade indicate that misoprostol is a potent anti-inflammatory therapeutic in LPS-stimulated equine leukocytes. This data is exciting, as safe and effective therapeutics designed to inhibit cytokine production and limit inflammation have yet to be identified in horses. Misoprostol is currently employed in horses as a gastroprotectant, and our data suggests that misoprostol could also be safely used as an anti-inflammatory therapeutic. Additionally, previous studies have shown that misoprostol can augment the anti-inflammatory effects of NSAIDs (Kitsis et al., 1991; Moraes et al., 2007), while also preventing GI injury by decreased gastric acid secretion and enhancing GI mucosal repair in horses (Sangiah et al., 1989; Tomlinson & Blikslager, 2005). Further studies will investigate the ability of misoprostol to inhibit cytokine production in *ex vivo* and

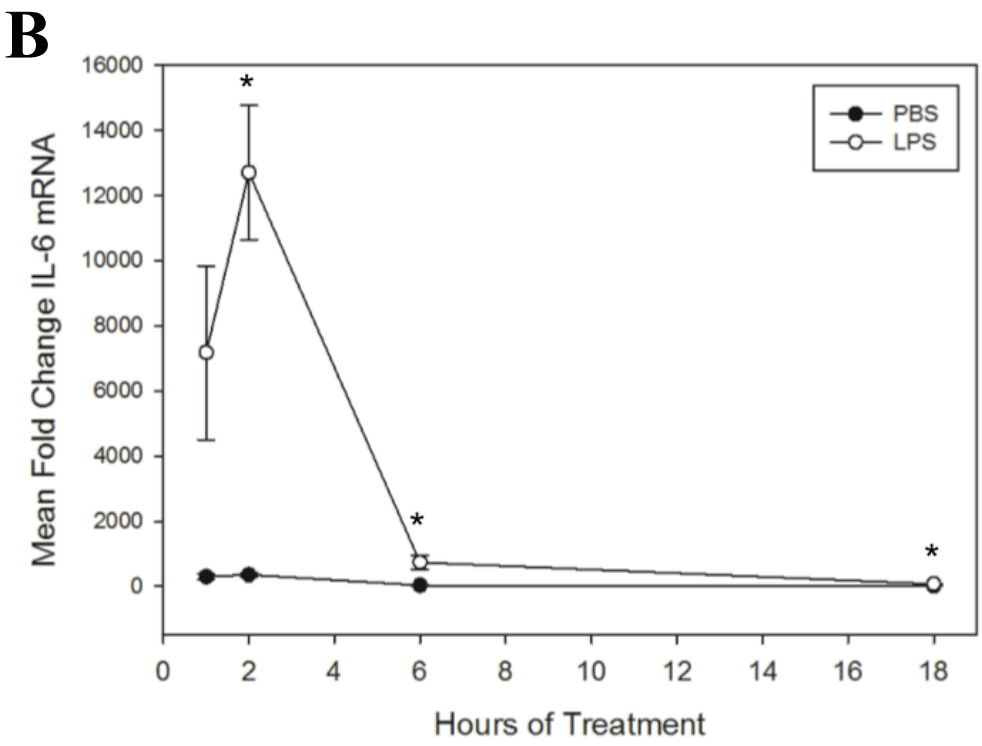
in vivo models of inflammation in order to further characterize misoprostol as an anti-inflammatory therapeutic in horses.

6. Figures

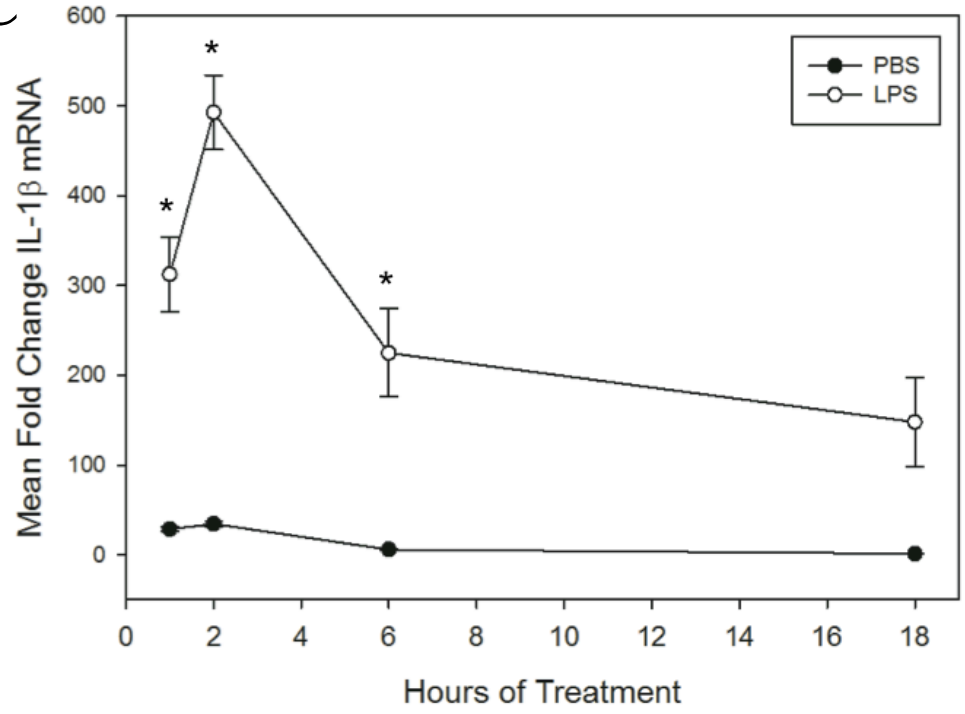
Figure 1: LPS increases TNFα, IL-6, IL-1β, and IL-8 mRNA levels in equine leukocytes.

Equine LRP was stimulated with 100ng/mL LPS or vehicle (PBS) for 1, 2, 6, or 18 hours. mRNA was isolated, and levels of (A) TNFα, (B) IL-6, (C), IL-1β, and (D) IL-8 mRNA were quantified using real-time PCR. Data are expressed as mean ± SEM fold change in mRNA levels over untreated cells at 0 hours (not shown), and represent four different horses. *$p<0.05$ indicates a significant difference between LPS-stimulated cells and time-matched unstimulated controls via paired t-test.


A
Mean Fold Change TNFα mRNA
70
60
50
40
30
20
10
0
PBS
LPS
*
*
*
0
2
4
6
8
10
12
14
16
18
Hours of Treatment


B
Mean Fold Change IL-6 mRNA
16000
14000
12000
10000
8000
6000
4000
2000
0
PBS
LPS
*
*
*
0
2
4
6
8
10
12
14
16
18
Hours of Treatment

C


Mean Fold Change IL-1β mRNA
600
500
400
300
200
100
0
PBS
LPS
*
*
*
0
2
4
6
8
10
12
14
16
18
Hours of Treatment

D

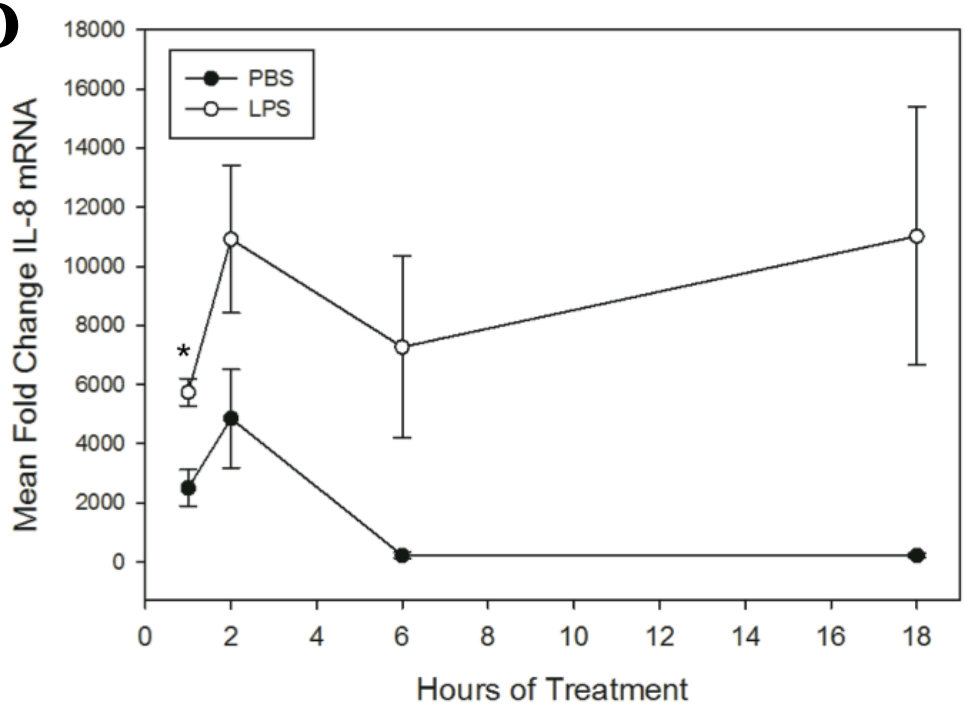
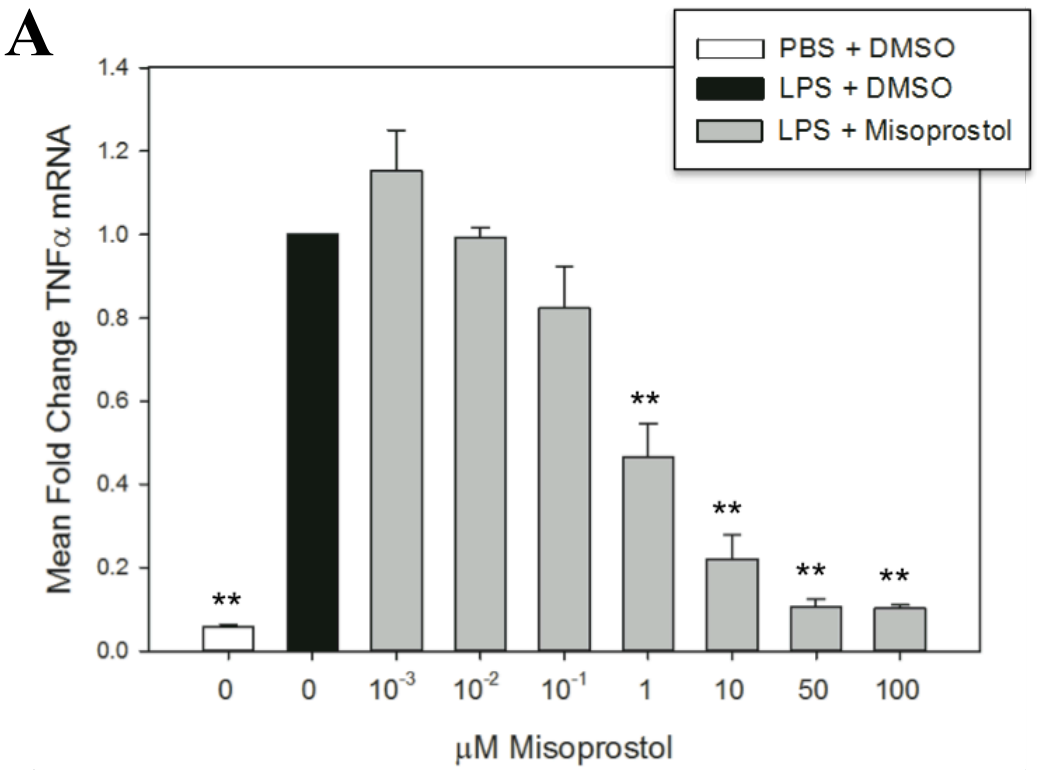
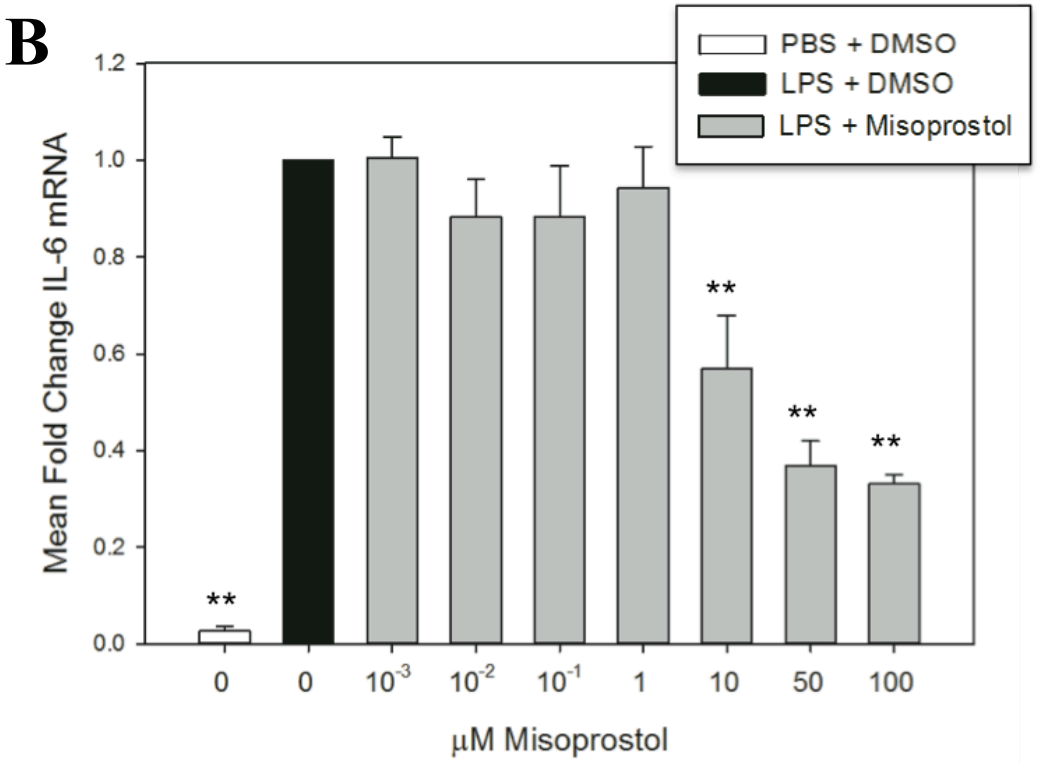
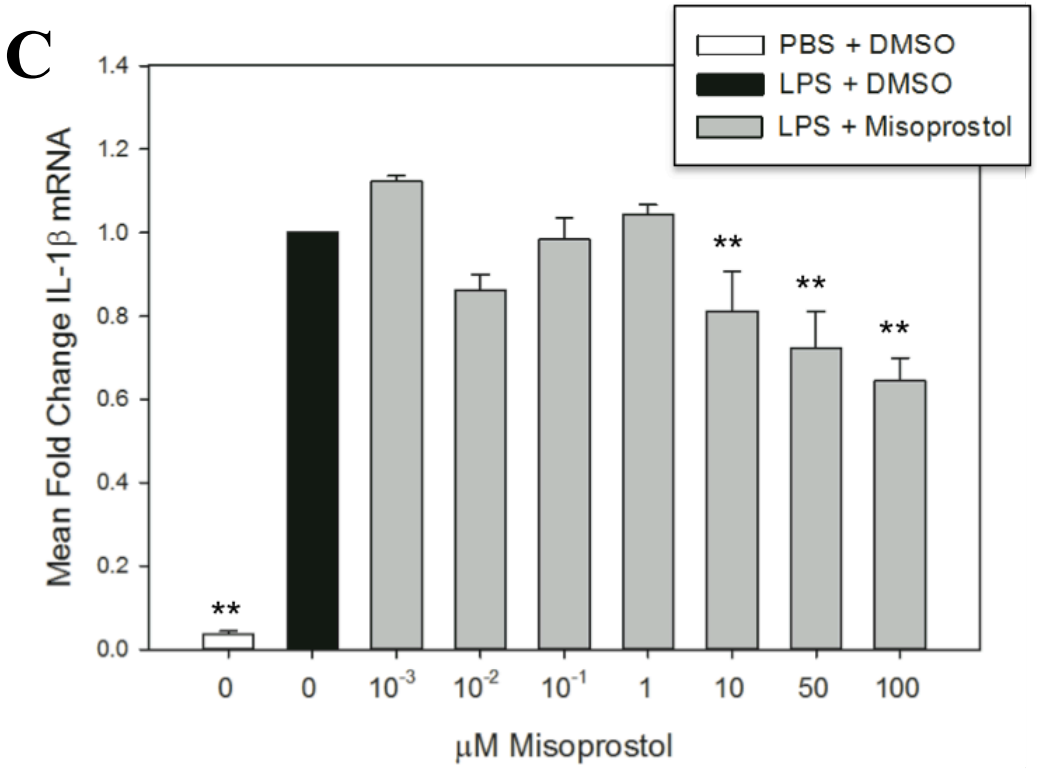

Mean Fold Change IL-8 mRNA
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
PBS
LPS
*
0
2
4
6
8
10
12
14
16
18
Hours of Treatment

Figure 2: Misoprostol pre-treatment decreases TNFα, IL-6, and IL-1β mRNA levels in LPS-stimulated equine leukocytes.

Equine LRP was pre-treated with various concentrations of misoprostol or vehicle (0.05% DMSO) for 30 minutes, followed by stimulation with 100ng/mL LPS or PBS for 2 hours. mRNA was isolated, and levels of (A) TNFα, (B) IL-6, (C), IL-1β, and (D) IL-8 mRNA were quantified via real-time PCR. Data are expressed as mean ± SEM fold change in mRNA levels versus LPS-stimulated cells pretreated with the misoprostol vehicle (denoted as 0μM misoprostol), and represent three different horses. $**p<0.001$ and $*p<0.05$ indicates a significant difference compared to LPS-stimulated cells pretreated with the misoprostol vehicle (black bar) via One-Way RM ANOVA.


A
PBS + DMSO
LPS + DMSO
LPS + Misoprostol
Mean Fold Change TNFα mRNA
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
**
**
**
**
0
0
10-3
10-2
10-1
1
10
50
100
μM Misoprostol


B
PBS + DMSO
LPS + DMSO
LPS + Misoprostol
Mean Fold Change IL-6 mRNA
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
**
**
**
0
0
10-3
10-2
10-1
1
10
50
100
μM Misoprostol


C
PBS + DMSO
LPS + DMSO
LPS + Misoprostol
Mean Fold Change IL-1β mRNA
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
**
**
**
0
0
10-3
10-2
10-1
1
10
50
100
μM Misoprostol

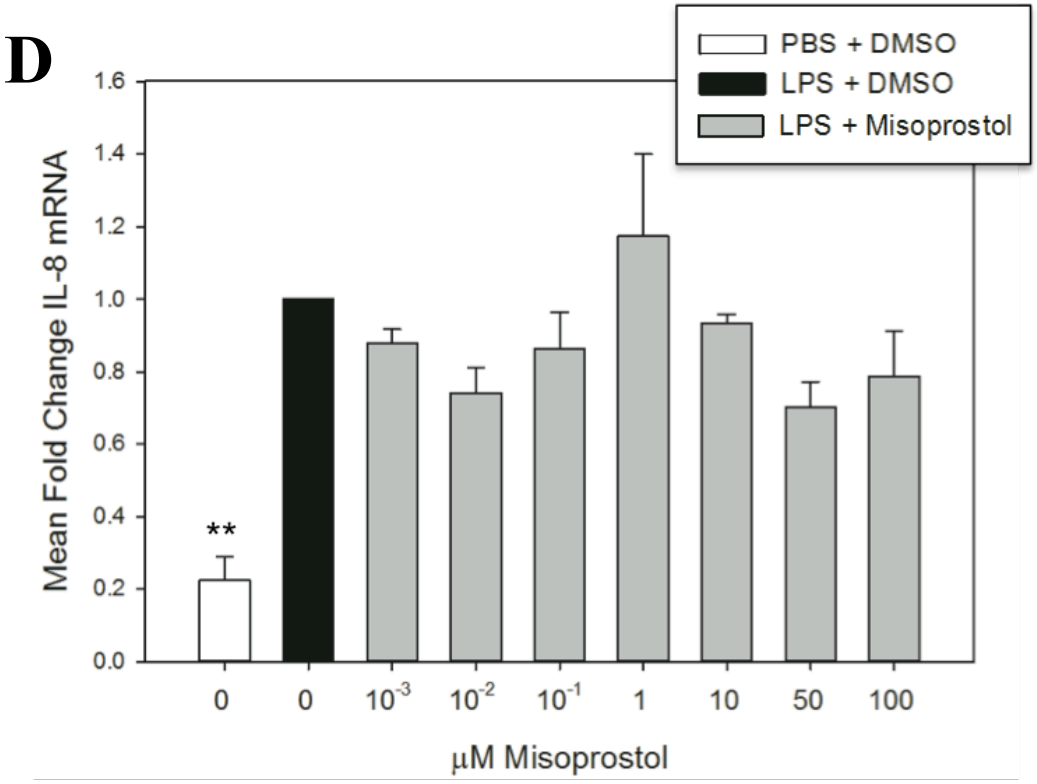
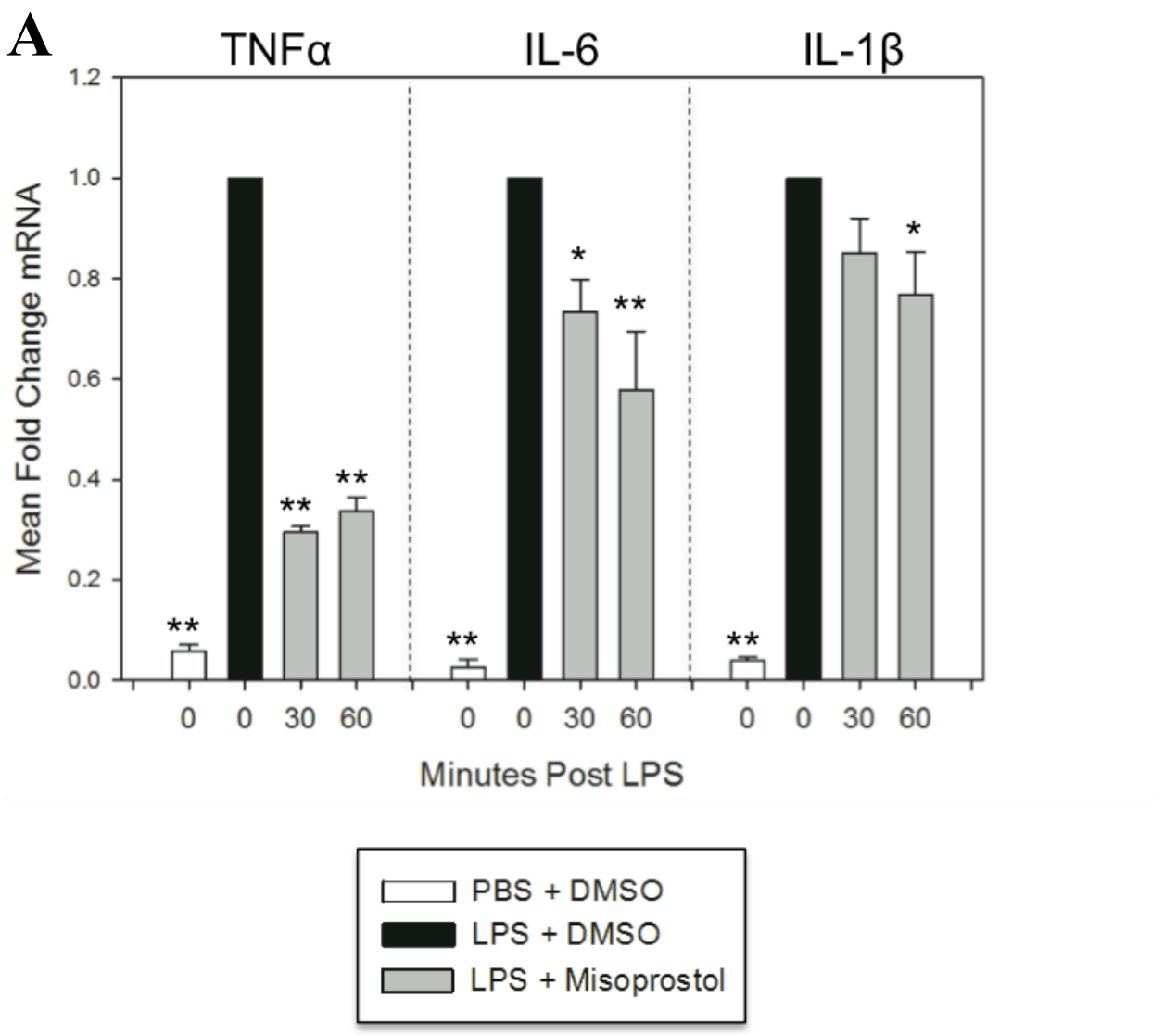
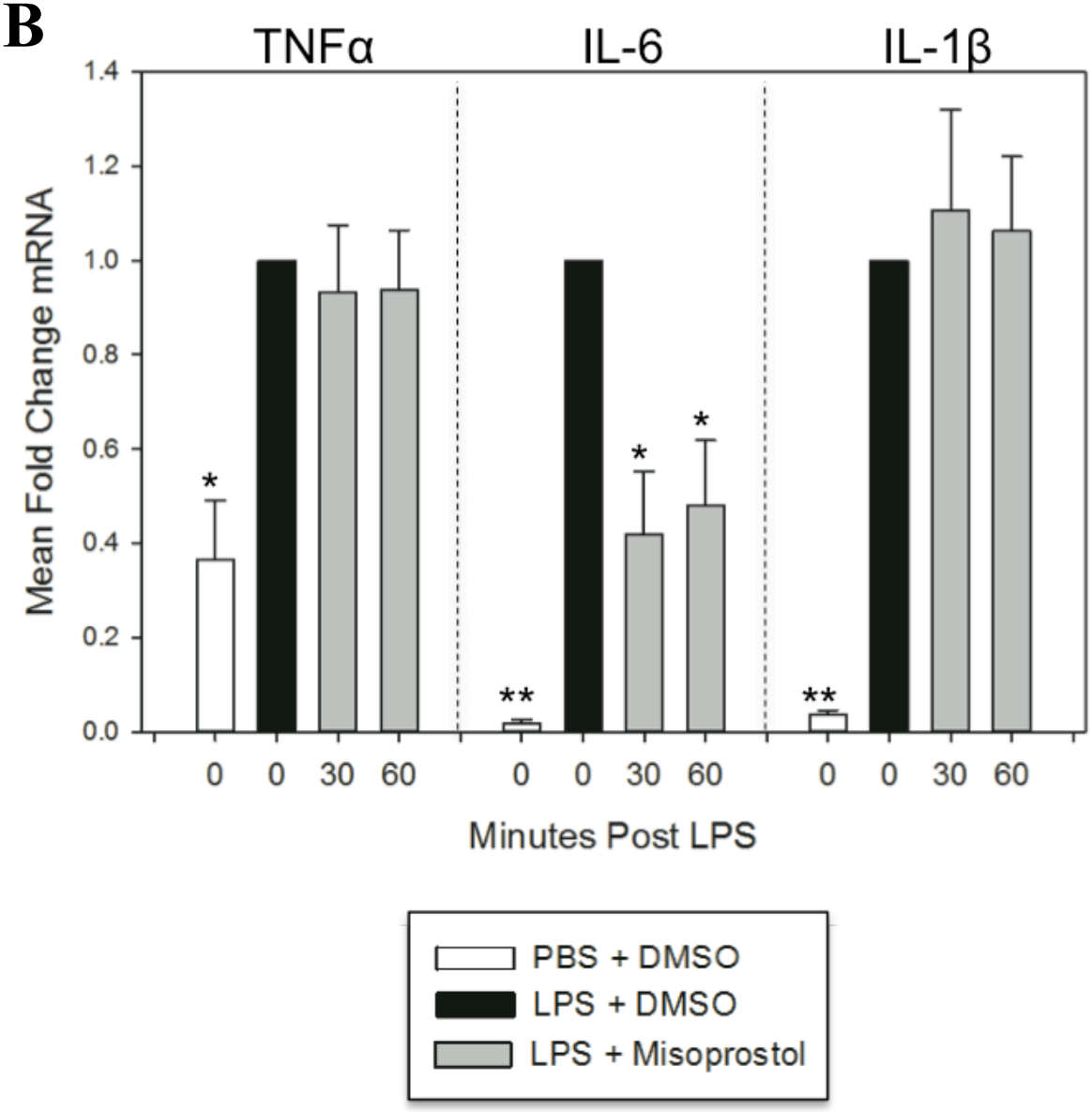

D
PBS + DMSO
LPS + DMSO
LPS + Misoprostol
Mean Fold Change IL-8 mRNA
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
0
0
10-3
10-2
10-1
1
10
50
100
μM Misoprostol

Figure 3: Misoprostol treatment 30 and 60 minutes following LPS stimulation inhibits TNFα, IL-6, and IL-1β mRNA production in equine leukocytes.

Equine LRP was stimulated with 100ng/mL LPS or PBS, followed 30 or 60 minutes later by treatment with 100μM misoprostol or vehicle (0.05% DMSO). Cells were incubated for (A) 2 hours, or (B) 6 hours, and mRNA was isolated. Levels of TNFα, IL-6, and IL-1β mRNA were assessed via real-time PCR. Data are expressed as mean ± SEM fold change in mRNA levels of six (A) or four (B) different horses versus LPS-stimulated cells that were post-treated with the misoprostol vehicle (denoted as 0μM misoprostol). $**p<0.001$ and $*p<0.05$ indicates a significant deference compared to LPS-stimulated cells that were post-treated with the misoprostol vehicle (black bars) via One-Way RM ANOVA.


A
TNFα
IL-6
IL-1β
Mean Fold Change mRNA
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
**
**
**
*
**
**
*
0
0
30
60
0
0
30
60
0
0
30
60
Minutes Post LPS
PBS + DMSO
LPS + DMSO
LPS + Misoprostol

B


TNFα
IL-6
IL-1β
Mean Fold Change mRNA
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
**
*
*
**
0 0 30 60 0 0 30 60 0 0 30 60
Minutes Post LPS
PBS + DMSO
LPS + DMSO
LPS + Misoprostol

7. Tables

Table 1: Effect of misoprostol treatment before or after LPS stimulation on equine leukocyte extracellular cytokine levels.

Four different treatment groups were analyzed: unstimulated equine LRP pre-treated with the misoprostol vehicle (0.05% DMSO), not shown; 0μM = equine LRP pre-treated for 30 minutes with misoprostol vehicle (0.05% DMSO) prior to LPS stimulation; 100μM Pre Treatment = equine LRP pre-treated for 30 minutes with 100μM misoprostol prior to LPS stimulation; 100μM Post Treatment = equine LRP was stimulated with 100ng/mL LPS, followed 30 minutes later by treatment with 100μM misoprostol. All samples were incubated at 37°C for 6 or 24 hours. Cell supernatants were analyzed for cytokine/chemokine levels using a multiplex bead-based immunoassay. Data are presented as mean ± SEM cytokine levels in pg/mL, and represent five different horses. Significant differences from unstimulated samples pre-treated with the misoprostol vehicle (not shown) via One Way RM ANOVA (*$p<0.05$) or Friedman RM ANOVA (§$p<0.05$) are indicated. Significant differences from LPS-stimulated samples pre-treated with the misoprostol vehicle (denoted as 0μM misoprostol) via One Way RM ANOVA (†$p<0.05$), or Friedman RM ANOVA on Ranks (‡$p<0.05$) are also indicated.

Table 1 (Continued): Effect of misoprostol treatment before or after LPS stimulation on equine leukocyte extracellular cytokine levels.

Analyte Levels 6 Hours Post LPS Treatment (pg/mL)			
Cytokine	**Misoprostol (µM)**		
	0µM	***100µM Pre Treatment***	***100µM Post Treatment***
TNF-α	894.064 (±179.221)*	106.758 (±18.060)†	123.098 (±30.886)†
IL-6	261.540 (±42.707)*	189.514 (±27.817)†	142.422 (±26.252)†
IL-1β	3150.524 (±1520.336)§	6027.748 (±2914.889)‡	3594.648 (±1682.129)
IL-8	160.750 (±33.802)*	158.802 (±30.795)	154.986 (±30.643)
IP-10	173.014 (±51.100)*	168.298 (±52.347)	166.196 (±50.940)†
IL-10	284.534 (±59.435)*	322.008 (±79.808)	269.366 (±53.206)
IL-4	1486.826 (±557.649)*	1468.370 (±550.651)	1460.358 (±543.439)
IFNγ	947.748 (±286.373)*	932.092 (±281.452)	918.024 (±285.689)
IL-2	12.988 (±2.803)*	12.984 (±2.635)	12.988 (±2.687)
IL-18	142.850 (±43.023)*	143.136 (±42.121)	142.644 (±42.195)
IL-5	56.785 (±14.970)*	58.262 (±14.692)	56.848 (±14.935)
Fractaline	1211.792 (±772.502)*	1210.620 (±795.108)	1203.280 (±776.168)
G-CSF	334.736 (±100.147)*	337.046 (±99.541)	329.058 (±101.121)
GRO	206.730 (±19.548)*	217.976 (±19.254)	210.944 (±19.566)
MCP-1	3767.000 (±1012.198)*	3770.200 (±1004.467)	3686.400 (±990.298)
Eotaxin	15.282 (±6.499)	15.618 (±6.714)	15.008 (±6.685)

Table 1 (Continued): Effect of misoprostol treatment before or after LPS stimulation on equine leukocyte extracellular cytokine levels.

Analyte Levels 24 Hours Post LPS Treatment (pg/mL)			
	Misoprostol (µM)		
Cytokine	***0µM***	***100µM Pre Treatment***	***100µM Post Treatment***
TNF-α	159.388 (±32.838)*	27.758 (±3.707)†	35.814 (±9.906)†
IL-6	93.298 (±14.794)*	59.310 (±12.267)†	52.858 (±10.413)†
IL-1β	1133.266 (±366.477)§	670.146 (±214.096)	575.286 (±200.162)‡
IL-8	67.866 (±10.138)*	68.048 (±9.256)	67.058 (±8.910)
IP-10	90.624 (±19.337)	82.244 (±23.320)	84.344 (±21.908)
IL-10	163.348 (±38.802)*	153.022 (±33.184)	147.664 (±35.273)
IL-4	389.620 (±121.471)	393.586 (±124.957)	395.160 (±125.627)
IFNγ	617.860 (±202.735)	283.524 (±67.384)	251.630 (±72.228)
IL-2	5.688 (±0.769)	5.694 (±0.677)	5.550 (±0.791)
IL-18	35.224 (±8.172)	35.786 (±8.486)	35.934 (±8.32)
IL-5	16.934 (±3.445)	17.280 (±3.481)	17.166 (±3.444)
Fractaline	2.294 (±0.233)	2.314 (±0.232)	2.306 (±0.243)
G-CSF	85.532 (±28.732)	89.294 (±29.413)	89.166 (±31.782)
GRO	62.574 (±7.753)*	61.424 (±6.904)	63.848 (±6.862)
MCP-1	778.114 (217.657)	797.044 (±215.033)	791.620 (±214.179)
Eotaxin	--	--	--

Chapter V

Pharmacokinetics and Anti-inflammatory Effects of Oral Misoprostol in Horses

Emily G. Martin[1], Jenna M. Schirmer[1], Samuel L. Jones[1,2], Jennifer L. Davis[1,2]

[1]Department of Clinical Sciences and, [2]Comparative Medicine Institute, College of Veterinary Medicine, North Carolina State University, Raleigh NC 27607 USA

1. Abstract

Misoprostol is an E prostanoid (EP) 2, 3, and 4 receptor agonist that is used to treat and prevent NSAID-induced GI injury in horses. In addition, misoprostol has also been shown to elicit anti-inflammatory effects *in vivo* by increasing production of leukocyte intracellular cyclic AMP (cAMP). cAMP-elevating agents inhibit production of the potent pro-inflammatory cytokine TNFα, which plays a key role in the pathophysiology of multiple equine inflammatory diseases. While previous studies from our lab reveal that misoprostol inhibits TNFα production in equine leukocytes *in vitro, ex vivo* studies are needed to assess the clinical relevance of this drug as an anti-inflammatory in horses. Furthermore, the pharmacokinetic profile of oral misoprostol administration has not been determined in horses. Thus, our goals were to define the pharmacokinetic parameters of orally administered misoprostol in horses and to evaluate misoprostol as a potential equine anti-inflammatory therapeutic. We hypothesized that misoprostol would be rapidly absorbed following oral administration at a clinically-relevant dose of 5ug/kg, and would achieve maximal plasma concentrations that inhibited TNFα mRNA production in lipopolysaccharide (LPS)-stimulated leukocytes *ex vivo.* For this study, we utilized leukocyte-rich plasma (LRP) to more closely reflect conditions experienced by equine leukocytes i*n vivo*. Oral administration of misoprostol at a dose of 5ug/kg produced a rapid Tmax of 23.4 ± 2.4 minutes, with a Cmax of 290 ± 70 pg/mL. LPS stimulation of equine LRP *ex vivo* before and after misoprostol administration elicited a significant increase in TNFα mRNA levels. At our approximate Tmax, a 23% decrease in TNFα mRNA levels was observed when compared to

LPS-stimulated LRP collected before misoprostol administration. However, this decrease was not statistically significant. This study indicates that misoprostol is rapidly absorbed following oral administration in horses, but that a single dose of 5ug/kg elicits only a mild inhibitory effect on LPS-stimulated TNFα mRNA production in equine leukocytes *ex vivo*. Further studies analyzing different misoprostol dosing strategies, including repeat administration or combination with other anti-inflammatory drugs, are warranted.

2. Introduction

Immune response to tissue injury and infection is mediated by an intricate series of inflammatory processes, including pro-inflammatory mediator production and subsequent leukocyte activation. While beneficial under homeostatic conditions, these inflammatory responses often become overabundant and disregulated in disease states and lead to unnecessary tissue damage. Of the milieu of pro-inflammatory mediators produced, tumor necrosis factor α (TNFα) is a particularly potent pro-inflammatory cytokine that plays an important role in the pathophysiology of multiple equine disorders, such as inflammatory airway disease (IAD) (Richard et al., 2014), endotoxemia (Morris et al., 1990), and laminitis (Kwon et al., 2013). TNFα induces multiple leukocyte functions that while helpful for defense against pathogens, can conversely exacerbate injury in multiple diseases states such as sepsis (Strassheim et al., 2002), equine laminitis (la RebiÃ re de Pouyade & Serteyn, 2011), and equine recurrent airway obstruction (RAO) (Bullone & Lavoie, 2015). Thus, anti-inflammatory therapeutics that inhibit TNFα activity are needed for treatment of equine inflammatory disease.

Current anti-inflammatory regimens used in equine medicine rely heavily on non-steroidal anti-inflammatory drugs (NSAIDS) that halt the inflammatory cascade through inhibition of prostaglandin production. While these drugs effectively mitigate inflammatory pain and edema, NSAIDs have conversely been demonstrated to increase TNFα production in LPS-stimulated monocytes, whole blood, and arthritic synovial membrane cultures (Page et al., 2010). In addition, NSAIDs are associated with dangerous gastrointestinal (GI) side

effects in horses that limit their use (Cook & Blikslager, 2014; Marshall & Blikslager, 2011). NSAID-induced GI injury is predominantly caused by inhibition of prostaglandin E_2 (PGE_2), which serves a gastroprotective role *in vivo.* Restoration of PGE levels using the PGE_1 analog misoprostol has been shown to aid in mucosal recovery of ischemia injured equine jejunum (Tomlinson & Blikslager, 2005) and reduce gastric acid secretion in the equine stomach (Sangiah er al., 1989). Thus, misoprostol is used clinically to treat and prevent NSAID-induced GI pathology in horses. Interestingly in addition to this role, misoprostol has also been shown to elicit anti-inflammatory effects on human leukocytes by agonizing E-type prostaglandin receptor EP2 (Armstrong, 1995; Talpain et al., 1995). EP2 agonists activate adenylate cyclase (AC), which in turn cyclizes intracellular AMP to form cyclic AMP (cAMP), a critical second messenger molecule that modulates multiple cellular mechanisms. (Smallwood & Malawista, 1995). Elevated intracellular cAMP dampens equine leukocyte effector functions including chemotaxis, adhesion, and production of reactive oxygen species that elicit substantial tissue injury (Brooks et al., 2011; Chilcoat et al., 2002; Sun et al., 2008; Wong et al., 2012). Furthermore, EP2 agonists that elevate intracellular cAMP have been shown to inhibit human leukocyte TNFα production *in vitro* (Meja et al., 1997). Thus, EP2 activation provides a potential alternative mechanism of decreasing TNFα synthesis and inhibiting inflammation in horses that is safer than NSAID use.

Our lab has recently shown that misoprostol inhibits multiple equine leukocyte functions *in vitro* including LPS-stimulated TNFα production (unpublished data). However, the therapeutic potential and anti-inflammatory benefits of oral misoprostol administration in horses have not yet been evaluated. Multiple studies have investigated the ability of

misoprostol to control inflammation *in vivo* in other species. Oral misoprostol administration reduces serum TNFα levels in rodents following intravenous administration of lipopolysaccharide (LPS) (Mahatma et al., 1991; Nakamura et al., 1992), as well as *ex vivo* in LPS-stimulated human whole blood (Gobejishvili et al., 2015). Oral administration of misoprostol also attenuates inflammation in rodent models of airway inflammation (Smith et al., 1996), GI injury (Yamada et al., 1991), and carragenan-induced paw inflammation (Moraes et al., 2007). Interestingly, misoprostol has also been shown to augment the anti-inflammatory benefits of NSAIDs when they are administered simultaneously (Kitsis et al., 1991; Moraes et al., 2007), potentially by dampening the stimulatory effect of NSAIDs on TNFα synthesis. Thus, misoprostol has the potential to provide not only gastroprotective benefits, but also anti-inflammatory effects when used alone or in combination with NSAID therapy in horses.

Misoprostol has been shown to have a relatively short half-life following oral administration, with a Tmax of 20-30 minutes in humans and a highly variable Cmax (Abdel-Aleem et al., 2003; Huang et al., 2012; Tang et al., 2007). The pharmacokinetics of misoprostol have not yet been evaluated in horses, but are critical to establishing the use of misoprostol as an anti-inflammatory therapeutic in this species. Additionally, the effects of oral misoprostol administration on equine leukocyte TNFα production have not yet been evaluated. Thus, our goal was to define the pharmacokinetics of oral misoprostol administration in horses, as well as the effects of orally dosed misoprostol on TNFα cytokine production of stimulated equine leukocytes. We hypothesized that administration of a single oral dose of 5ug/kg misoprostol, which is used clinically in horses (Jacobson et al., 2013),

would significantly inhibit TNFα mRNA production in LPS-stimulated equine leukocytes in an *ex vivo* inflammation model.

3. Materials and Methods

Equine Subjects

All experiments performed in this study were approved by the North Carolina State University (NCSU) Institutional Animal Care and Use Committee. Six adult, castrated male horses of various breeds were used. Horses weighed between 400-600kg, and ages ranged from 5-15 years. All horses were deemed healthy based on normal physical exam, blood serum chemistry, and complete blood count findings prior to inclusion in the study. Horses were housed in indoor box stalls for the duration of the study and offered free choice water and hay, with the exception of a 12 hour fasting period prior to administration of misoprostol.

3.1 Methods: Pharmacokinetic Study

Misoprostol Administration

One day prior to drug administration, an area over the right jugular vein of each horse was clipped and aseptically prepared. A local subcutaneous block was performed with 0.5mL of 2% lidocaine, followed by percutaneous placement of a 14-guage 13.3cm intravenous catheter into the right jugular vein as previously described (Messenger et al., 2011). Extension tubing was secured to each catheter, and a three-way stopcock was utilized to cap the tubing and facilitate blood collection. The total internal volume of the catheter plus

extension tubing was 3.8mL. Catheters and extension tubing were then secured in place using 2-0 Monosof suture, and routine monitoring of the catheter and catheterization site was performed throughout the study. Horses were then fasted overnight. The next day, misoprostol tablets (Cytotec 200ug, Pfizer) at a dose of 5ug/kg were dissolved in 30ml of warm water in a 60mL catheter tip syringe for 5-10 minutes. 10mL of Karo syrup was added to enhance palatability. Misoprostol solutions were then immediately administered to horses in the interdental space onto the back of the tongue, and the head was elevation for approximately 30 seconds to ensure adequate delivery of the drug. Physical exams were conducted prior to drug administration, as well as 1, 2, 4, 6, 8, 10, 12, and 24 hours following misoprostol administration to assess horse's mentation, rectal temperature, heart and respiratory rates, capillary refill time, mucous membrane color and hydration, digital pulse, as well as intestinal borborygmi in all four gut quadrants in response to the drug. All abnormalities were noted, and none required intervention by a veterinarian. Horses were fed one flake of hay 2 hours following drug delivery, and fecal piles were quantified at each sampling point.

Blood Sampling

Thirty seconds prior to blood sample collection, 10mL of blood waste was collected through the catheter and discarded to ensure adequate removal of contaminants from the extension line. 7mL of blood was then sampled through the catheter at 10, 20, 30, 45, 60, and 90 minutes, and 2, 4, 6, 8, 12, and 24 hours following oral misoprostol administration and immediately placed into lithium heparin vacutainer tubes. Catheters were flushed with 10mL

heparinized saline following each blood collection. Blood samples were kept on ice for a maximum of 1 hour prior to centrifugation at 2,000 *g* for 10 minutes. Plasma was collected, placed into cryogenic plastic tubes, and frozen at -80°C until analysis.

Chromatographic Assay – Misoprostol Free Acid

Misoprostol is quickly metabolized in plasma to the stable metabolite, misoprostol free acid (MFA). Plasma concentrations of MFA were determined by ultra-high pressure liquid chromatography with mass spectrometry. Calibration curves were prepared by fortifying blank equine plasma with stock solutions of misoprostol free acid (Cayman Chemical, Ann Arbor, MI, USA) and the internal standard, misoprostol free acid-d5, (Cayman Chemical, Ann Arbor, MI, USA) dissolved in 100% methanol. Samples and standards were then prepared by adding 1 mL plasma to 1 mL of 1% formic acid in water in a glass tube, and vortexing for 15 seconds. The sample mixture was then added to supported liquid extraction cartridges (Isolute SLE+ 2 mL, Biotage, Charlotte, NC, USA) and a light vacuum was applied to initiate absorption. Two aliquots of 5 mL of methyl tert-butyl ether were added to the cartridges, allowed to sit for 5 minutes, and then slowly eluted under light vacuum. The resulting eluate was then placed in an evaporator and dried under a 20 psi stream of nitrogen for 30 minutes at 44°C. Samples were reconstituted in 100 µl of 50:50 water:methanol (v/v). Volumes of 80 µL for samples and standards were injected on an Agilent Infinity 1290 system coupled with an Agilent G6530A Q-TOF Mass Spectrometer (Agilent Technologies, Santa Clara, CA, USA) run in ESI negative mode. Parameters for the ESI dual source were: drying gas, 9 L/min of nitrogen; nebulization gas, 30 psi; sheath gas flow rate, 11 L/min;

sheath gas temperature, 350°C; drying gas temperature, 350°C; capillary voltage, 4000 V; nozzle voltage, 1000 V; fragmentor voltage, 120 V; and CID collision gas, nitrogen. A gradient was used and the initial mobile phase was 5mM ammonium acetate buffer and 95:5 water:acetonitrile (50:50 v/v) for the first 10 minutes. The last 2 minutes of the run, the mobile phase was (5:95v/v). Flow rate was maintained at 0.5 mL/min. The SIRS used for misoprostol free acid and misoprostol free acid-d5 were 367.25 and 372.29 respectively. Separation was achieved using an Agilent Zorbax SB C18 (3.5 μm, 4.6 x 50 mm) and guard column (Waters Corporation, Milford, MA, USA) maintained at 30°C. Under these conditions, the retention time for misoprostol free acid and misoprostol free aid-d5 were 8.4 and 8.0 minutes respectively. Standard curves were linear over a concentration range of 0.05-50 ng/mL. The lower limit of quantification was 0.05 ng/mL, with an $R^2 \geq 0.99$.

Data Analysis

Data analysis was performed using a one-compartmental model, which was chosen based on visual analysis of the mean plasma concentration versus time curve to determine the model of best fit. The maximal plasma concentration of MFA (Cmax) and the time at which MFA reached maximal concentration (Tmax) were derived from the plasma concentration curve and are presented as mean ± SD. Additional parameters reported here are the total systemic drug exposure, as calculated by the area under the MFA serum concentration versus time curve extrapolated to infinity ($AUC_{0-\infty}$), the first-order absorption and elimination rate constants (k_{01} and k_{10}, respectively), and the half-life of absorption and elimination (k_{01} $t_{½}$ and k_{10} $t_{½}$, respectively) of misoprostol. As no intravenous administration of misoprostol was

performed for comparison, the true bioavailability (F) of the drug could not be calculated. Thus, the primary pharmacokinetic parameters of volume of distribution (Vd) and clearance (Cl) are reported as Vd/F and Cl/F and assumes that F is 100%.

3.2 Methods: Ex Vivo Inflammation Model Study

Misoprostol Administration and Blood Sampling

In a separate phase of this study, conducted 9 months following the pharmacokinetics trial, the same group of horses was utilized to evaluate the effects of misoprostol in an *ex vivo* equine inflammation model. Misoprostol was orally administered and physical exams and monitoring were completed as described in the pharmacokinetic study methods section. Thirty milliliters of heparinized whole blood was obtained via jugular venipuncture prior to drug administration (time 0, T0), and 30 minutes, 1 hour, and 4 hours following drug administration. An additional EDTA whole blood sample was obtained at the T0 and 30 minute time points and used for a complete blood count analysis. Samples were taken from alternating sides, as well as alternating upper and lower quadrants of the vein to avoid excessive jugular trauma. Following collection, heparinized whole blood was aliquoted into sterile 15 mL polypropylene conical tubes and allowed to settle at room temperature for 1 hour. After erythrocytes settled out of solution, and the leukocyte-rich plasma (LRP) layer was collected and leukocytes were examined for viability via trypan blue exclusion. Leukocyte viability was routinely above 95% prior to and following oral misoprostol administration.

Ex Vivo LPS Stimulation

One milliliter aliquots of LRP were placed into sterile polypropylene micro centrifuge tubes and treated with LPS (*E. coli* 055:B5, Sigma-Aldrich, St. Louis, MO) at 100ng/mL final concentration, or the vehicle for LPS (sterile PBS) for 2 hours at 37°C. Leukocyte viability was assessed via trypan blue exclusion and was routinely >95%.

RNA Isolation and First-Strand cDNA Synthesis

RNA isolation and DNase materials were obtained from Qiagen (Valencia, CA). RNA was isolated using an RNEasy Mini Kit per manufacturer's instructions, with homogenation using a QIAshredder. One on-column DNAse digestion was performed prior to RNA elution, as well as following RNA elution using an RNase-Free DNAse set. RNA was then cleaned up using an RNEasy mini kit per manufacturer's protocol. Final eluted RNA concentration was determined using a Nanodrop Spectrometer. First-strand cDNA synthesis was carried out with equal quantities of mRNA from each sample using the Superscript III Reverse Transcription System (Invitrogen, Thermo-Fischer Scientific, Grand Island, NY) per manufacturer's instructions using random hexamers (50ng/mL).

Real-Time PCR

Real-time PCR was performed using a BioRad MyIQ Single-Color Real-Time PCR Detection System (Biorad, Hercules, CA). A PCR master mix was prepared for each sample, and each was dispensed onto PCR plates in triplicate. Each well contained 1 x Taqman Gene Expression Master Mix, 1 x Taqman primer and probe (Applied Biosystems, Thermo-Fischer

Scientific), equal quantities of cDNA (ranging from 6-10ng per experiment), and RNase-free water up to a final volume of 25ul. No-reverse-transcriptase and no-template controls were included to confirm the absence of genomic DNA and DNA contamination, respectively. Equine-specific Taqman primers and probes were obtained from Invitrogen's proprietary database (Assay ID for TNFα: Ec03467871, and β2M: Ec03468699). Invitrogen validates these primers and provides the NCBI target sequence used to design the primer and probe, the 25 base pair region of probe binding, and predicted size of the amplicon for each set (Canales et al., 2006; Shi et al., 2006). In preliminary experiments, products of the PCR reaction were run on a 2% agarose gel and visualized using EZ Vision Three DNA Dye (Amresco, Solon, OH) to verify product specificity. Amplification cycles were carried out per manufacturer's protocol as follows: 50°C for 2 minutes, one time; 95°C for 10 minutes, one time; 95°C for 15 seconds, followed by 60°C for 1 minute (with enabled real-time data collection), 40 times. Fold change in TNFα mRNA levels was calculated using the ΔΔCt method of data analysis using β2M as the housekeeping gene. We have previously identified β2M as a stably expressed housekeeping gene in equine leukocytes using the method developed by Radonic *et. al* (Radonić et al., 2004) (data not shown).

Statistical Analysis

All data were normally distributed as determined by the Shapiro-Wilk method. mRNA data are expressed as mean fold change TNFα mRNA ± SEM, and were analyzed using One Way RM ANOVA with Holm-Sidak multiple comparisons *post hoc* testing. Mean pulse, respiration rate, temperature, complete blood cell counts of total white blood cells (WBCs)

and individual leukocyte populations were compared before and after misoprostol administration via paired t-test, and were not significantly different.

4. Results

4.1 Misoprostol Dosing and Tolerance

Oral misoprostol administration at a dose of 5ug/kg is routinely used in clinical settings at the NCSU College of Veterinary Medicine Teaching Hospital and is generally well tolerated. Misoprostol was administered to horses orally by a veterinarian who is board-certified in equine internal medicine and pharmacology (JLD). Minimal loss of drug occurred during each oral dosing procedure. In the first phase of this study, horses showed few side effects to this dose of misoprostol, with the exception of one horse who displayed signs of abdominal discomfort, soft manure, and depressed mentation 30 minutes post dosing. This episode did not require veterinary intervention and resolved before conclusion of the 24-hour study period. One additional horse developed soft manure approximately 2 hours post dosing that also resolved within 24 hours. In the second phase of the study, 4 of the 6 horses had one soft manure pile over the 24-hour study period but did not display any signs of discomfort or colic. Physical exam parameters including pulse, respiratory rate, and temperature were not significantly different before and after misoprostol administration (data not shown). Complete blood counts reflected no significant changes in white blood cell (WBC) number, or individual populations of neutrophils, monocytes, or lymphocytes following misoprostol administration in the second phase of the trial (Table 2).

4.2 Misoprostol Pharmacokinetics

Misoprostol is quickly metabolized *in vivo* via rapid presystemic de-esterification in the stomach to form the pharmacologically active carboxylic acid, misoprostol acid (Davies et al., 2001). Thus, misoprostol free acid (MFA) was measured in equine plasma as a reflection of misoprostol absorption. The mean plasma concentration of misoprostol free acid versus time following oral administration of misoprostol in five horses is depicted in Figure 1. A single dose of misoprostol was absorbed quickly and resulted in detectable MFA levels in the plasma by 15 minutes following oral drug administration. The time of peak MFA plasma concentration (Tmax) occurred at 23.4 ± 2.4 minutes following administration, and the maximal MFA plasma concentration (Cmax) was 290 ± 70 pg/mL. Plasma MFA concentration increased with an absorption half-life (k_{01} $t_{1/2}$) of 7.2 ± 2.4 minutes, and declined with an elimination half-life (k_{10} $t_{1/2}$) of 40.2 ± 12 minutes. The mean total systemic exposure to the drug as extrapolated by $AUC_{0-\infty}$ was found to be 400 ± 120 pg h/mL (Table 2).

4.3 Oral misoprostol administration leads to a trending, but insignificant inhibition of TNFα mRNA levels ex vivo in LPS stimulated equine leukocytes

Misoprostol has been demonstrated to decrease TNFα production *in vivo* in mouse models (Mahatma et al., 1991; Nakamura et al., 1992), and *in vitro* in human neutrophils (Meja et al., 1997). Additionally, a recent study in human subjects demonstrated that a 14-day course of orally administered misoprostol at doses of 100-300μg four times daily significantly inhibited TNFα levels by approximately 29% in an *ex vivo* LPS-stimulated whole blood

model (Gobejishvili et al., 2015). Thus, we were interested in evaluating the effect of oral misoprostol administration on TNFα production in equine leukocytes using a similar model of inflammation in horses. Stimulation of equine leukocytes with 100ng/mL of LPS led to significant increase in TNF-α mRNA levels compared to vehicle controls at all four time points sampled: prior to dosing (time 0, or T0), at the approximate Tmax (30m), and 1 and 4 hours after misoprostol administration (Figure 2A). Administration of 5μg/kg misoprostol decreased LPS-stimulated TNF-α mRNA expression at 30 minutes by approximately 23%, however this did not reach statistical significance (Figure 2B). High inter horse variability was seen, with misoprostol decreasing TNFα production in four of the six horses at 30 minutes (in one horse (A) by approximately 75%), and slightly increasing TNFα mRNA levels in two horses tested (Figure 2C).

5. Discussion

TNFα plays a destructive role in many equine inflammatory diseases including IAD, RAO, endotoxemia, and laminitis (Bullone & Lavoie, 2015; Kwon et al., 2013; Morris et al., 1990; Richard et al., 2014). Currently, equine veterinary medicine relies heavily on NSAID therapies to reduce inflammation and pain, however these drugs are associated with detrimental GI side effects in horses and can increase TNFα production (Marshall & Blikslager, 2011; Page et al., 2010). In human medicine, specific TNFα-targeting strategies have been developed that effectively inhibit inflammation, but are associated with multiple side effects (Lichtenstein et al., 2012). Thus, there is a critical need for safer therapies to

inhibit TNFα production in both equine and human inflammatory disease. Misoprostol, a PGE_1 analog that is currently used as a gastroprotectant in horses and humans, has been shown to decrease pro-inflammatory cytokine expression *in vitro* and *in vivo* in human cells and rodent models (Mahatma et al., 1991; Meja et al., 1997; Nakamura et al., 1992; Widomski et al., 1997). Furthermore, previous studies in our lab have shown that misoprostol inhibits TNFα mRNA production and secretion in a concentration-dependent manner in equine leukocytes *in vitro* (unpublished data). Thus, we sought to evaluate the effect of orally-administered misoprostol on TNFα mRNA production in an *ex vivo* equine leukocyte model of inflammation. We first defined the pharmacokinetics of orally administered misoprostol in horses, and found that the Cmax and Tmax of the misoprostol metabolite, misoprostol free acid (MFA), was comparable to data from human trials (Abdel-Aleem et al., 2003; Huang et al., 2012; Tang et al., 2002). A recent study reports that repeat oral dosing of misoprostol in humans significantly inhibited LPS-stimulated TNFα levels in whole blood by 29% in an *ex vivo* inflammation model (Gobejishvili et al., 2015). Therefore, we hypothesized that orally-administered misoprostol would inhibit TNFα mRNA production in our equine *ex vivo* model as well. Our study demonstrates that a single oral dose of 5μg/kg misoprostol led to a 23% decrease in TNFα mRNA levels in LPS-stimulated equine leukocytes *ex vivo*, however this decrease was not statistically significant (Figure 2).

This study is the first to define the pharmacokinetics of oral misoprostol administration in horses. A single dose of 5μg /kg misoprostol produced a Cmax of 290 ± 70 pg/mL and a Tmax of 23.4 ± 2.4 minutes. Additionally, this dose of misoprostol resulted in an absorption half-life (k_{01} $t_{1/2}$) of 7.2 ± 2.4 minutes, an elimination half-life (k_{10} $t_{1/2}$) of 40.2

± 12 minutes, and an $AUC_{0-\infty}$ of 400 ± 120 pg h/mL (Table 2). These kinetics are similar to those previously reported in human literature. Misoprostol is a lipophilic methyl ester drug that is rapidly and extensively absorbed from the GI tract (Tang et al., 2007). While high lipophilicity promotes quick absorption, it is also associated with rapid metabolism. In accordance with these properties, orally administered misoprostol displays a consistent mean Tmax in humans of 20-29 minutes and elimination half-life of 40-70 minutes (Abdel-Aleem et al., 2003; Foote et al., 1995; Huang et al., 2012; Tang et al., 2002). However, other pharmacokinetic parameters have been shown to be inconsistent between human studies. Reported values for Cmax range from 280-2047 pg/mL, and $AUC_{0-\infty}$ values range from 420-1700 pg h/mL (Foote et al., 1995; Huang et al., 2012; Tang et al., 2002). These human studies report a coefficient of variation between Cmax values in different subjects of up to 50%, indicating that misoprostol is a highly variable drug (Tang et al., 2002). These wide-ranging differences may be accounted for by well-known concomitant parameters such as age, weight, genetic differences, and race/breed differences that affect absorption, elimination, or metabolism of the drug between subjects. These factors could also explain the inter-horse variability of Cmax and $AUC_{0-\infty}$ seen in the current study following identical dosing strategies. This variability could potentially cause differences in the anti-inflammatory effects of misoprostol in horses within the same population.

In this study, we evaluated the effect of oral administration of misoprostol on equine leukocyte TNFα mRNA levels following LPS stimulation *ex vivo*. TNFα is a potent pro-inflammatory cytokine and key player in the pathophysiology of equine inflammatory disease. TNFα signals through two distinct transmembrane receptors, TNF Receptor 1

(TNFR1) and TNFR2, which can elicit diverging intracellular signaling pathways. TNFR activation recruits various proteins to the receptor cytoplasmic tails to direct downstream signaling events ranging from apoptosis to inflammation and enhanced cell survival. Pro-inflammatory effects elicited by TNFα are primarily mediated through activation of the transcription factor NF-κβ, and include increased expression of endothelial adhesion molecules needed for leukocyte extravasation and emigration into tissues, and induction of pro-inflammatory prostaglandins, chemokines, and cytokines that mediate immune cell activation and responses (Meager, 1999; Sedger & McDermott, 2014). TNFα has also been associated with downregulation of neutrophil motility, enhanced release of reactive oxygen species (ROS) and proteases, and has been implicated in dysregulated immune responses during sepsis (Beutler et al., 2008; Binder et al., 1999). Thus, inhibition of TNFα has been intensely investigated for treatment of inflammatory disease in humans. Anti-TNFα monoclonal antibody therapies elicit a small but significant increase in survival of human septic patients (Lv et al., 2014). Additionally, anti-TNFα therapy inhibits inflammation in inflammatory bowel disease in humans, but is also associated with loss of efficacy in a large percentage of patients (Ben-Horin et al., 2014). Furthermore, side effects of anti-TNF therapy have been reported that include serious and potentially life-threatening infections (Lichtenstein et al., 2012). Reviews of these therapies indicate the importance of TNFα in mediating inflammatory disease and indicate a need for continued study of mechanisms to modulate TNFα.

One potential mechanism of inhibiting TNFα production is elevation of intracellular cyclic AMP (cAMP), a cyclic nucleotide that regulates multiple innate immune cell

functions. Elevation of cAMP is induced by activation of stimulatory G-protein coupled receptor (GPCR) signaling, leading to activation of intracellular adenylate cyclase (AC) and cyclization of AMP to form cAMP (Serezani et al., 2008). Agonists of the PGE GPCRs EP2 and EP4, including misoprostol and naturally-occurring PGE_1, lead to elevation of intracellular cAMP and have been demonstrated to dampen LPS-stimulated cytokine production in leukocytes (Haynes et al., 1992; Smallwood & Malawista, 1995; Talpain et al., 1995). LPS is a pathogen-associated molecular pattern (PAMP) that induces TNFα mRNA production by interaction with toll-like receptor 4 (TLR4) on leukocytes. TLR4 signaling stimulates multiple pathways that culminate in activation of transcription factors such as NF-κβ and AP-1 that mediate pro-inflammatory cytokine synthesis. Activated AP-1 and NF-κβ translocate into the nucleus and bind promoter regions of proinflammatory genes such as TNF-a, IL-6, and IL-1β, to induce gene transcription. It has been proposed that misoprostol can inhibit pro-inflammatory cytokine production through cAMP activation of protein kinase A (PKA) and subsequent inhibition of transcription factor CREB (cAMP response element-binding protein) (Gobejishvili et al., 2015). Phosphorylated CREB inhibits NF-κβ transcriptional activity through competition for coactivators that are needed for NF-κβ binding and function in the promoter region of proinflammatory genes (Wen et al., 2010). Thus, we hypothesized that oral administration of the EP2, EP3, and EP4 receptor agonist and cAMP-elevating agent misoprostol would inhibit LPS-stimulated equine leukocyte TNFα mRNA production in an *ex vivo* model of equine inflammation.

The plasma concentration of misoprostol that elicits anti-inflammatory activity *in vivo* has not been determined. However, *in vitro* studies report IC50s of 750nM and 955nM for

misoprostol-induced TNFα suppression in human monocytes (Haynes et al., 1992; Meja et al., 1997). Our mean Cmax of 290 pg/mL (758pM) in horses is one order of magnitude lower than these predicted IC50s, however it is important to remember results obtained from *in vitro* studies are not always an accurate representation of the *in vivo* environment. *In vivo*, leukocytes interact with other cell types such as endothelial cells that are critical to innate immune cell responses during inflammation. In rodent models of ischemia-reperfusion injury, misoprostol provides tissue protective effects that are mediated through EP2 and EP4 receptors on endothelial cells (Li et al., 2008; Natori et al., 1997). Therefore, *in vivo* administration of misoprostol affects leukocytes directly and indirectly through their actions on other cell types.

In our study, a single oral dose of misoprostol (5µg /kg) led to a 23% decrease in LPS-induced TNFα mRNA production *ex vivo* at 30 minutes when compared to baseline controls (0 hours). However, this trend was not statistically significant. We hypothesized that lack of significance was due to high inter-horse variability, as well as a low maximal plasma concentration of misoprostol obtained following oral administration. Our results are similar to a recently published trial in which oral misoprostol administration in humans resulted in a significant decrease (29%) in TNFα levels in an *ex vivo* inflammation model similar to our study design (Gobejishvili et al., 2015). This human trial differed from our study in that they utilized a repeat dosing strategy of 1-300ug misoprostol four times daily for 14 days prior to *ex vivo* LPS stimulation of whole blood. Repeat dosing of misoprostol is unlikely to achieve higher Cmax values in horses, as peak plasma concentrations have been shown to remain relatively stable after a repeat dosing regimen every 3 hours in humans; thus no drug

accumulation in the plasma is detected. However, this study did demonstrate that the $AUC_{0-\infty}$ and therefore total systemic drug exposure was significantly increased following this repeat dosing regimen (Tang et al., 2009). Therefore, repeat dosing could exert a cumulative effect on cells exposed to misoprostol for longer periods and has yet to be explored in horses.

Additional methods of increasing peak plasma concentrations of misoprostol, and thus anti-inflammatory effects *in vivo*, include administration of a higher dose of misoprostol. The Cmax of MFA in this study was relatively low, and detection of MFA plasma concentrations in horses required a highly sensitive quantification method (time of flight quantification). This is most likely due to the short half-life, high plasma protein binding (85%), and poor systemic bioavailability (7%) of orally administered misoprostol. However, administering a higher dose of misoprostol is likely to result in gastrointestinal side effects in horses. Administering greater than 400-800ug misoprostol per day in humans is associated with diarrhea and abdominal cramping (Davies et al., 2001). The dose utilized in this study (5µg /kg) is routinely and safely used in horses clinically (Jacobson et al., 2013), however it is unknown if higher doses of misoprostol would elicit these abdominal side effects in equine patients. One horse in the first phase of our study developed signs of colic within 30 minutes of dosing, as evidenced by abdominal discomfort, soft feces, and dull mentation. While no medical intervention was needed, it is likely that increasing the dose of misoprostol could potentially lead to additional or more severe side effects.

If repeat or increased dosing strategies prove to be ineffective, misoprostol could be used to augment the anti-inflammatory effects of NSAIDs while preserving GI homeostasis in horses. Previous studies have demonstrated that misoprostol enhances the anti-

inflammatory effects of the NSAIDs piroxicam, indomethacin, and sodium salicylate on neutrophil ROS production, degranulation, and aggregation *in vitro* (Kitsis et al., 1991). Additionally, misoprostol augments the anti-inflammatory effects of the COX-2 selective drug etoricoxib in rodent models of carrageen-induced paw inflammation (Moraes et al., 2007). The augmentative anti-inflammatory effects of misoprostol are potentially mediated by inhibition of NSAID-induced TNFα production, as has been demonstrated in human monocytes and arthritic synovial membrane explants (Page et al., 2010). Conversely, some studies have shown that oral administration of NSAIDs significantly reduces TNFα levels in osteoarthritic human synovial joints (Gallelli et al., 2013). Additionally, the COX-2 inhibitor nimesulide has been shown to decrease TNFα mRNA production in murine brain tissues following LPS challenge (Teeling et al., 2010). Taken together, these studies indicate that addition of misoprostol to existing NSAID therapy could strengthen anti-inflammatory effects by either reducing NSAID-induced TNFα production, or by augmenting the inhibitory effects of NSAIDs on TNFα synthesis that occur in certain disease processes.

This study demonstrates that orally administered cAMP-elevating agents and EP2 receptor agonists elicit moderate anti-inflammatory effects on equine leukocytes *ex vivo.* This paves the way for use of other potentially more effective cAMP-elevating agents and EP2 receptor agonists in horses as they become available. Previous studies have shown that oral administration of pentoxifylline (PTX), a phosphodiesterase inhibitor that increases intracellular cAMP, decreases severity of RAO in equine models but does not inhibit leukocyte influx (Leguillette et al., 2002). Additionally, intravenous PTX produces mild beneficial effects in models of equine endotoxemia by decreasing rectal temperature,

respiratory rate, and prolonging time to recalcification of whole blood, however substantial benefit was not observed (Barton et al., 1997b). Taken together with our study, oral administration of existing as well as future cAMP-elevating agents have the potential to decrease inflammation in equine disease. Therapeutic EP2 receptor agonists are not yet commercially available, although multiple groups have developed small molecule EP2 agonists that produce beneficial anti-inflammatory effects in preclinical stroke and glaucoma models (Ganesh, 2014). Thus, future EP2 receptor agonists could exert beneficial effects in equine inflammatory diseases.

Use of misoprostol as an anti-inflammatory in specific types of diseases warrants further study. For example, misoprostol has been shown to enhance T17-cell mediated inflammation in disease models of rheumatoid arthritis and inflammatory bowel disease (Sheibanie et al., 2007). Previous studies in our lab have demonstrated that misoprostol inhibits production of multiple cytokines in equine leukocytes, as well as decreases equine neutrophil functions in a concentration-dependent manner (unpublished data). Together with the current study, this data suggests that misoprostol would provide moderate anti-inflammatory effects *in vivo* to inhibit inflammation in leukocyte-mediated diseases in horses. However, different misoprostol dosing strategies warrant further study in this *ex vivo* model prior to *in vivo* testing.

6. Figures

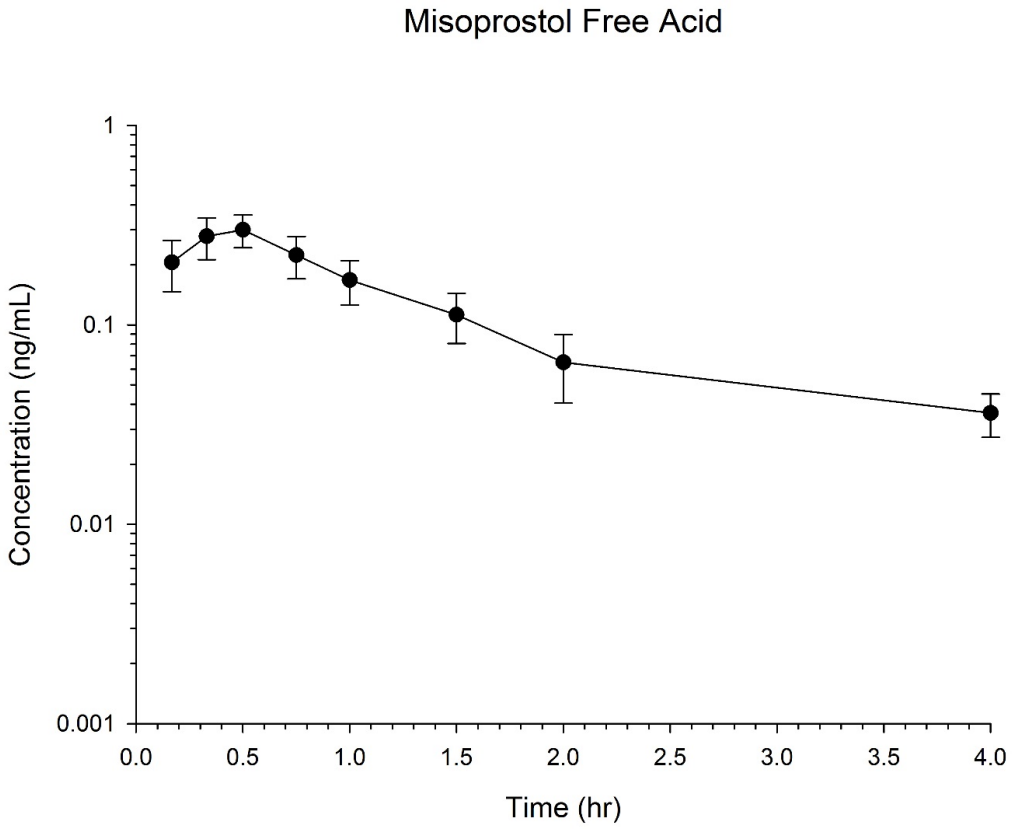
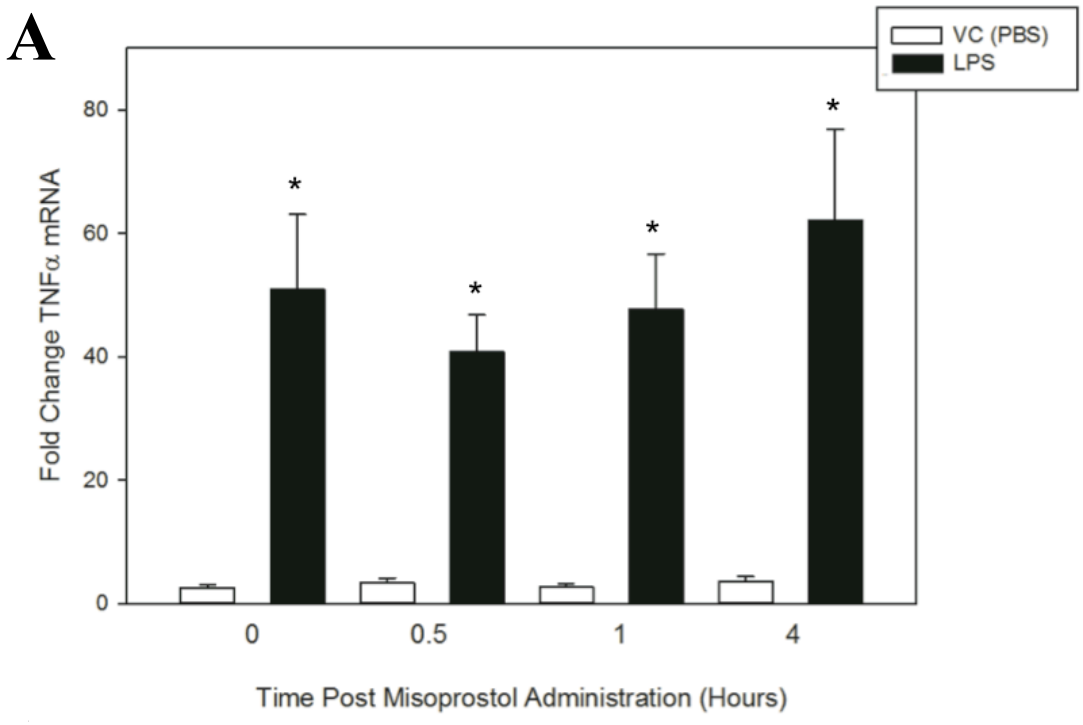


Figure 1: Mean plasma concentration of misoprostol free acid (pg/mL) versus time (hours) after oral administration of misoprostol at a dose of 5ug/kg. Data are represented as mean ± SD and include five different horses

Figure 2: Effect of oral misoprostol administration on LPS-stimulated equine leukocyte TNFα mRNA levels *ex vivo*.

Heparinized whole blood samples were obtained via jugular venipuncture before oral misoprostol administration (0 hours), as well as 0.5, 1, and 4 hours after administration. One leukocyte-rich plasma (LRP) sample was immediately processed per horse as a baseline measurement of TNFα mRNA levels. The remaining LRP was divided into groups and stimulated with LPS (100ng/mL) or vehicle (PBS) for 2 hours. mRNA was isolated for real-time PCR. **(A)** LPS significantly increased TNFα mRNA levels at each time point evaluated. Data are presented as mean fold change TNFα mRNA ± SEM versus unstimulated baseline cells (not shown, equal to 1) and represent 6 horses. *$p<0.05$ versus time-matched vehicle treated cells (white bar) via paired t-test. **(B)** Misoprostol did not exert a statistically significant effect on LPS-stimulated TNFα mRNA levels in equine leukocyte rich plasma. Data are presented as mean fold change TNFα mRNA ± SEM versus time-matched vehicle treated unstimulated cells (not shown, equal to 1) and represent 6 different horses. **(C)** Inter-horse variability of the effect of misoprostol on LPS-stimulated TNFα mRNA levels. Data are presented as in (B).


A
VC (PBS)
LPS
Fold Change TNFα mRNA
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80
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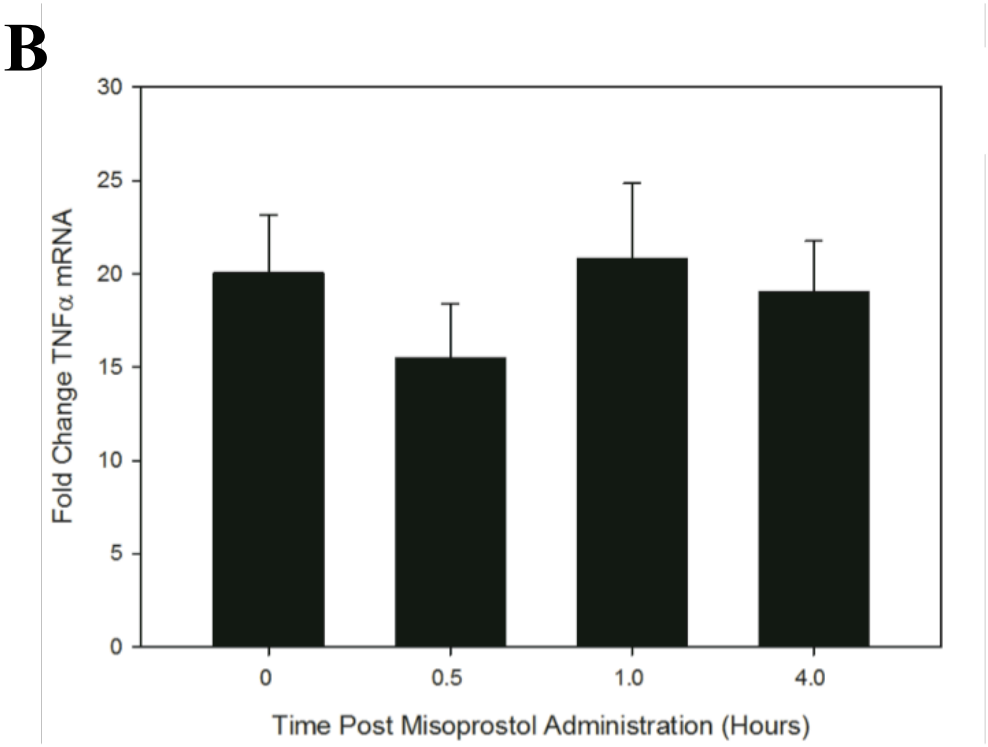
4
Time Post Misoprostol Administration (Hours)


B
Fold Change TNFα mRNA
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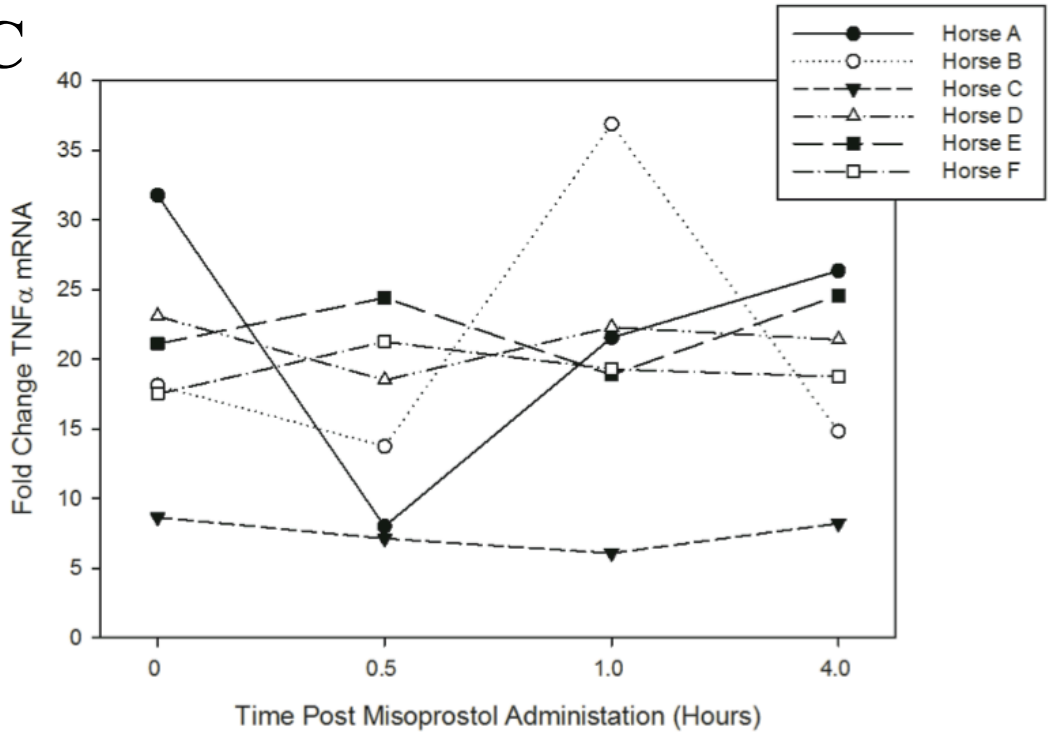
Time Post Misoprostol Administration (Hours)

C


Horse A
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Horse D
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35
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5
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0
0.5
1.0
4.0
Time Post Misoprostol Administation (Hours)

7. Tables

Table 1: Pharmacokinetics of oral administration of 5ug/kg misoprostol in 5 horses.

Data represent 5 different horses. $T_{max,}$ time to maximum concentration; C_{max}, maximum concentration; k_{01}, first-order absorption rate constant; k_{10}, first-order elimination rate constant; k_{01} $t_{1/2\alpha,}$ half-life of absorption; k_{10} $t_{1/2,}$ half-life of elimination; $AUC_{0-\infty}$, area under the concentration-time curve extrapolated to infinity; Vd/F, apparent volume of distribution dependent on bioavailability; Cl/F, clearance dependent on bioavailability.

Pharmacokinetic Parameter	**Value ± SD**
T_{max} (hr)	0.39 ± 0.04
C_{max} (ng/mL)	0.29 ± 0.07
k_{01} (hr^{-1})	6.27 ± 1.57
k_{10} (hr^{-1})	1.10 ± 0.31
k_{01} $t_{1/2}$ (hr)	0.12 ± 0.04
k_{10} $t_{1/2}$ (hr)	0.67 ± 0.20
$AUC_{0-\infty}$ (hr*ng/mL)	0.40 ± 0.12
Vd/F	12.49 ± 2.38
Cl/F	224.84 ± 59.89

Table 2: Effects of 5ug/kg oral misoprostol on leukocyte counts before (0 hours) and 0.5 hours following administration. Values were not significantly different before or after oral misoprostol administration (via pared t-test or signed rank t-test, where appropriate).

	Cell Number (10^3/µL)							
	Total WBC		Neutrophils		Monocytes		Lymphocytes	
Min	0	30	0	30	0	30	0	30
Horse A	6.18	6.20	4.264	4.526	0.062	0.00	1.669	1.550
Horse B	6.72	6.00	2.755	3.00	0.067	0.18	3.696	2.160
Horse C	6.62	6.25	4.237	4.813	0.066	0.313	2.052	0.875
Horse D	6.72	6.46	5.174	4.845	0.067	0.065	1.142	1.292
Horse E	5.79	5.93	3.532	3.973	0.116	0.059	2.084	1.779
Horse F	5.54	5.65	3.158	2.543	0.166	0.113	2.161	2.656

REFERENCES

Abdel-Aleem, H., Villar, J., Metin Gülmezoglu, A., Mostafa, S. A., Youssef, A. A., Shokry, M., & Watzer, B. (2003). The pharmacokinetics of the prostaglandin E1 analogue misoprostol in plasma and colostrum after postpartum oral administration. *European Journal of Obstetrics & Gynecology and Reproductive Biology*, *108*(1), 25–28. http://doi.org/10.1016/S0301-2115(02)00355-X

Abdelnour, A., Bremell, T., Holmdahl, R., & Tarkowski, A. (1994). Role of T lymphocytes in experimental Staphylococcus aureus arthritis. Scandinavian Journal of Immunology, 39(4), 403–408.

Abram, C. L., & Lowell, C. A. (2009). The Ins and Outs of Leukocyte Integrin Signaling. *Annual Review of Immunology*, *27*(1), 339–362. http://doi.org/10.1146/annurev.immunol.021908.132554

Ahluwalia, A., Baatar, D., Jones, M. K., & Tarnawski, A. S. (2014). Novel mechanisms and signaling pathways of esophageal ulcer healing: the role of prostaglandin EP2 receptors, cAMP, and pCREB. *AJP: Gastrointestinal and Liver Physiology*, *307*(6), G602–G610. http://doi.org/10.1152/ajpgi.00177.2014

Amin, A. R., Attur, M., Patel, R. N., Thakker, G. D., Marshall, P. J., Rediske, J., et al. (1997). Superinduction of cyclooxygenase-2 activity in human osteoarthritis-affected cartilage. Influence of nitric oxide. *Journal of Clinical Investigation*, *99*(6), 1231–1237. http://doi.org/10.1172/JCI119280

Anderson, B. O., Brown, J. M., & Harken, A. H. (1991). Mechanisms of neutrophil-mediated tissue injury. *The Journal of Surgical Research*, *51*(2), 170–179.

Arhancet, G. B., Walker, D. P., Metz, S., Fobian, Y. M., Heasley, S. E., Carter, J. S., et al. (2013). Discovery and SAR of PF-4693627, a potent, selective and orally bioavailable mPGES-1 inhibitor for the potential treatment of inflammation. *Bioorganic & Medicinal Chemistry Letters*, *23*(4), 1114–1119. http://doi.org/10.1016/j.bmcl.2012.11.109

Arikawa, T., Omura, K., & Morita, I. (2004). Regulation of bone morphogenetic protein-2 expression by endogenous prostaglandin E2 in human mesenchymal stem cells. *Journal of Cellular Physiology*, *200*(3), 400–406. http://doi.org/10.1002/jcp.20031

Armstrong, R. A. (1995). Investigation of the inhibitory effects of PGE2 and selective EP agonists on chemotaxis of human neutrophils. *British Journal of Pharmacology*, *116*(7), 2903–2908.

Arslan, F., de Kleijn, D. P. V., Timmers, L., Doevendans, P. A., & Pasterkamp, G. (2008). Bridging innate immunity and myocardial ischemia/reperfusion injury: the search for therapeutic targets. *Current Pharmaceutical Design*, *14*(12), 1205–1216.

Atay, S., Tarnawski, A. S., & Dubois, A. (2000). Eicosanoids and the stomach. *Prostaglandins and Other Lipid Mediators*, *61*(3-4), 105–124.

Attur, M., Al-Mussawir, H. E., Patel, J., Kitay, A., Dave, M., Palmer, G., Pillinger, M. H., & Abramson, S. B. (2008). Prostaglandin E2 Exerts Catabolic Effects in Osteoarthritis Cartilage: Evidence for Signaling via the EP4 Receptor. *The Journal of Immunology*, *181*(7), 5082–5088. http://doi.org/10.4049/jimmunol.181.7.5082

Auer, J. A., Fackelman, G. E., Gingerich, D. A., & Fetter, A. W. (1980). Effect of hyaluronic acid in naturally occurring and experimentally induced osteoarthritis. *American Journal of Veterinary Research*, *41*(4), 568–574.

Aversano, T., Zhou, W., Nedelman, M., Nakada, M., & Weisman, H. (1995). A chimeric IgG4 monoclonal antibody directed against CD18 reduces infarct size in a primate model of myocardial ischemia and reperfusion. *Journal of the American College of Cardiology*, *25*(3), 781–788. http://doi.org/10.1016/0735-1097(94)00443-T

Ayral, X., Ravaud, P., Bonvarlet, J. P., Simonnet, J., Lecurieux, R., Nguyen, M., et al. (1999). Arthroscopic evaluation of post-traumatic patellofemoral chondropathy. *The Journal of Rheumatology*, *26*(5), 1140–1147.

Bailey, S. R., Adair, H. S., Reinemeyer, C. R., Morgan, S. J., Brooks, A. C., Longhofer, S. L., & Elliott, J. (2009). Plasma concentrations of endotoxin and platelet activation in the developmental stage of oligofructose-induced laminitis. *Veterinary Immunology and Immunopathology*, *129*(3-4), 167–173. http://doi.org/10.1016/j.vetimm.2008.11.009

Baran, K. W., Nguyen, M., McKendall, G. R., Lambrew, C. T., Dykstra, G., Palmeri, S. T., et al. (2001). Double-blind, randomized trial of an anti-CD18 antibody in conjunction with recombinant tissue plasminogen activator for acute myocardial infarction: limitation of myocardial infarction following thrombolysis in acute myocardial infarction (LIMIT AMI) study. *Circulation*, *104*(23), 2778–2783.

Baratelli, F., Lin, Y., Zhu, L., Yang, S. C., Heuze-Vourc'h, N., Zeng, G., et al. (2005). Prostaglandin E2 Induces FOXP3 Gene Expression and T Regulatory Cell Function in Human CD4+ T Cells. *The Journal of Immunology*, *175*(3), 1483–1490. http://doi.org/10.4049/jimmunol.175.3.1483

Barton, M. H., & Moore, J. N. (1994). Pentoxifylline inhibits mediator synthesis in an equine in vitro whole blood model of endotoxemia. *Circulatory Shock*, *44*(4), 216–220.

Barton, M. H., Ferguson, D., Davis, P. J., & Moore, J. N. (1997a). The effects of pentoxifylline infusion on plasma 6-keto-prostaglandin F1 alpha and ex vivo endotoxin-induced tumour necrosis factor activity in horses. *Journal of Veterinary Pharmacology and Therapeutics*, *20*(6), 487–492.

Barton, M. H., Moore, J. N., & Norton, N. (1997b). Effects of pentoxifylline infusion on response of horses to in vivo challenge exposure with endotoxin. *American Journal of Veterinary Research*, *58*(11), 1300–1307.

Basu, S., Whiteman, M., Mattey, D. L., & Halliwell, B. (2001). Raised levels of F(2)-isoprostanes and prostaglandin F(2alpha) in different rheumatic diseases. *Annals of the Rheumatic Diseases*, *60*(6), 627–631.

Bauer, T. R. J., Adler, R. L., & Hickstein, D. D. (2009). Potential large animal models for gene therapy of human genetic diseases of immune and blood cell systems. *ILAR Journal / National Research Council, Institute of Laboratory Animal Resources*, *50*(2), 168–186.

Båge, T., Kats, A., Lopez, B. S., Morgan, G., Nilsson, G., Burt, I., et al. (2011). Expression of Prostaglandin E Synthases in Periodontitis. *Ajpa*, *178*(4), 1676–1688. http://doi.org/10.1016/j.ajpath.2010.12.048

Belknap, J. K., GIGUÈRE, S., Pettigrew, A., Cochran, A. M., Van Eps, A. W., & Pollitt, C. C. (2007). Lamellar pro-inflammatory cytokine expression patterns in laminitis at the developmental stage and at the onset of lameness: innate vs. adaptive immune response. *Equine Veterinary Journal*, *39*(1), 42–47.

Belknap, J. K., Moore, J. N., & Crouser, E. C. (2009). Sepsis—From human organ failure to laminar failure. *Veterinary Immunology and Immunopathology*, *129*(3-4), 155–157. http://doi.org/10.1016/j.vetimm.2008.11.013

Beluche, L. A., Bertone, A. L., Anderson, D. E., & Rohde, C. (2001). Effects of oral administration of phenylbutazone to horses on in vitro articular cartilage metabolism. *American Journal of Veterinary Research*, *62*(12), 1916–1921.

Ben-Av, P., Crofford, L. J., Wilder, R. L., & Hla, T. (1995). Induction of vascular endothelial growth factor expression in synovial fibroblasts by prostaglandin E and interleukin-1: a potential mechanism for inflammatory angiogenesis. *FEBS Letters*, *372*(1), 83–87.

Ben-Horin, S., Kopylov, U., & Chowers, Y. (2014). Optimizing anti-TNF treatments in inflammatory bowel disease. *Autoimmunity Reviews*, *13*(1), 24–30. http://doi.org/10.1016/j.autrev.2013.06.002

Benbarek, H., Deby-Dupont, G., Deby, C., Caudron, I., Mathy-Hartert, M., Lamy, M., & Serteyn, D. (1996). Experimental model for the study by chemiluminescence of the activation of isolated equine leucocytes. *Research in Veterinary Science*, *61*(1), 59–64.

Bengis-Garber, C., & Gruener, N. (1996). Protein kinase A downregulates the phosphorylation of p47 phox in human neutrophils: a possible pathway for inhibition of the respiratory burst. *Cellular Signalling*, *8*(4), 291–296.

Benito, M. J. (2005). Synovial tissue inflammation in early and late osteoarthritis. *Annals of the Rheumatic Diseases*, *64*(9), 1263–1267. http://doi.org/10.1136/ard.2004.025270

Beretta, C., Garavaglia, G., & Cavalli, M. (2005a). COX-1 and COX-2 inhibition in horse blood by phenylbutazone, flunixin, carprofen and meloxicam: An in vitro analysis. *Pharmacological Research*, *52*(4), 302–306. http://doi.org/10.1016/j.phrs.2005.04.004

Bergmeier, W., Goerge, T., Wang, H.-W., Crittenden, J. R., Baldwin, A. C. W., Cifuni, S. M., et al. (2007). Mice lacking the signaling molecule CalDAG-GEFI represent a model for leukocyte adhesion deficiency type III. *Journal of Clinical Investigation*, *117*(6), 1699–1707. http://doi.org/10.1172/JCI30575

Bertolotto, M., Contini, P., Ottonello, L., Pende, A., Dallegri, F., & Montecucco, F. (2014). Neutrophil migration towards C5a and CXCL8 is prevented by non-steroidal anti-inflammatory drugs via inhibition of different pathways. *British Journal of Pharmacology*, *171*(14), 3376–3393. http://doi.org/10.1111/bph.12670

Bertone, A. L., Palmer, J. L., & Jones, J. (2001). Synovial fluid cytokines and eicosanoids as markers of joint disease in horses. *Veterinary Surgery*, *30*(6), 528–538. http://doi.org/10.1053/jvet.2001.28430

Betz, M., & Fox, B. S. (1991). Prostaglandin E2 inhibits production of Th1 lymphokines but not of Th2 lymphokines. *The Journal of Immunology*, *146*(1), 108–113.

Beutler, B., Milsark, I. W., & Cerami, A. C. (2008). Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. Science, 1985, 229(4716):869-871. Classical article. *The Journal of Immunology*, *181*(1), 7–9.

Binder, R., Kress, A., Kan, G., Herrmann, K., & Kirschfink, M. (1999). Neutrophil priming by cytokines and vitamin D binding protein (Gc-globulin): impact on C5a-mediated chemotaxis, degranulation and respiratory burst. *Molecular Immunology*, *36*(13-14), 885–892.

Birukova, A. A., Burdette, D., Moldobaeva, N., Xing, J., Fu, P., & Birukov, K. G. (2010). Rac GTPase is a hub for protein kinase A and Epac signaling in endothelial barrier

protection by cAMP. *Microvascular Research*, *79*(2), 128–138. http://doi.org/10.1016/j.mvr.2009.11.007

Blackwell, K. A., Raisz, L. G., & Pilbeam, C. C. (2010). Prostaglandins in bone: bad cop, good cop? *Trends in Endocrinology & Metabolism*, *21*(5), 294–301. http://doi.org/10.1016/j.tem.2009.12.004

Blikslager, A. T. (2012). Misoprostol: Is it safety or a lack of understanding that prevents its more frequent usage? *Equine Veterinary Journal*, *45*(1), 8–8. http://doi.org/10.1111/evj.12017

Blikslager, A. T., Roberts, M. C., Young, K. M., Rhoads, J. M., & Argenzio, R. A. (2000). Genistein augments prostaglandin-induced recovery of barrier function in ischemia-injured porcine ileum. *AJP: Gastrointestinal and Liver Physiology*, *278*(2), G207–16.

Bloemen, P. G., van den Tweel, M. C., Henricks, P. A., Engels, F., Kester, M. H., van de Loo, P. G., et al. (1997). Increased cAMP levels in stimulated neutrophils inhibit their adhesion to human bronchial epithelial cells. *The American Journal of Physiology*, *272*(4 Pt 1), L580–7.

Bombardier, C., Laine, L., Reicin, A., Shapiro, D., Burgos-Vargas, R., Davis, B., et al. (2000). Comparison of upper gastrointestinal toxicity of rofecoxib and naproxen in patients with rheumatoid arthritis. VIGOR Study Group. *The New England Journal of Medicine*, *343*(21), 1520–8– 2 p following 1528. http://doi.org/10.1056/NEJM200011233432103

Bondeson, J., Wainwright, S. D., Lauder, S., Amos, N., & Hughes, C. E. (2006). The role of synovial macrophages and macrophage-produced cytokines in driving aggrecanases, matrix metalloproteinases, and other destructive and inflammatory responses in osteoarthritis. *Arthritis Research & Therapy*, *8*(6), R187–14. http://doi.org/10.1186/ar2099

Bonnet, C. S. (2005). Osteoarthritis, angiogenesis and inflammation. *Rheumatology*, *44*(1), 7–16. http://doi.org/10.1093/rheumatology/keh344

Borregaard, N. (2010). Neutrophils, from Marrow to Microbes. *Immunity*, *33*(5), 657–670. http://doi.org/10.1016/j.immuni.2010.11.011

Boulet, L., Ouellet, M., Bateman, K. P., Ethier, D., Percival, M. D., Riendeau, D., et al. (2004). Deletion of Microsomal Prostaglandin E2 (PGE2) Synthase-1 Reduces Inducible and Basal PGE2 Production and Alters the Gastric Prostanoid Profile. *Journal of Biological Chemistry*, *279*(22), 23229–23237. http://doi.org/10.1074/jbc.M400443200

Brash, A. R. (2001). Arachidonic acid as a bioactive molecule. *Journal of Clinical Investigation*, *107*(11), 1339–1345. http://doi.org/10.1172/JCI13210

Brideau, C., Van Staden, C., & Chan, C. C. (2001). In vitro effects of cyclooxygenase inhibitors in whole blood of horses, dogs, and cats. *American Journal of Veterinary Research*, *62*(11), 1755–1760.

Briston, L., DUDHIA, J., & LEES, P. (2009). Age-related differences in prostaglandin E2 synthesis by equine cartilage explants and synoviocytes. *Journal of Veterinary Pharmacology and Therapeutics*, *33*(3), 268–276. http://doi.org/10.1111/j.1365-2885.2009.01131.x

Bromley, M., Bertfield, H., Evanson, J. M., & Woolley, D. E. (1985). Bidirectional erosion of cartilage in the rheumatoid knee joint. *Annals of the Rheumatic Diseases*, *44*(10), 676–681.

Brooks, A. C., Menzies-Gow, N. J., Wheeler-Jones, C., Bailey, S. R., Cunningham, F. M., & Elliott, J. (2009). Endotoxin-induced activation of equine digital vein endothelial cells: Role of p38 MAPK. *Veterinary Immunology and Immunopathology*, *129*(3-4), 174–180. http://doi.org/10.1016/j.vetimm.2008.11.008

Brooks, A. C., Rickards, K. J., & Cunningham, F. M. (2011). CXCL8 attenuates chemoattractant-induced equine neutrophil migration. *Veterinary Immunology and Immunopathology*, *139*(2-4), 141–147. http://doi.org/10.1016/j.vetimm.2010.09.008

Brown, G. E., Stewart, M. Q., Bissonnette, S. A., Elia, A. E. H., Wilker, E., & Yaffe, M. B. (2004). Distinct Ligand-dependent Roles for p38 MAPK in Priming and Activation of the Neutrophil NADPH Oxidase. *Journal of Biological Chemistry*, *279*(26), 27059–27068. http://doi.org/10.1074/jbc.M314258200

Bryant, C. E., Appleton, I., & Mitchell, J. A. (1998). Vascular endothelial growth factor upregulates constitutive cyclooxygenase 1 in primary bovine and human endothelial cells. *Life Sciences*, *62*(24), 2195–2201.

Bullone, M., & Lavoie, J.-P. (2015). Asthma "of horses and men"—How can equine heaves help us better understand human asthma immunopathology and its functional consequences? *Molecular Immunology*, *66*(1), 97–105. http://doi.org/10.1016/j.molimm.2014.12.005

Burelout, C., Thibault, N., Levasseur, S., Simard, S., Naccache, P. H., & Bourgoin, S. G. (2004). Prostaglandin E2 inhibits the phospholipase D pathway stimulated by formyl-methionyl-leucyl-phenylalanine in human neutrophils. Involvement of EP2 receptors and phosphatidylinositol 3-kinase gamma. *Molecular Pharmacology*, *66*(2), 293–301.

Burns, P.J., Morrow, C., Gilley, R.M., Papich, M.G. (2010). Evaluation of Pharmacokinetic-Pharmacodynamic Relationships for BioRelease Meloxicam Formulations in Horses. *Journal of Equine Veterinary Science*, *30*(10), 539–544. http://doi.org/10.1016/j.jevs.2010.09.004

Burns, T. A., Westerman, T., Nuovo, G. J., Watts, M. R., Pettigrew, A., YIN, C., & Belknap, J. K. (2011). Role of Oxidative Tissue Injury in the Pathophysiology of Experimentally Induced Equine Laminitis: A Comparison of 2 Models. *Journal of Veterinary Internal Medicine*, *25*(3), 540–548. http://doi.org/10.1111/j.1939-1676.2011.0706.x

Burr, D. B., & Gallant, M. A. (2012). Bone remodelling in osteoarthritis. *Nature Publishing Group*, *8*(11), 665–673. http://doi.org/10.1038/nrrheum.2012.130

Byron, C. R., Stewart, M. C., Stewart, A. A., & Pondenis, H. C. (2008). Effects of clinically relevant concentrations of glucosamine on equine chondrocytes and synoviocytes in vitro. *American Journal of Veterinary Research*, *69*(9), 1129–1134. http://doi.org/10.2460/ajvr.69.9.1129

Calich, A. L. G., Domiciano, D. S., & Fuller, R. (2010). Osteoarthritis: can anti-cytokine therapy play a role in treatment? *Clinical Rheumatology*, *29*(5), 451–455. http://doi.org/10.1007/s10067-009-1352-3

Canales, R. D., Luo, Y., Willey, J. C., Austermiller, B., Barbacioru, C. C., Boysen, C., et al. (2006). Evaluation of DNA microarray results with quantitative gene expression platforms. *Nature Biotechnology*, *24*(9), 1115–1122. http://doi.org/10.1038/nbt1236

Capsoni, F., Ongari, A. M., Reali, E., Bose, F., & Altomare, G. F. (2012). The protein kinase C inhibitor AEB071 (sotrastaurin) modulates migration and superoxide anion production by human neutrophils in vitro. *International Journal of Immunopathology and Pharmacology*, *25*(3), 617–626.

Carmona, J. U., Argüelles, D., Deulofeu, R., Martínez-Puig, D., & Prades, M. (2009). Effect of the administration of an oral hyaluronan formulation on clinical and biochemical parameters in young horses with osteochondrosis. *Veterinary and Comparative Orthopaedics and Traumatology*, 1–5. http://doi.org/10.3415/VCOT-09-01-0001

Carter, S. D., Osborne, A. C., May, S. A., & Bennett, D. (1995). Rheumatoid factor, anti-heat shock protein (65 kDa) antibodies and anti-nuclear antibodies in equine joint diseases. *Equine Veterinary Journal*, *27*(4), 288–295.

Chakravarti, A., Raquil, M.-A., Tessier, P., & Poubelle, P. E. (2009). Surface RANKL of Toll-like receptor 4-stimulated human neutrophils activates osteoclastic bone resorption.

Blood, *114*(8), 1633–1644. http://doi.org/10.1182/blood-2008-09-178301

Chandrasekharan, N. V., Dai, H., Roos, K. L. T., Evanson, N. K., Tomsik, J., Elton, T. S., & Simmons, D. L. (2002). COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: cloning, structure, and expression. *Proceedings of the National Academy of Sciences*, *99*(21), 13926–13931.

Chaudhry, H., Zhou, J., Zhong, Y., Ali, M. M., McGuire, F., Nagarkatti, P. S., & Nagarkatti, M. (2013). Role of cytokines as a double-edged sword in sepsis. *In Vivo (Athens, Greece)*, *27*(6), 669–684.

Chen, Q., Powell, D. W., Rane, M. J., Singh, S., Butt, W., Klein, J. B., & McLeish, K. R. (2003). Akt phosphorylates p47phox and mediates respiratory burst activity in human neutrophils. *The Journal of Immunology*, *170*(10), 5302–5308.

Cheng, X., Ji, Z., Tsalkova, T., & Mei, F. (2008). Epac and PKA: a tale of two intracellular cAMP receptors. *Acta Biochimica Et Biophysica Sinica*, *40*(7), 651–662.

Cheng, Y. (2006). Cyclooxygenases, microsomal prostaglandin E synthase-1, and cardiovascular function. *Journal of Clinical Investigation*, *116*(5), 1391–1399. http://doi.org/10.1172/JCI27540

Cheon, H., Rho, Y. H., Choi, S. J., Lee, Y. H., Song, G. G., Sohn, J., et al. (2006). Prostaglandin E2 Augments IL-10 Signaling and Function. *The Journal of Immunology*, *177*(2), 1092–1100. http://doi.org/10.4049/jimmunol.177.2.1092

Chilcoat, C. D., Rowlingson, K. A., & Jones, S. L. (2002). The effects of cAMP modulation upon the adhesion and respiratory burst activity of immune complex-stimulated equine neutrophils. *Veterinary Immunology and Immunopathology*, *88*(1-2), 65–77.

Choudhary, S., Wadhwa, S., Raisz, L. G., Alander, C., & Pilbeam, C. C. (2003). Extracellular calcium is a potent inducer of cyclo-oxygenase-2 in murine osteoblasts through an ERK signaling pathway. *Journal of Bone and Mineral Research*, *18*(10), 1813–1824. http://doi.org/10.1359/jbmr.2003.18.10.1813

Chung, K. F. (2005). Evaluation of selective prostaglandin E2 (PGE2) receptor agonists as therapeutic agents for the treatment of asthma. *Science's STKE : Signal Transduction Knowledge Environment*, *2005*(303), pe47. http://doi.org/10.1126/stke.3032005pe47

Clark, P., Rowland, S. E., Denis, D., Mathieu, M. C., Stocco, R., Poirier, H., et al. (2008). MF498 [N- -2-(2-methoxyphenyl)acetamide], a Selective E Prostanoid Receptor 4 Antagonist, Relieves Joint Inflammation and Pain in Rodent Models of Rheumatoid and Osteoarthritis. *Journal of Pharmacology and Experimental Therapeutics*, *325*(2), 425–

434. http://doi.org/10.1124/jpet.107.134510

Claudino, R. F. (2006). Pharmacological and Molecular Characterization of the Mechanisms Involved in Prostaglandin E2-Induced Mouse Paw Edema. *Journal of Pharmacology and Experimental Therapeutics*, *318*(2), 611–618. http://doi.org/10.1124/jpet.106.102806

Coggins, K. G., Latour, A., Nguyen, M. S., Audoly, L., Coffman, T. M., & Koller, B. H. (2002). Metabolism of PGE2 by prostaglandin dehydrogenase is essential for remodeling the ductus arteriosus. *Nature Medicine*, *8*(2), 91–92.

Convery, F. R., Akeson, W. H., & Keown, G. H. (1972). The repair of large osteochondral defects. An experimental study in horses. *Clinical Orthopaedics and Related Research*, *82*, 253–262.

Cook, V. L., & Blikslager, A. T. (2014). The use of nonsteroidal anti-inflammatory drugs in critically ill horses. *Journal of Veterinary Emergency and Critical Care*, *25*(1), 76–88. http://doi.org/10.1111/vec.12271

Cook, V. L., Meyer, C. T., Campbell, N. B., & Blikslager, A. T. (2009a). Effect of firocoxib or flunixin meglumine on recovery of ischemic-injured equine jejunum. *American Journal of Veterinary Research*, *70*(8), 992–1000. http://doi.org/10.2460/ajvr.70.8.992

Cook, V. L., Neuder, L. E., Blikslager, A. T., & Jones, S. L. (2009b). The effect of lidocaine on in vitro adhesion and migration of equine neutrophils. *Veterinary Immunology and Immunopathology*, *129*(1-2), 137–142. http://doi.org/10.1016/j.vetimm.2008.12.017

Costantini, T. W., Deree, J., Peterson, C. Y., Putnam, J. G., Woon, T., Loomis, W. H., et al. (2010). Pentoxifylline modulates p47phox activation and downregulates neutrophil oxidative burst through PKA-dependent and -independent mechanisms. *Immunopharmacology and Immunotoxicology*, *32*(1), 82–91. http://doi.org/10.3109/08923970903183557

Coyne, M. J., Cousin, H., Loftus, J. P., Johnson, P. J., Belknap, J. K., Gradil, C. M., et al. (2009). Cloning and expression of ADAM-related metalloproteases in equine laminitis. *Veterinary Immunology and Immunopathology*, *129*(3-4), 231–241. http://doi.org/10.1016/j.vetimm.2008.11.022

Côté, B., Boulet, L., Brideau, C., Claveau, D., Ethier, D., Frenette, R., et al. (2007). Substituted phenanthrene imidazoles as potent, selective, and orally active mPGES-1 inhibitors. *Bioorganic & Medicinal Chemistry Letters*, *17*(24), 6816–6820. http://doi.org/10.1016/j.bmcl.2007.10.033

Cudmore, L. A., Muurlink, T., Whittem, T., & Bailey, S. R. (2013). Effects of oral

clenbuterol on the clinical and inflammatory response to endotoxaemia in the horse. *Research in Veterinary Science*, *94*(3), 682–686. http://doi.org/10.1016/j.rvsc.2013.01.003

Curiel, R. V., & Katz, J. D. (2013). Mitigating the cardiovascular and renal effects of NSAIDs. *Pain Medicine (Malden, Mass.)*, *14 Suppl 1*, S23–8. http://doi.org/10.1111/pme.12275

D'Hellencourt, C. L., Diaw, L., Cornillet, P., & Guenounou, M. (1996). Differential regulation of TNF alpha, IL-1 beta, IL-6, IL-8, TNF beta, and IL-10 by pentoxifylline. *International Journal of Immunopharmacology*, *18*(12), 739–748.

Dahlgren, C., & Karlsson, A. (1999). Respiratory burst in human neutrophils. *Journal of Immunological Methods*, *232*(1-2), 3–14.

Dang, P. M., Dewas, C., Gaudry, M., Fay, M., Pedruzzi, E., Gougerot-Pocidalo, M. A., & Benna, El, J. (1999). Priming of human neutrophil respiratory burst by granulocyte/macrophage colony-stimulating factor (GM-CSF) involves partial phosphorylation of p47(phox). *Journal of Biological Chemistry*, *274*(29), 20704–20708.

Davies, N. M., Longstreth, J., & Jamali, F. (2001). Misoprostol therapeutics revisited. *Pharmacotherapy*, *21*(1), 60–73.

de Grauw, J. C., Brama, P. A., Wiemer, P., Brommer, H., van de Lest, C. H., & van Weeren, P. R. (2006). Cartilage-derived biomarkers and lipid mediators of inflammation in horses with osteochondritis dissecans of the distal intermediate ridge of the tibia. *American Journal of Veterinary Research*, *67*(7), 1156–1162. http://doi.org/10.2460/ajvr.67.7.1156

de Grauw, J. C., van de Lest, C. H. A., Brama, P. A. J., Rambags, B. P. B., & van Weeren, P. R. (2009). In vivo effects of meloxicam on inflammatory mediators, MMP activity and cartilage biomarkers in equine joints with acute synovitis. *Equine Veterinary Journal*, *41*(7), 693–699.

de Lange-Brokaar, B. J. E., Ioan-Facsinay, A., van Osch, G. J. V. M., Zuurmond, A. M., Schoones, J., Toes, R. E. M., et al. (2012). Synovial inflammation, immune cells and their cytokines in osteoarthritis: a review. *Osteoarthritis and Cartilage*, *20*(12), 1484–1499. http://doi.org/10.1016/j.joca.2012.08.027

De Rensis, F., Saleri, R., Tummaruk, P., Techakumphu, M., & Kirkwood, R. N. (2012). Prostaglandin F2α and control of reproduction in female swine: A review. *The*, *77*(1), 1–11. http://doi.org/10.1016/j.theriogenology.2011.07.035

DeForge, L. E., & Remick, D. G. (1991). Kinetics of TNF, IL-6, and IL-8 gene expression in

LPS-stimulated human whole blood. *Biochemical and Biophysical Research Communications*, *174*(1), 18–24.

Dekker, L. V., Leitges, M., Altschuler, G., Mistry, N., McDermott, A., Roes, J., & Segal, A. W. (2000). Protein kinase C-beta contributes to NADPH oxidase activation in neutrophils. *Biochemical Journal*, *347 Pt 1*, 285–289.

Deligne, C., Casulli, S., Pigenet, A., Bougault, C., Campillo-Gimenez, L., Nourissat, G., et al. (2015). Differential expression of interleukin-17 and interleukin-22 in inflamed and non-inflamed synovium from osteoarthritis patients. *Osteoarthritis and Cartilage*, *23*(11), 1843–1852. http://doi.org/10.1016/j.joca.2014.12.007

Dendorfer, U., Oettgen, P., & Libermann, T. A. (1994). Multiple regulatory elements in the interleukin-6 gene mediate induction by prostaglandins, cyclic AMP, and lipopolysaccharide. *Molecular and Cellular Biology*, *14*(7), 4443–4454.

Deng, G. M., Nilsson, I. M., Verdrengh, M., Collins, L. V., & Tarkowski, A. (1999). Intra-articularly localized bacterial DNA containing CpG motifs induces arthritis. *Nature Medicine*, *5*(6), 702–705. http://doi.org/10.1038/9554

Dey, I., Lejeune, M., & Chadee, K. (2006). Prostaglandin E 2receptor distribution and function in the gastrointestinal tract. *British Journal of Pharmacology*, *149*(6), 611–623. http://doi.org/10.1038/sj.bjp.0706923

Diaz-Gonzalez, F., Gonzalez-Alvaro, I., Campanero, M. R., Mollinedo, F., del Pozo, M. A., Munoz, C., et al. (1995). Prevention of in vitro neutrophil-endothelial attachment through shedding of L-selectin by nonsteroidal antiinflammatory drugs. *Journal of Clinical Investigation*, *95*(4), 1756–1765. http://doi.org/10.1172/JCI117853

DiBattista, J. A., Martel-Pelletier, J., Cloutier, J. M., & Pelletier, J. P. (1991). Modulation of glucocorticoid receptor expression in human articular chondrocytes by cAMP and prostaglandins. *The Journal of Rheumatology. Supplement*, *27*, 102–105.

DiBattista, J. A., Martel-Pelletier, J., Fujimoto, N., Obata, K., Zafarullah, M., & Pelletier, J. P. (1994). Prostaglandins E2 and E1 inhibit cytokine-induced metalloprotease expression in human synovial fibroblasts. Mediation by cyclic-AMP signalling pathway. *Laboratory Investigation*, *71*(2), 270–278.

DiCarlo, E. F., & Kahn, L. B. (2011). Inflammatory diseases of the bones and joints. *Ysdia*, *28*(1), 53–64. http://doi.org/10.1053/j.semdp.2011.02.012

Dinarello, C. A. (2003). Anti-cytokine therapeutics and infections. *Vaccine*, *21 Suppl 2*, S24–34.

Ding, M., Kinoshita, Y., Kishi, K., Nakata, H., Hassan, S., Kawanami, C., et al. (1997). Distribution of prostaglandin E receptors in the rat gastrointestinal tract. *Prostaglandins*, *53*(3), 199–216.

Dingle, J. T. (1993). Prostaglandins in human cartilage metabolism. *Journal of Lipid Mediators*, *6*(1-3), 303–312.

Doucet, M. Y., Bertone, A. L., Hendrickson, D., Hughes, F., Macallister, C., McClure, S., et al. (2008). Comparison of efficacy and safety of paste formulations of firocoxib and phenylbutazone in horses with naturally occurring osteoarthritis. *Journal of the American Veterinary Medical Association*, *232*(1), 91–97. http://doi.org/10.2460/javma.232.1.91

Dubin, A., Potempa, J., & Travis, J. (1994). Structural and functional characterization of elastases from horse neutrophils. *Biochemical Journal*, *300 (Pt 2)*, 401–406.

Dufour, J. H., Dziejman, M., Liu, M. T., Leung, J. H., Lane, T. E., & Luster, A. D. (2002). IFN-gamma-inducible protein 10 (IP-10; CXCL10)-deficient mice reveal a role for IP-10 in effector T cell generation and trafficking. *The Journal of Immunology*, *168*(7), 3195–3204.

Eades, S. C., Stokes, A. M., & Moore, R. M. (2006). Effects of an endothelin receptor antagonist and nitroglycerin on digital vascular function in horses during the prodromal stages of carbohydrate overload-induced laminitis. *American Journal of Veterinary Research*, *67*(7), 1204–1211. http://doi.org/10.2460/ajvr.67.7.1204

Eaton, S. A., Allen, D., Eades, S. C., & Schneider, D. A. (1995). Digital Starling forces and hemodynamics during early laminitis induced by an aqueous extract of black walnut (Juglans nigra) in horses. *American Journal of Veterinary Research*, *56*(10), 1338–1344.

Eckert, R. E., Neuder, L. E., Bell, J. L., Trujillo, J. C., & Jones, S. L. (2007). The role of p38 mitogen-activated kinase (MAPK) in the mechanism regulating cyclooxygenase gene expression in equine leukocytes. *Veterinary Immunology and Immunopathology*, *118*(3-4), 294–303. http://doi.org/10.1016/j.vetimm.2007.06.001

Eckert, R. E., Sharief, Y., & Jones, S. L. (2009). p38 mitogen-activated kinase (MAPK) is essential for equine neutrophil migration. *Veterinary Immunology and Immunopathology*, *129*(3-4), 181–191. http://doi.org/10.1016/j.vetimm.2008.11.007

Eigler, A., Siegmund, B., Emmerich, U., Baumann, K. H., Hartmann, G., & Endres, S. (1998). Anti-inflammatory activities of cAMP-elevating agents: enhancement of IL-10 synthesis and concurrent suppression of TNF production. *Journal of Leukocyte Biology*, *63*(1), 101–107.

El-Benna, J., Dang, P. M.-C., Gougerot-Pocidalo, M. A., Marie, J. C., & Braut-Boucher, F. (2009). p47phox, the phagocyte NADPH oxidase/NOX2 organizer: structure, phosphorylation and implication in diseases. *Experimental & Molecular Medicine*, *41*(4), 217–225. http://doi.org/10.3858/emm.2009.41.4.058

Elferink, J. G., & de Koster, B. M. (1993). The effect of cyclic GMP and cyclic AMP on migration by electroporated human neutrophils. *European Journal of Pharmacology*, *246*(2), 157–161.

Eliasson, P., Andersson, T., & Aspenberg, P. (2012). Influence of a single loading episode on gene expression in healing rat Achilles tendons. *Journal of Applied Physiology*, *112*(2), 279–288. http://doi.org/10.1152/japplphysiol.00858.2011

Elshabrawy, H. A., Chen, Z., Volin, M. V., Ravella, S., Virupannavar, S., & Shahrara, S. (2015). The pathogenic role of angiogenesis in rheumatoid arthritis. *Angiogenesis*, *18*(4), 433–448. http://doi.org/10.1007/s10456-015-9477-2

Engblom, D., Saha, S., Engström, L., Westman, M., Audoly, L. P., Jakobsson, P.-J., & Blomqvist, A. (2003). Microsomal prostaglandin E synthase-1 is the central switch during immune-induced pyresis. *Nature Neuroscience*, *6*(11), 1137–1138. http://doi.org/10.1038/nn1137

Eruslanov, E., Kaliberov, S., Daurkin, I., Kaliberova, L., Buchsbaum, D., Vieweg, J., & Kusmartsev, S. (2009). Altered Expression of 15-Hydroxyprostaglandin Dehydrogenase in Tumor-Infiltrated CD11b Myeloid Cells: A Mechanism for Immune Evasion in Cancer. *The Journal of Immunology*, *182*(12), 7548–7557. http://doi.org/10.4049/jimmunol.0802358

Escolar, D. M., Zimmerman, A., Bertorini, T., Clemens, P. R., Connolly, A. M., Mesa, L., et al. (2012). Pentoxifylline as a rescue treatment for DMD: a randomized double-blind clinical trial. *Neurology*, *78*(12), 904–913. http://doi.org/10.1212/WNL.0b013e31824c46be

Evans, J. H., Spencer, D. M., Zweifach, A., & Leslie, C. C. (2001). Intracellular Calcium Signals Regulating Cytosolic Phospholipase A2 Translocation to Internal Membranes. *Journal of Biological Chemistry*, *276*(32), 30150–30160. http://doi.org/10.1074/jbc.M100943200

Eymard, F., Pigenet, A., Citadelle, D., Flouzat-Lachaniette, C.-H., Poignard, A., Benelli, C., et al. (2014). Induction of an Inflammatory and Prodegradative Phenotype in Autologous Fibroblast-like Synoviocytes by the Infrapatellar Fat Pad From Patients With Knee Osteoarthritis. *Arthritis & Rheumatology*, *66*(8), 2165–2174. http://doi.org/10.1002/art.38657

Faleiros, R. R., Johnson, P. J., Nuovo, G. J., Messer, N. T., Black, S. J., & Belknap, J. K. (2010). Laminar Leukocyte Accumulation in Horses with Carbohydrate Overload-Induced Laminitis. *Journal of Veterinary Internal Medicine*, *25*(1), 107–115. http://doi.org/10.1111/j.1939-1676.2010.0650.x

Faleiros, R. R., Nuovo, G. J., & Belknap, J. K. (2009). Calprotectin in Myeloid and Epithelial Cells of Laminae from Horses with Black Walnut Extract-Induced Laminitis. *Journal of Veterinary Internal Medicine*, *23*(1), 174–181. http://doi.org/10.1111/j.1939-1676.2008.0241.x

Faleiros, R. R., Stokes, A. M., Eades, S. C., Kim, D. Y., Paulsen, D. B., & Moore, R. M. (2004). Assessment of apoptosis in epidermal lamellar cells in clinically normal horses and those with laminitis. *American Journal of Veterinary Research*, *65*(5), 578–585.

Faour, W. H. (2001). Prostaglandin E2 Regulates the Level and Stability of Cyclooxygenase-2 mRNA through Activation of p38 Mitogen-activated Protein Kinase in Interleukin-1beta -treated Human Synovial Fibroblasts. *Journal of Biological Chemistry*, *276*(34), 31720–31731. http://doi.org/10.1074/jbc.M104036200

Faour, W. H., Mancini, A., He, Q. W., & Di Battista, J. A. (2003). T-cell-derived Interleukin-17 Regulates the Level and Stability of Cyclooxygenase-2 (COX-2) mRNA through Restricted Activation of the p38 Mitogen-activated Protein Kinase Cascade: ROLE OF DISTAL SEQUENCES IN THE 3'-UNTRANSLATED REGION OF COX-2 mRNA. *Journal of Biological Chemistry*, *278*(29), 26897–26907. http://doi.org/10.1074/jbc.M212790200

Farley, J., Sirois, J., MacFarlane, P.-H., Kombe, A., & Laverty, S. (2005). Evaluation of coexpression of microsomal prostaglandin E synthase-1 and cyclooxygenase-2 in interleukin-1-stimulated equine articular chondrocytes. *American Journal of Veterinary Research*, *66*(11), 1985–1991.

Farmer, J. C., Burkey, T. H., Kew, R. R., & Webster, R. O. (1991). Concentration-dependent regulatory effects of prostaglandin E1 on human neutrophil function in vitro. *The American Review of Respiratory Disease*, *144*(3 Pt 1), 593–599. http://doi.org/10.1164/ajrccm/144.3_Pt_1.593

Fasano, M. B. E. A. (1998). Human Neutrophils Express the Prostaglandin G/H Synthase 2 Gene When Stimulated with Bacterial Lipopolysaccharide, 1–5.

Faxon, D. P., Gibbons, R. J., Chronos, N. A. F., Gurbel, P. A., & Sheehan, F. (2002). The effect of blockade of the CD11/CD18 integrin receptor on infarct size in patients with acute myocardial infarction treated with direct angioplasty: the results of the HALT-MI

study. *Journal of the American College of Cardiology*, *40*(7), 1199–1204.

Ferrer, R., & Moreno, J. J. (2010). Role of eicosanoids on intestinal epithelial homeostasis. *Biochemical Pharmacology*, *80*(4), 431–438. http://doi.org/10.1016/j.bcp.2010.04.033

Fiuza, C. (2002). Inflammation-promoting activity of HMGB1 on human microvascular endothelial cells. *Blood*, *101*(7), 2652–2660. http://doi.org/10.1182/blood-2002-05-1300

Flaherty, J. T., Pitt, B., Gruber, J. W., Heuser, R. R., Rothbaum, D. A., Burwell, L. R., et al. (1994). Recombinant human superoxide dismutase (h-SOD) fails to improve recovery of ventricular function in patients undergoing coronary angioplasty for acute myocardial infarction. *Circulation*, *89*(5), 1982–1991.

Foote, E. F., Lee, D. R., Karim, A., Keane, W. F., & Halstenson, C. E. (1995). Disposition of misoprostol and its active metabolite in patients with normal and impaired renal function. *Journal of Clinical Pharmacology*, *35*(4), 384–389.

Forwood, M. R., Kelly, W. L., & Worth, N. F. (1998). Localisation of prostaglandin endoperoxide H synthase (PGHS)-1 and PGHS-2 in bone following mechanical loading in vivo. *The Anatomical Record*, *252*(4), 580–586. http://doi.org/10.1002/(SICI)1097-0185(199812)252:4<580::AID-AR8>3.0.CO;2-S

Frean, S. P. E. A. (1999). In vitro stimulation of equine articular cartilage proteoglycan synthesis by hyaluronan and carprofen, 1–8.

Fries, J. W., Williams, A. J., Atkins, R. C., Newman, W., Lipscomb, M. F., & Collins, T. (1993). Expression of VCAM-1 and E-selectin in an in vivo model of endothelial activation. *Ajpa*, *143*(3), 725–737.

Frisbie, D. D., Al-Sobayil, F., Billinghurst, R. C., Kawcak, C. E., & McIlwraith, C. W. (2008). Changes in synovial fluid and serum biomarkers with exercise and early osteoarthritis in horses. *Osteoarthritis and Cartilage*, *16*(10), 1196–1204. http://doi.org/10.1016/j.joca.2008.03.008

Frisbie, D. D., Cross, M. W., & McIlwraith, C. W. (2006). A comparative study of articular cartilage thickness in the stifle of animal species used in human pre-clinical studies compared to articular cartilage thickness in the human knee. *Veterinary and Comparative Orthopaedics and Traumatology*, *19*(3), 142–146.

Frisbie, D. D., Ghivizzani, S. C., Robbins, P. D., Evans, C. H., & McIlwraith, C. W. (2002). Treatment of experimental equine osteoarthritis by in vivo delivery of the equine interleukin-1 receptor antagonist gene. *Gene Therapy*, *9*(1), 12–20. http://doi.org/10.1038/sj.gt.3301608

Frisbie, D. D., Lu, Y., Kawcak, C. E., DiCarlo, E. F., Binette, F., & McIlwraith, C. W. (2009). In Vivo Evaluation of Autologous Cartilage Fragment-Loaded Scaffolds Implanted Into Equine Articular Defects and Compared With Autologous Chondrocyte Implantation. *The American Journal of Sports Medicine*, *37*(1_suppl), 71S–80S. http://doi.org/10.1177/0363546509348478

Frisbie, D. D., Trotter, G. W., Powers, B. E., Rodkey, W. G., Steadman, J. R., Howard, R. D., et al. (1999). Arthroscopic subchondral bone plate microfracture technique augments healing of large chondral defects in the radial carpal bone and medial femoral condyle of horses. *Veterinary Surgery : vs*, *28*(4), 242–255.

Frobøse, H., Groth Rønn, S., Heding, P. E., Mendoza, H., Cohen, P., Mandrup-Poulsen, T., & Billestrup, N. (2006). Suppressor of Cytokine Signaling-3 Inhibits Interleukin-1 Signaling by Targeting the TRAF-6/TAK1 Complex. *Molecular Endocrinology*, *20*(7), 1587–1596. http://doi.org/10.1210/me.2005-0301

Funk, C. D. (2001). Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science*, *294*(5548), 1871–1875.

Furuzawa-Carballeda, J., Macip-Rodriguez, P. M., & Cabral, A. R. (2008). Osteoarthritis and rheumatoid arthritis pannus have similar qualitative metabolic characteristics and pro-inflammatory cytokine response. *Clinical and Experimental Rheumatology*, *26*(4), 554–560.

Futosi, K., Fodor, S., & Mócsai, A. (2013). Reprint of Neutrophil cell surface receptors and their intracellular signal transduction pathways. *International Immunopharmacology*, *17*(4), 1185–1197. http://doi.org/10.1016/j.intimp.2013.11.010

Gallelli, L., Galasso, O., Falcone, D., Southworth, S., Greco, M., Ventura, V., et al. (2013). The effects of nonsteroidal anti-inflammatory drugs on clinical outcomes, synovial fluid cytokine concentration and signal transduction pathways in knee osteoarthritis. A randomized open label trial. *Osteoarthritis and Cartilage*, *21*(9), 1400–1408. http://doi.org/10.1016/j.joca.2013.06.026

Ganesh, T. (2014). Prostanoid Receptor EP2 as a Therapeutic Target. *Journal of Medicinal Chemistry*, *57*(11), 4454–4465. http://doi.org/10.1021/jm401431x

Gasperini, S., Crepaldi, L., Calzetti, F., Gatto, L., Berlato, C., Bazzoni, F., et al. (2002). Interleukin-10 and cAMP-elevating agents cooperate to induce suppressor of cytokine signaling-3 via a protein kinase A-independent signal. *European Cytokine Network*, *13*(1), 47–53.

Geissmann, F., Manz, M. G., Jung, S., Sieweke, M. H., Merad, M., & Ley, K. (2010). Development of Monocytes, Macrophages, and Dendritic Cells. *Science*, *327*(5966), 656–661. http://doi.org/10.1126/science.1178331

Gemmell, C. G., Goutcher, S. C., Reid, R., & Sturrock, R. D. (1997). Role of certain virulence factors in a murine model of Staphylococcus aureus arthritis. *Journal of Medical Microbiology*, *46*(3), 208–213. http://doi.org/10.1099/00222615-46-3-208

Ghosh, A. K. (2003). Regulation by prostaglandin E2 and histamine of angiogenesis in inflammatory granulation tissue. *Yakugaku Zasshi : Journal of the Pharmaceutical Society of Japan*, *123*(5), 295–303.

Gibson, K. T., Hodge, H., & Whittem, T. (1996). Inflammatory mediators in equine synovial fluid. *Australian Veterinary Journal*, *73*(4), 148–151.

Giroux, A., Boulet, L., Brideau, C., Chau, A., Claveau, D., Côté, B., et al. (2009). Discovery of disubstituted phenanthrene imidazoles as potent, selective and orally active mPGES-1 inhibitors. *Bioorganic & Medicinal Chemistry Letters*, *19*(20), 5837–5841. http://doi.org/10.1016/j.bmcl.2009.08.085

Gobejishvili, L., Ghare, S., Khan, R., Cambon, A., Barker, D. F., Barve, S., et al. (2015). Misoprostol modulates cytokine expression through a cAMP pathway: Potential therapeutic implication for liver disease. *Clinical Immunology*, *161*(2), 291–299. http://doi.org/10.1016/j.clim.2015.09.008

Gold, K. N., Weyand, C. M., & Goronzy, J. J. (1994). Modulation of helper T cell function by prostaglandins. *Arthritis & Rheumatism*, *37*(6), 925–933.

Goldring, M. B., Birkhead, J., Sandell, L. J., Kimura, T., & Krane, S. M. (1988). Interleukin 1 suppresses expression of cartilage-specific types II and IX collagens and increases types I and III collagens in human chondrocytes. *Journal of Clinical Investigation*, *82*(6), 2026–2037. http://doi.org/10.1172/JCI113823

Gomi, K., Zhu, F. G., & Marshall, J. S. (2000). Prostaglandin E2 Selectively Enhances the IgE-Mediated Production of IL-6 and Granulocyte-Macrophage Colony-Stimulating Factor by Mast Cells Through an EP1/EP3-Dependent Mechanism. *The Journal of Immunology*, *165*(11), 6545–6552. http://doi.org/10.4049/jimmunol.165.11.6545

Goodrich, L. R., & Nixon, A. J. (2006). Medical treatment of osteoarthritis in the horse – A review. *The Veterinary Journal*, *171*(1), 51–69. http://doi.org/10.1016/j.tvjl.2004.07.008

Gosset, M., Berenbaum, F., Levy, A., Pigenet, A., Thirion, S., Cavadias, S., & Jacques, C. (2008). Mechanical stress and prostaglandin E2 synthesis in cartilage. *Biorheology*, *45*(3-

4), 301–320.

Gómez-Hernández, A., Martín-Ventura, J. L., Sánchez-Galán, E., Vidal, C., Ortego, M., Blanco-Colio, L. M., et al. (2006). Overexpression of COX-2, Prostaglandin E Synthase-1 and Prostaglandin E Receptors in blood mononuclear cells and plaque of patients with carotid atherosclerosis: Regulation by nuclear factor-κB. *Atherosclerosis*, *187*(1), 139–149. http://doi.org/10.1016/j.atherosclerosis.2005.08.035

Grasa, L., Arruebo, M. P., Plaza, M. A., & Murillo, M. D. (2006). PGE2 receptors and their intracellular mechanisms in rabbit small intestine. *Prostaglandins and Other Lipid Mediators*, *79*(3-4), 206–217. http://doi.org/10.1016/j.prostaglandins.2006.01.001

Gregory, M. H., Capito, N., Kuroki, K., Stoker, A. M., Cook, J. L., & Sherman, S. L. (2012). A Review of Translational Animal Models for Knee Osteoarthritis. *Arthritis*, *2012*(7), 1–14. http://doi.org/10.1155/2012/764621

Grimmer, N.M., Gimbar, R.P., Bursua, A., Patel, M. (2016). Rhabdomyolysis Secondary to Clenbuterol Use and Exercise. *Journal of Emergency Medicine*, *50*(2), e71–e74. http://doi.org/10.1016/j.jemermed.2015.09.006

Gross, S., Tilly, P., Hentsch, D., Vonesch, J.-L., & Fabre, J.-E. (2007). Vascular wall–produced prostaglandin E2 exacerbates arterial thrombosis and atherothrombosis through platelet EP3 receptors. *The Journal of Experimental Medicine*, *204*(2), 311–320. http://doi.org/10.1084/jem.20061617

Grosser, T. (2005). Biological basis for the cardiovascular consequences of COX-2 inhibition: therapeutic challenges and opportunities. *Journal of Clinical Investigation*, *116*(1), 4–15. http://doi.org/10.1172/JCI27291

Gu, J., Hutchins, C. W., Zhu, R., Shen, J., Harris, M. C., Belanger, E., et al. (2014). Prostaglandin E2 binding proteins and uses thereof.

Guerrero, M. D., Aquino, M., Bruno, I., Riccio, R., Terencio, M. C., & Payá, M. (2009). Anti-inflammatory and analgesic activity of a novel inhibitor of microsomal prostaglandin E synthase-1 expression. *European Journal of Pharmacology*, *620*(1-3), 112–119. http://doi.org/10.1016/j.ejphar.2009.08.007

Han, J., Luby-Phelps, K., Das, B., Shu, X., Xia, Y., Mosteller, R. D., et al. (1998). Role of substrates and products of PI 3-kinase in regulating activation of Rac-related guanosine triphosphatases by Vav. *Science*, *279*(5350), 558–560.

Han, R., & Smith, T. J. (2002). Cytoplasmic Prostaglandin E2 Synthase Is Dominantly Expressed in Cultured KAT-50 Thyrocytes, Cells That Express Constitutive

Prostaglandin-endoperoxide H Synthase-2: BASIS FOR LOW PROSTAGLANDIN E2PRODUCTION. *Journal of Biological Chemistry*, *277*(39), 36897–36903. http://doi.org/10.1074/jbc.M206949200

Hao, C.-M., & Breyer, M. D. (2008). Physiological Regulation of Prostaglandins in the Kidney. *Annual Review of Physiology*, *70*(1), 357–377. http://doi.org/10.1146/annurev.physiol.70.113006.100614

Hara, S., Kamei, D., Sasaki, Y., Tanemoto, A., Nakatani, Y., & Murakami, M. (2010). Prostaglandin E synthases: Understanding their pathophysiological roles through mouse genetic models. *Biochimie*, *92*(6), 651–659. http://doi.org/10.1016/j.biochi.2010.02.007

Harada, A., Sekido, N., Akahoshi, T., Wada, T., Mukaida, N., & Matsushima, K. (1994). Essential involvement of interleukin-8 (IL-8) in acute inflammation. *Journal of Leukocyte Biology*, *56*(5), 559–564.

Harada, S. I., Balena, R., Rodan, G. A., & Rodan, S. B. (1995). The role of prostaglandins in bone formation. *Connective Tissue Research*, *31*(4), 279–282.

Hardy, J., Bertone, A. L., & Muir, W. W. (1996). Joint pressure influences synovial tissue blood flow as determined by colored microspheres. *Journal of Applied Physiology (Bethesda, Md. : 1985)*, *80*(4), 1225–1232.

Hardy, M. M., Seibert, K., Manning, P. T., Currie, M. G., Woerner, B. M., Edwards, D., et al. (2002). Cyclooxygenase 2-dependent prostaglandin E2 modulates cartilage proteoglycan degradation in human osteoarthritis explants. *Arthritis & Rheumatism*, *46*(7), 1789–1803. http://doi.org/10.1002/art.10356

Haringman, J. J. (2005). Synovial tissue macrophages: a sensitive biomarker for response to treatment in patients with rheumatoid arthritis. *Annals of the Rheumatic Diseases*, *64*(6), 834–838. http://doi.org/10.1136/ard.2004.029751

Harirforoosh, S., Asghar, W., & Jamali, F. (2013). Adverse effects of nonsteroidal antiinflammatory drugs: an update of gastrointestinal, cardiovascular and renal complications. *Journal of Pharmacy & Pharmaceutical Sciences : a Publication of the Canadian Society for Pharmaceutical Sciences, Societe Canadienne Des Sciences Pharmaceutiques*, *16*(5), 821–847.

Harizi, H., Corcuff, J.-B., & Gualde, N. (2008). Arachidonic-acid-derived eicosanoids: roles in biology and immunopathology. *Trends in Molecular Medicine*, *14*(10), 461–469. http://doi.org/10.1016/j.molmed.2008.08.005

Harvath, L., Robbins, J. D., Russell, A. A., & Seamon, K. B. (1991). cAMP and human

neutrophil chemotaxis. Elevation of cAMP differentially affects chemotactic responsiveness. *The Journal of Immunology*, *146*(1), 224–232.

Haselmayer, P., Daniel, M., Tertilt, C., Salih, H. R., Stassen, M., Schild, H. O. R., & Radsak, M. P. (2009). Signaling Pathways of the TREM-1- and TLR4-Mediated Neutrophil Oxidative Burst. *Journal of Innate Immunity*, *1*(6), 582–591. http://doi.org/10.1159/000231973

Hayami, T., Pickarski, M., Wesolowski, G. A., McLane, J., Bone, A., Destefano, J., et al. (2004). The role of subchondral bone remodeling in osteoarthritis: reduction of cartilage degeneration and prevention of osteophyte formation by alendronate in the rat anterior cruciate ligament transection model. *Arthritis & Rheumatism*, *50*(4), 1193–1206. http://doi.org/10.1002/art.20124

Hayami, T., Pickarski, M., Zhuo, Y., Wesolowski, G. A., Rodan, G. A., & Duong, L. T. (2006). Characterization of articular cartilage and subchondral bone changes in the rat anterior cruciate ligament transection and meniscectomized models of osteoarthritis. *Bone*, *38*(2), 234–243. http://doi.org/10.1016/j.bone.2005.08.007

Haynes, D. R., Whitehouse, M. W., & Vernon-Roberts, B. (1992). The prostaglandin E1 analogue, misoprostol, regulates inflammatory cytokines and immune functions in vitro like the natural prostaglandins E1, E2 and E3. *Immunology*, *76*(2), 251–257.

Hikiji, H., Takato, T., Shimizu, T., & Ishii, S. (2008). The roles of prostanoids, leukotrienes, and platelet-activating factor in bone metabolism and disease. *Progress in Lipid Research*, *47*(2), 107–126. http://doi.org/10.1016/j.plipres.2007.12.003

Hilal, G., Martel-Pelletier, J., Pelletier, J. P., Ranger, P., & Lajeunesse, D. (1998). Osteoblast-like cells from human subchondral osteoarthritic bone demonstrate an altered phenotype in vitro: possible role in subchondral bone sclerosis. *Arthritis & Rheumatism*, *41*(5), 891–899. http://doi.org/10.1002/1529-0131(199805)41:5<891::AID-ART17>3.0.CO;2-X

Hilal, G., Massicotte, F., Martel-Pelletier, J., Fernandes, J. C., Pelletier, J. P., & Lajeunesse, D. (2001). Endogenous prostaglandin E2 and insulin-like growth factor 1 can modulate the levels of parathyroid hormone receptor in human osteoarthritic osteoblasts. *Journal of Bone and Mineral Research*, *16*(4), 713–721. http://doi.org/10.1359/jbmr.2001.16.4.713

Hillmann, A., Ahrberg, A. B., Brehm, W., Heller, S., Josten, C., Paebst, F., & Burk, J. (2016). Comparative Characterization of Human and Equine Mesenchymal Stromal Cells: A Basis for Translational Studies in the Equine Model. *Cell Transplantation*, *25*(1), 109–124. http://doi.org/10.3727/096368915X687822

Hirata, T., & Narumiya, S. (2012). Chapter 5 - Prostanoids as Regulators of Innate and Adaptive Immunity. *Advances in Immunology* (1st ed., Vol. 116, pp. 143–174). Elsevier Inc. http://doi.org/10.1016/B978-0-12-394300-2.00005-3

Hirsch, G., Lavoie-Lamoureux, A., Beauchamp, G., & Lavoie, J.-P. (2012). Neutrophils Are Not Less Sensitive Than Other Blood Leukocytes to the Genomic Effects of Glucocorticoids. *Plos One*, *7*(9), e44606–11. http://doi.org/10.1371/journal.pone.0044606

Hong, Y. S. (1996). Selective inhibition of polymorphonuclear leukocytes by immunosuppressive concentration of prostaglandin E2. *Journal of Korean Medical Science*, *11*(1), 8–16. http://doi.org/10.3346/jkms.1996.11.1.8

Hood, D. M., Grosenbaugh, D. A., Mostafa, M. B., Morgan, S. J., & Thomas, B. C. (1993). The role of vascular mechanisms in the development of acute equine laminitis. *Journal of Veterinary Internal Medicine / American College of Veterinary Internal Medicine*, *7*(4), 228–234.

Hoper, M. M., Voelkel, N. F., Bates, T. O., Allard, J. D., Horan, M., Shepherd, D., & Tuder, R. M. (1997). Prostaglandins induce vascular endothelial growth factor in a human monocytic cell line and rat lungs via cAMP. *American Journal of Respiratory Cell and Molecular Biology*, *17*(6), 748–756. http://doi.org/10.1165/ajrcmb.17.6.2888

Huang, J., Chen, R., Li, R., Wei, C. M., Yuan, G. Y., Liu, X. Y., et al. (2012). Bioequivalence of Two Misoprostol Tablets in Healthy Chinese Female Volunteers: A Single-Dose, Two-Period, Double Crossover Study. *Arzneimittelforschung*, *62*(01), 35–39. http://doi.org/10.1055/s-0031-1295426

Hulkower, K. I., Wertheimer, S. J., Levin, W., Coffey, J. W., Anderson, C. M., Chen, T., et al. (1994). Interleukin-1 beta induces cytosolic phospholipase A2 and prostaglandin H synthase in rheumatoid synovial fibroblasts. Evidence for their roles in the production of prostaglandin E2. *Arthritis & Rheumatism*, *37*(5), 653–661.

Humby, F., Bombardieri, M., Manzo, A., Kelly, S., Blades, M. C., Kirkham, B., et al. (2009). Ectopic Lymphoid Structures Support Ongoing Production of Class-Switched Autoantibodies in Rheumatoid Synovium. *PLoS Medicine*, *6*(1), e1–17. http://doi.org/10.1371/journal.pmed.0060001

Hunter, C. A., & Jones, S. A. (2015). IL-6 as a keystone cytokine in health and disease. *Nature Publishing Group*, *16*(5), 448–457. http://doi.org/10.1038/ni.3153

Hurley, D. J., Berghaus, L. J., Hurley, K. A. E., & Moore, J. N. (2011a). Evaluation of the in

vitro effects of aqueous black walnut extract on equine mononuclear cells. *American Journal of Veterinary Research*, *72*(3), 318–325. http://doi.org/10.2460/ajvr.72.3.318

Hurley, D. J., Hurley, K. A. E., Galland, K. L., Baker, B., Berghaus, L. J., Moore, J. N., & Majerle, R. S. K. (2011b). Evaluation of the ability of aqueous black walnut extracts to induce the production of reactive oxygen species. *American Journal of Veterinary Research*, *72*(3), 308–317. http://doi.org/10.2460/ajvr.72.3.308

Inada, M., Matsumoto, C., Uematsu, S., Akira, S., & Miyaura, C. (2006). Membrane-Bound Prostaglandin E Synthase-1-Mediated Prostaglandin E2 Production by Osteoblast Plays a Critical Role in Lipopolysaccharide-Induced Bone Loss Associated with Inflammation. *The Journal of Immunology*, *177*(3), 1879–1885. http://doi.org/10.4049/jimmunol.177.3.1879

Jackson, L. M., Wu, K. C., Mahida, Y. R., Jenkins, D., & Hawkey, C. J. (2000). Cyclooxygenase (COX) 1 and 2 in normal, inflamed, and ulcerated human gastric mucosa. *Gut*, *47*(6), 762–770.

Jacobson, C. C., Sertich, P. L., & Mc Donnell, S. M. (2013). Mid-gestation pregnancy is not disrupted by a 5-day gastrointestinal mucosal cytoprotectant oral regimen of misoprostol. *Equine Veterinary Journal*, *45*(1), 91–93. http://doi.org/10.1111/j.2042-3306.2012.00572.x

Jones, S. L., Knaus, U. G., Bokoch, G. M., & Brown, E. J. (1998). Two signaling mechanisms for activation of alphaM beta2 avidity in polymorphonuclear neutrophils. *Journal of Biological Chemistry*, *273*(17), 10556–10566.

Kadri, A., Ea, H. K., Bazille, C., Hannouche, D., Liote, F., & Cohen-Solal, M. E. (2008). Osteoprotegerin inhibits cartilage degradation through an effect on trabecular bone in murine experimental osteoarthritis. *Arthritis & Rheumatism*, *58*(8), 2379–2386. http://doi.org/10.1002/art.23638

Kalinski, P. (2011). Regulation of Immune Responses by Prostaglandin E2. *The Journal of Immunology*, *188*(1), 21–28. http://doi.org/10.4049/jimmunol.1101029

Kalinski, P., Hilkens, C. M., Snijders, A., Snijdewint, F. G., & Kapsenberg, M. L. (1997). Dendritic cells, obtained from peripheral blood precursors in the presence of PGE2, promote Th2 responses. *Advances in Experimental Medicine and Biology*, *417*, 363–367.

Kamei, D., Yamakawa, K., Takegoshi, Y., Mikami-Nakanishi, M., Nakatani, Y., Oh-ishi, S., et al. (2004). Reduced Pain Hypersensitivity and Inflammation in Mice Lacking Microsomal Prostaglandin E Synthase-1. *Journal of Biological Chemistry*, *279*(32), 33684–33695. http://doi.org/10.1074/jbc.M400199200

Kamm, J. L., Nixon, A. J., & WITTE, T. H. (2010). Cytokine and catabolic enzyme expression in synovium, synovial fluid and articular cartilage of naturally osteoarthritic equine carpi. *Equine Veterinary Journal*, *42*(8), 693–699. http://doi.org/10.1111/j.2042-3306.2010.00140.x

Kapoor, M., Kojima, F., Qian, M., Yang, L., & Crofford, L. J. (2006). Shunting of prostanoid biosynthesis in microsomal prostaglandin E synthase-1 null embryo fibroblasts: regulatory effects on inducible nitric oxide synthase expression and nitrite synthesis. *The FASEB Journal*, *20*(13), 2387–2389. http://doi.org/10.1096/fj.06-6366fje

Kapoor, M., Martel-Pelletier, J., Lajeunesse, D., Pelletier, J.-P., & Fahmi, H. (2010). Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. *Nature Publishing Group*, *7*(1), 33–42. http://doi.org/10.1038/nrrheum.2010.196

Katz, L. M., & Bailey, S. R. (2012). A review of recent advances and current hypotheses on the pathogenesis of acute laminitis. *Equine Veterinary Journal*, *44*(6), 752–761. http://doi.org/10.1111/j.2042-3306.2012.00664.x

Kawabata, A. (2011). Prostaglandin E2 and pain--an update. *Biological & Pharmaceutical Bulletin*, *34*(8), 1170–1173.

Kawcak, C. E., Frisbie, D. D., & McIlwraith, C. W. (2011). Effects of extracorporeal shock wave therapy and polysulfated glycosaminoglycan treatment on subchondral bone, serum biomarkers, and synovial fluid biomarkers in horses with induced osteoarthritis. *American Journal of Veterinary Research*, *72*(6), 772–779. http://doi.org/10.2460/ajvr.72.6.772

Kawcak, C. E., McIlwraith, C. W., Norrdin, R. W., Park, R. D., & James, S. P. (2001). The role of subchondral bone in joint disease: a review. *Equine Veterinary Journal*, *33*(2), 120–126.

Kearns, C. F., & McKeever, K. H. (2009). Clenbuterol and the horse revisited. *The Veterinary Journal*, *182*(3), 384–391. http://doi.org/10.1016/j.tvjl.2008.08.021

Khandpur, R., Carmona-Rivera, C., Vivekanandan-Giri, A., Gizinski, A., Yalavarthi, S., Knight, J. S., et al. (2013). NETs Are a Source of Citrullinated Autoantigens and Stimulate Inflammatory Responses in Rheumatoid Arthritis. *Science Translational Medicine*, *5*(178), 178ra40–178ra40. http://doi.org/10.1126/scitranslmed.3005580

Kim, D., & Haynes, C. L. (2012). Neutrophil Chemotaxis within a Competing Gradient of Chemoattractants. *Analytical Chemistry*, *84*(14), 6070–6078. http://doi.org/10.1021/ac3009548

Kim, H. A., Cho, M.-L., Choi, H. Y., Yoon, C. S., Jhun, J. Y., Oh, H. J., & Kim, H.-Y. (2006). The catabolic pathway mediated by Toll-like receptors in human osteoarthritic chondrocytes. *Arthritis & Rheumatism*, *54*(7), 2152–2163. http://doi.org/10.1002/art.21951

Kirker-Head, C. A., Chandna, V. K., Agarwal, R. K., Morris, E. A., Tidwell, A., O'Callaghan, M. W., et al. (2000). Concentrations of substance P and prostaglandin E2 in synovial fluid of normal and abnormal joints of horses. *American Journal of Veterinary Research*, *61*(6), 714–718.

Kirkham, B. W., Lassere, M. N., Edmonds, J. P., Juhasz, K. M., Bird, P. A., Lee, C. S., et al. (2006). Synovial membrane cytokine expression is predictive of joint damage progression in rheumatoid arthritis: A two-year prospective study (the DAMAGE study cohort). *Arthritis & Rheumatism*, *54*(4), 1122–1131. http://doi.org/10.1002/art.21749

Kitsis, E. A., Weissmann, G., & Abramson, S. B. (1991). The prostaglandin paradox: additive inhibition of neutrophil function by aspirin-like drugs and the prostaglandin E1 analog misoprostol. *The Journal of Rheumatology*, *18*(10), 1461–1465.

Knight, D. A., Stewart, G. A., & Thompson, P. J. (1994). The respiratory epithelium and airway smooth muscle homeostasis: its relevance to asthma. *Clinical Experimental Allergy*, *24*(8), 698–706.

Kobayashi, Y., Mizoguchi, T., Take, I., Kurihara, S., Udagawa, N., & Takahashi, N. (2005). Prostaglandin E2 Enhances Osteoclastic Differentiation of Precursor Cells through Protein Kinase A-dependent Phosphorylation of TAK1. *Journal of Biological Chemistry*, *280*(12), 11395–11403. http://doi.org/10.1074/jbc.M411189200

Kocher, M., Siegel, M. E., Edberg, J. C., & Kimberly, R. P. (1997). Cross-linking of Fc gamma receptor IIa and Fc gamma receptor IIIb induces different proadhesive phenotypes on human neutrophils. *The Journal of Immunology*, *159*(8), 3940–3948.

Koeberle, A., & Werz, O. (2015). Perspective of microsomal prostaglandin E2 synthase-1 as drug target in inflammation-related disorders. *Biochemical Pharmacology*, *98*(1), 1–15. http://doi.org/10.1016/j.bcp.2015.06.022

Koeberle, A., Rossi, A., Zettl, H., Pergola, C., Dehm, F., Bauer, J., et al. (2010). The Molecular Pharmacology and In Vivo Activity of 2-(4-Chloro-6-(2,3-dimethylphenylamino)pyrimidin-2-ylthio)octanoic acid (YS121), a Dual Inhibitor of Microsomal Prostaglandin E2 Synthase-1 and 5-Lipoxygenase. *Journal of Pharmacology and Experimental Therapeutics*, *332*(3), 840–848. http://doi.org/10.1124/jpet.109.160663

Kojima, F., Naraba, H., Miyamoto, S., Beppu, M., Aoki, H., & Kawai, S. (2004). Membrane-associated prostaglandin E synthase-1 is upregulated by proinflammatory cytokines in chondrocytes from patients with osteoarthritis. *Arthritis Research & Therapy*, *6*(4), R355–11. http://doi.org/10.1186/ar1195

Kojima, F., Naraba, H., Sasaki, Y., Okamoto, R., Koshino, T., & Kawai, S. (2002). Coexpression of microsomal prostaglandin E synthase with cyclooxygenase-2 in human rheumatoid synovial cells. *The Journal of Rheumatology*, *29*(9), 1836–1842.

Kolaczkowska, E., & Kubes, P. (2013). Neutrophil recruitment and function in health and inflammation. *Nature Reviews Immunology*, *13*(3), 159–175. http://doi.org/10.1038/nri3399

Kolsch, V., Charest, P. G., & Firtel, R. A. (2008). The regulation of cell motility and chemotaxis by phospholipid signaling. *Journal of Cell Science*, *121*(Pt 5), 551–559. http://doi.org/10.1242/jcs.023333

Komatsu, N., & Takayanagi, H. (2015). The International Journal of Biochemistry & Cell Biology. *International Journal of Biochemistry and Cell Biology*, *58*, 92–96. http://doi.org/10.1016/j.biocel.2014.11.008

Korotkova, M., & Jakobsson, P.-J. (2011). Microsomal Prostaglandin E Synthase-1 in Rheumatic Diseases. *Frontiers in Pharmacology*, *1*, 1–8. http://doi.org/10.3389/fphar.2010.00146

Korotkova, M., & Jakobsson, P.-J. (2013). Characterization of Microsomal Prostaglandin E Synthase 1 Inhibitors. *Basic & Clinical Pharmacology & Toxicology*, *114*(1), 64–69. http://doi.org/10.1111/bcpt.12162

Korotkova, M., & Jakobsson, P.-J. (2014). Persisting eicosanoid pathways in rheumatic diseases. *Nature Reviews. Rheumatology*, *10*(4), 229–241. http://doi.org/10.1038/nrrheum.2014.1

Kotake, S., Sato, K., Kim, K. J., Takahashi, N., Udagawa, N., Nakamura, I., et al. (1996). Interleukin-6 and soluble interleukin-6 receptors in the synovial fluids from rheumatoid arthritis patients are responsible for osteoclast-like cell formation. *Journal of Bone and Mineral Research*, *11*(1), 88–95. http://doi.org/10.1002/jbmr.5650110113

Kruger, P., Saffarzadeh, M., Weber, A. N. R., Rieber, N., Radsak, M., Bernuth, von, H., Benarafa, C., Roos, D., Skokowa, J., & Hartl, D. (2015). Neutrophils: Between Host Defence, Immune Modulation, and Tissue Injury. *PLOS Pathogens*, *11*(3), e1004651–23. http://doi.org/10.1371/journal.ppat.1004651

Kulkarni, S., Sitaru, C., Jakus, Z., Anderson, K. E., Damoulakis, G., Davidson, K., et al. (2011). PI3Kbeta plays a critical role in neutrophil activation by immune complexes. *Science Signaling*, *4*(168), ra23. http://doi.org/10.1126/scisignal.2001617

Kwon, S., Moore, J. N., Robertson, T. P., Hurley, D. J., Wagner, B., & Vandenplas, M. L. (2013). Disparate effects of LPS infusion and carbohydrate overload on inflammatory gene expression in equine laminae. *Veterinary Immunology and Immunopathology*, *155*(1-2), 1–8. http://doi.org/10.1016/j.vetimm.2013.05.001

la RebiÃ re de Pouyade, de, G., & Serteyn, D. (2011). The role of activated neutrophils in the early stage of equine laminitis. *The Veterinary Journal*, *189*(1), 27–33. http://doi.org/10.1016/j.tvjl.2010.06.008

la RebiÃ re de Pouyade, de, G., Riggs, L. M., Moore, J. N., Franck, T., Deby-Dupont, G., Hurley, D. J., & Serteyn, D. (2010). Equine neutrophil elastase in plasma, laminar tissue, and skin of horses administered black walnut heartwood extract. *Veterinary Immunology and Immunopathology*, *135*(3-4), 181–187. http://doi.org/10.1016/j.vetimm.2009.10.024

Laan, T. T. J. M., Bull, S., Pirie, R. S., & Fink-Gremmels, J. (2006). The anti-inflammatory effects of IV administered clenbuterol in horses with recurrent airway obstruction. *The Veterinary Journal*, *171*(3), 429–437. http://doi.org/10.1016/j.tvjl.2005.02.019

Lacourt, M., Gao, C., Li, A., Girard, C., Beauchamp, G., Henderson, J. E., & Laverty, S. (2012). Relationship between cartilage and subchondral bone lesions in repetitive impact trauma-induced equine osteoarthritis. *Osteoarthritis and Cartilage*, *20*(6), 572–583. http://doi.org/10.1016/j.joca.2012.02.004

Lader, C. S., & Flanagan, A. M. (1998). Prostaglandin E2, interleukin 1alpha, and tumor necrosis factor-alpha increase human osteoclast formation and bone resorption in vitro. *Endocrinology*, *139*(7), 3157–3164. http://doi.org/10.1210/endo.139.7.6085

Landoni, M. F., Foot, R., Frean, S., & LEES, P. (1996). Effects of flunixin, tolfenamic acid, R(-) and S(+) ketoprofen on the response of equine synoviocytes to lipopolysaccharide stimulation. *Equine Veterinary Journal*, *28*(6), 468–475.

Lang, P., Gesbert, F., Delespine-Carmagnat, M., Stancou, R., Pouchelet, M., & Bertoglio, J. (1996). Protein kinase A phosphorylation of RhoA mediates the morphological and functional effects of cyclic AMP in cytotoxic lymphocytes. *The EMBO Journal*, *15*(3), 510–519.

Langereis, J. D. (2013). Neutrophil integrin affinity regulation in adhesion, migration, and bacterial clearance. *Cell Adhesion & Migration*, *7*(6), 476–481. http://doi.org/10.4161/cam.27293

Lapointe, B. M., Frenette, J., & Côté, C. H. (2002). Lengthening contraction-induced inflammation is linked to secondary damage but devoid of neutrophil invasion. *Journal of Applied Physiology*, *92*(5), 1995–2004. http://doi.org/10.1152/japplphysiol.00803.2001

Larena, M., Holmgren, J., Lebens, M., Terrinoni, M., & Lundgren, A. (2015). Cholera Toxin, and the Related Nontoxic Adjuvants mmCT and dmLT, Promote Human Th17 Responses via Cyclic AMP-Protein Kinase A and Inflammasome-Dependent IL-1 Signaling. *The Journal of Immunology*, *194*(8), 3829–3839. http://doi.org/10.4049/jimmunol.1401633

Larsen, R., Hansen, M. B., & Bindslev, N. (2005). Duodenal secretion in humans mediated by the EP4 receptor subtype. *Acta Physiologica Scandinavica*, *185*(2), 133–140. http://doi.org/10.1111/j.1365-201X.2005.01471.x

Laudanna, C., Campbell, J. J., & Butcher, E. C. (1996). Role of Rho in chemoattractant-activated leukocyte adhesion through integrins. *Science*, *271*(5251), 981–983.

Laudanna, C., Campbell, J. J., & Butcher, E. C. (1997). Elevation of intracellular cAMP inhibits RhoA activation and integrin-dependent leukocyte adhesion induced by chemoattractants. *Journal of Biological Chemistry*, *272*(39), 24141–24144.

Laverty, S., Okouneff, S., Ionescu, M., Reiner, A., Pidoux, I., Webber, C., et al. (2002). Excessive degradation of type II collagen in articular cartilage in equine osteochondrosis. *Journal of Orthopaedic Research*, *20*(6), 1282–1289. http://doi.org/10.1016/S0736-0266(02)00053-0

Leclerc, P., Idborg, H., Spahiu, L., Larsson, C., Nekhotiaeva, N., Wannberg, J., et al. (2013). Characterization of a human and murine mPGES-1 inhibitor and comparison to mPGES-1 genetic deletion in mouse models of inflammation. *Prostaglandins and Other Lipid Mediators*, *107*, 26–34. http://doi.org/10.1016/j.prostaglandins.2013.09.001

Leclere, M., Lavoie-Lamoureux, A., & Lavoie, J.-P. (2011). Heaves, an asthma-like disease of horses. *Respirology*, *16*(7), 1027–1046. http://doi.org/10.1111/j.1440-1843.2011.02033.x

Lefebvre, V., Peeters-Joris, C., & Vaes, G. (1990). Modulation by interleukin 1 and tumor necrosis factor alpha of production of collagenase, tissue inhibitor of metalloproteinases and collagen types in differentiated and dedifferentiated articular chondrocytes. *Biochimica Et Biophysica Acta*, *1052*(3), 366–378.

Lefer, D. J., Flynn, D. M., Phillips, M. L., Ratcliffe, M., & Buda, A. J. (1994). A novel sialyl

LewisX analog attenuates neutrophil accumulation and myocardial necrosis after ischemia and reperfusion. *Circulation*, *90*(5), 2390–2401.

Leguillette, R., Desevaux, C., & Lavoie, J.-P. (2002). Effects of pentoxifylline on pulmonary function and results of cytologic examination of bronchoalveolar lavage fluid in horses with recurrent airway obstruction. *American Journal of Veterinary Research*, *63*(3), 459–463.

Leise, B. S., Faleiros, R. R., WATTS, M., Johnson, P. J., Black, S. J., & Belknap, J. K. (2010). Laminar inflammatory gene expression in the carbohydrate overload model of equine laminitis. *Equine Veterinary Journal*, *43*(1), 54–61. http://doi.org/10.1111/j.2042-3306.2010.00122.x

Leistad, L., Feuerherm, A. J., Faxvaag, A., & Johansen, B. (2011). Multiple phospholipase A2 enzymes participate in the inflammatory process in osteoarthritic cartilage. *Scandinavian Journal of Rheumatology*, *40*(4), 308–316. http://doi.org/10.3109/03009742.2010.547872

Leslie, C. C. (2004). Regulation of arachidonic acid availability for eicosanoid production. *Biochemistry and Cell Biology*, *82*(1), 1–17. http://doi.org/10.1139/o03-080

Lewis, R. A., Soter, N. A., Diamond, P. T., Austen, K. F., Oates, J. A., & Roberts, L. J. 2. (1982). Prostaglandin D2 generation after activation of rat and human mast cells with anti-IgE. *The Journal of Immunology*, *129*(4), 1627–1631.

Ley, C., Ekman, S., Ronéus, B., & Eloranta, M. L. (2009). Interleukin-6 and high mobility group box protein-1 in synovial membranes and osteochondral fragments in equine osteoarthritis. *Research in Veterinary Science*, *86*(3), 490–497. http://doi.org/10.1016/j.rvsc.2008.10.008

Li, Jun, Liang, X., Wang, Q., Breyer, R. M., McCullough, L., & Andreasson, K. (2008). Misoprostol, an anti-ulcer agent and PGE2 receptor agonist, protects against cerebral ischemia. *Neuroscience Letters*, *438*(2), 210–215. http://doi.org/10.1016/j.neulet.2008.04.054

Li, L., PETTIT, A., GREGORY, L., & FORWOOD, M. (2006). Regulation of bone biology by prostaglandin endoperoxide H synthases (PGHS): A rose by any other name…. *Cytokine & Growth Factor Reviews*, *17*(3), 203–216. http://doi.org/10.1016/j.cytogfr.2006.01.005

Li, X, Okada, Y., Pilbeam, C. C., Lorenzo, J. A., Kennedy, C. R., Breyer, R. M., & Raisz, L. G. (2000). Knockout of the murine prostaglandin EP2 receptor impairs osteoclastogenesis in vitro. *Endocrinology*, *141*(6), 2054–2061.

http://doi.org/10.1210/endo.141.6.7518

Li, Xin, Ellman, M., Muddasani, P., Wang, J. H. C., Cs-Szabo, G., van Wijnen, A. J., & Im, H.-J. (2009). Prostaglandin E 2and its cognate EP receptors control human adult articular cartilage homeostasis and are linked to the pathophysiology of osteoarthritis. *Arthritis & Rheumatism*, *60*(2), 513–523. http://doi.org/10.1002/art.24258

Li, Xinfang, Afif, H., Cheng, S., Martel-Pelletier, J., Pelletier, J.-P., Ranger, P., & Fahmi, H. (2005). Expression and regulation of microsomal prostaglandin E synthase-1 in human osteoarthritic cartilage and chondrocytes. *The Journal of Rheumatology*, *32*(5), 887–895.

Lichtenstein, G. R., Feagan, B. G., Cohen, R. D., Salzberg, B. A., Diamond, R. H., Price, S., et al. (2012). Serious Infection and Mortality in Patients With Crohn's Disease: More Than 5 Years of Follow-Up in the TREAT™ Registry, *107*(9), 1409–1422. http://doi.org/10.1038/ajg.2012.218

Lieberthal, J., Sambamurthy, N., & Scanzello, C. R. (2015). Inflammation in joint injury and post-traumatic osteoarthritis. *Osteoarthritis and Cartilage*, *23*(11), 1825–1834. http://doi.org/10.1016/j.joca.2015.08.015

Lim, J., Hotchin, N. A., & Caron, E. (2011). Ser 756of β2 integrin controls Rap1 activity during inside-out activation of αMβ2. *Biochemical Journal*, *437*(3), 461–467. http://doi.org/10.1042/BJ20101666

Lin, C. R., Amaya, F., Barrett, L., Wang, H., Takada, J., Samad, T. A., & Woolf, C. J. (2006). Prostaglandin E2 Receptor EP4 Contributes to Inflammatory Pain Hypersensitivity. *Journal of Pharmacology and Experimental Therapeutics*, *319*(3), 1096–1103. http://doi.org/10.1124/jpet.106.105569

Lin, L. L., Wartmann, M., Lin, A. Y., Knopf, J. L., Seth, A., & Davis, R. J. (1993). cPLA2 is phosphorylated and activated by MAP kinase. *Cell*, *72*(2), 269–278.

Little, D., Brown, S. A., Campbell, N. B., Moeser, A. J., Davis, J. L., & Blikslager, A. T. (2007). Effects of the cyclooxygenase inhibitor meloxicam on recovery of ischemia-injured equine jejunum. *American Journal of Veterinary Research*, *68*(6), 614–624. http://doi.org/10.2460/ajvr.68.6.614

Loftus, J. P., Belknap, J. K., Stankiewicz, K. M., & Black, S. J. (2010). Laminar xanthine oxidase, superoxide dismutase and catalase activities in the prodromal stage of black-walnut induced equine laminitis. *Equine Veterinary Journal*, *39*(1), 48–53. http://doi.org/10.2746/042516406X151320

Loftus, J. P., Black, S. J., Pettigrew, A., Abrahamsen, E. J., & Belknap, J. K. (2007). Early

laminar events involving endothelial activation in horses with black walnut- induced laminitis. *American Journal of Veterinary Research*, *68*(11), 1205–1211. http://doi.org/10.2460/ajvr.68.11.1205

Loftus, J. P., Johnson, P. J., Belknap, J. K., Pettigrew, A., & Black, S. J. (2009). Leukocyte-derived and endogenous matrix metalloproteinases in the lamellae of horses with naturally acquired and experimentally induced laminitis. *Veterinary Immunology and Immunopathology*, *129*(3-4), 221–230. http://doi.org/10.1016/j.vetimm.2008.11.003

Lucia, J. L., Coverdale, J. A., Arnold, C. E., & Winsco, K. N. (2013). Influence of an intra-articular lipopolysaccharide challenge on markers of inflammation and cartilage metabolism in young horses. *Journal of Animal Science*, *91*(6), 2693–2699. http://doi.org/10.2527/jas.2012-5981

Lv, S., Han, M., Yi, R., Kwon, S., Dai, C., & Wang, R. (2014). Anti-TNF-alpha therapy for patients with sepsis: a systematic meta-analysis. *International Journal of Clinical Practice*, *68*(4), 520–528. http://doi.org/10.1111/ijcp.12382

Ma, A. D., Metjian, A., Bagrodia, S., Taylor, S., & Abrams, C. S. (1998). Cytoskeletal reorganization by G protein-coupled receptors is dependent on phosphoinositide 3-kinase gamma, a Rac guanosine exchange factor, and Rac. *Molecular and Cellular Biology*, *18*(8), 4744–4751.

Madison, J. B., Sommer, M., & Spencer, P. A. (1991). Relations among synovial membrane histopathologic findings, synovial fluid cytologic findings, and bacterial culture results in horses with suspected infectious arthritis: 64 cases (1979-1987). *Journal of the American Veterinary Medical Association*, *198*(9), 1655–1661.

Mahatma, M., Agrawal, N., Dajani, E. Z., Nelson, S., Nakamura, C., & Sitton, J. (1991). Misoprostol but not antacid prevents endotoxin-induced gastric mucosal injury: role of tumor necrosis factor-alpha. *Digestive Diseases and Sciences*, *36*(11), 1562–1568.

Maloney, C. G., Kutchera, W. A., Albertine, K. H., McIntyre, T. M., Prescott, S. M., & Zimmerman, G. A. (1998). Inflammatory agonists induce cyclooxygenase type 2 expression by human neutrophils. *The Journal of Immunology*, *160*(3), 1402–1410.

Marshall, J. F., & Blikslager, A. T. (2011). The effect of nonsteroidal anti-inflammatory drugs on the equine intestine. *Equine Veterinary Journal*, *43*, 140–144. http://doi.org/10.1111/j.2042-3306.2011.00398.x

Martel-Pelletier, J., Di Battista, J. A., Lajeunesse, D., & Pelletier, J. P. (1998). IGF/IGFBP axis in cartilage and bone in osteoarthritis pathogenesis. *Inflammation Research*, *47*(3), 90–100. http://doi.org/10.1007/s000110050288

Martel-Pelletier, J., Pelletier, J.-P., & Fahmi, H. (2003). Cyclooxygenase-2 and prostaglandins in articular tissues. *Seminars in Arthritis and Rheumatism*, *33*(3), 155–167. http://doi.org/10.1016/S0049-0172(03)00134-3

Martínez-Calatrava, M. J., Prieto-Potín, I., Roman-Blas, J. A., Tardio, L., Largo, R., & Herrero-Beaumont, G. (2012). RANKL synthesized by articular chondrocytes contributes to juxta-articular bone loss in chronic arthritis. *Arthritis Research & Therapy*, *14*(3), R149. http://doi.org/10.1186/ar3884

Massicotte, F., Fernandes, J. C., Martel-Pelletier, J., Pelletier, J.-P., & Lajeunesse, D. (2006). Modulation of insulin-like growth factor 1 levels in human osteoarthritic subchondral bone osteoblasts. *Bone*, *38*(3), 333–341. http://doi.org/10.1016/j.bone.2005.09.007

Massicotte, F., Lajeunesse, D., Benderdour, M., Pelletier, J. P., Hilal, G., Duval, N., & Martel-Pelletier, J. (2002). Can altered production of interleukin-1β, interleukin-6, transforming growth factor-β and prostaglandin E2 by isolated human subchondral osteoblasts identify two subgroups of osteoarthritic patients. *Osteoarthritis and Cartilage*, *10*(6), 491–500. http://doi.org/10.1053/joca.2002.0528

Masuko-Hongo, K., Berenbaum, F., Humbert, L., Salvat, C., Goldring, M. B., & Thirion, S. (2004). Up-regulation of microsomal prostaglandin E synthase 1 in osteoarthritic human cartilage: Critical roles of the ERK-1/2 and p38 signaling pathways. *Arthritis & Rheumatism*, *50*(9), 2829–2838. http://doi.org/10.1002/art.20437

May, S. A., Hooke, R. E., & LEES, P. (1988). Bone fragments stimulate equine synovial lining cells to produce the inflammatory mediator prostaglandin E2. *Equine Veterinary Journal. Supplement*, (6), 131–132.

May, S. A., Hooke, R. E., & LEES, P. (1991). Adverse conditions in vitro stimulate chondrocytes to produce prostaglandin E2 and stromelysin. *Equine Veterinary Journal*, *23*(5), 380–382.

Mayadas, T. N., Johnson, R. C., Rayburn, H., Hynes, R. O., & Wagner, D. D. (1993). Leukocyte rolling and extravasation are severely compromised in P selectin-deficient mice. *Cell*, *74*(3), 541–554.

Mayeenuddin, L. H., & Garrison, J. C. (2006). Phosphorylation of P-Rex1 by the cyclic AMP-dependent protein kinase inhibits the phosphatidylinositiol (3,4,5)-trisphosphate and Gbetagamma-mediated regulation of its activity. *Journal of Biological Chemistry*, *281*(4), 1921–1928. http://doi.org/10.1074/jbc.M506035200

Mbalaviele, G., Pauley, A. M., Shaffer, A. F., Ben S Zweifel, Mathialagan, S., Mnich, S. J.,

et al. (2010). Distinction of microsomal prostaglandin E synthase-1 (mPGES-1) inhibition from cyclooxygenase-2 inhibition in cells using a novel, selective mPGES-1 inhibitor. *Biochemical Pharmacology*, *79*(10), 1445–1454. http://doi.org/10.1016/j.bcp.2010.01.003

McCoy, A. M., Toth, F., Dolvik, N. I., Ekman, S., Ellermann, J., Olstad, K., et al. (2013). Articular osteochondrosis: a comparison of naturally-occurring human and animal disease. *Osteoarthritis and Cartilage*, *21*(11), 1638–1647. http://doi.org/10.1016/j.joca.2013.08.011

McCoy, J. M., Wicks, J. R., & Audoly, L. P. (2002). The role of prostaglandin E2 receptors in the pathogenesis of rheumatoid arthritis. *Journal of Clinical Investigation*, *110*(5), 651–658. http://doi.org/10.1172/JCI200215528

McIlwraith, C. W., & Frisbie, D. D. (2015). 11 - Nonsteroidal Antiinflammatory Drugs. *Joint Disease in the Horse* (Second Edition, pp. 192–201). Elsevier Inc. http://doi.org/10.1016/B978-1-4557-5969-9.00011-5

McIlwraith, C. W., Fortier, L. A., Frisbie, D. D., & Nixon, A. J. (2011). Equine Models of Articular Cartilage Repair. *Cartilage*, *2*(4), 317–326. http://doi.org/10.1177/1947603511406531

McInnes, I. B., & Schett, G. (2007). Cytokines in the pathogenesis of rheumatoid arthritis. *Nature Reviews Immunology*, *7*(6), 429–442. http://doi.org/10.1038/nri2094

McInnes, I. B., & Schett, G. (2011). The pathogenesis of rheumatoid arthritis. *The New England Journal of Medicine*, *365*(23), 2205–2219. http://doi.org/10.1056/NEJMra1004965

Meager, A. (1999). Cytokine regulation of cellular adhesion molecule expression in inflammation. *Cytokine & Growth Factor Reviews*, *10*(1), 27–39.

Meja, K. K., Barnes, P. J., & Giembycz, M. A. (1997). Characterization of the prostanoid receptor(s) on human blood monocytes at which prostaglandin E2 inhibits lipopolysaccharide-induced tumour necrosis factor-alpha generation. *British Journal of Pharmacology*, *122*(1), 149–157. http://doi.org/10.1038/sj.bjp.0701360

Mengshol, J. A., Vincenti, M. P., Coon, C. I., Barchowsky, A., & Brinckerhoff, C. E. (2000). Interleukin-1 induction of collagenase 3 (matrix metalloproteinase 13) gene expression in chondrocytes requires p38, c-Jun N-terminal kinase, and nuclear factor kappaB: differential regulation of collagenase 1 and collagenase 3. *Arthritis & Rheumatism*, *43*(4), 801–811. http://doi.org/10.1002/1529-0131(200004)43:4<801::AID-ANR10>3.0.CO;2-4

Mertens, P., Maes, A., Nuyts, J., Belmans, A., Desmet, W., Esplugas, E., et al. (2006). Recombinant P-selectin glycoprotein ligand–immunoglobulin, a P-selectin antagonist, as an adjunct to thrombolysis in acute myocardial infarction. The P-Selectin Antagonist Limiting Myonecrosis (PSALM) trial. *American Heart Journal*, *152*(1), 125.e1–125.e8. http://doi.org/10.1016/j.ahj.2006.04.020

Messenger, K. M., Davis, J. L., LaFevers, D. H., Barlow, B. M., & Posner, L. P. (2011). Intravenous and sublingual buprenorphine in horses: pharmacokinetics and influence of sampling site. *Veterinary Anaesthesia and Analgesia*, *38*(4), 374–384. http://doi.org/10.1111/j.1467-2995.2011.00613.x

Midwood, K., Sacre, S., Piccinini, A. M., Inglis, J., Trebaul, A., Chan, E., et al. (2009). Tenascin-C is an endogenous activator of Toll-like receptor 4 that is essential for maintaining inflammation in arthritic joint disease. *Nature Medicine*, *15*(7), 774–780. http://doi.org/10.1038/nm.1987

Miles, R. R., Sluka, J. P., Halladay, D. L., Santerre, R. F., Hale, L. V., Bloem, L., et al. (2000). ADAMTS-1: A cellular disintegrin and metalloprotease with thrombospondin motifs is a target for parathyroid hormone in bone. *Endocrinology*, *141*(12), 4533–4542. http://doi.org/10.1210/endo.141.12.7817

Milinovich, G. J., Burrell, P. C., Pollitt, C. C., Klieve, A. V., Blackall, L. L., Ouwerkerk, D., et al. (2008). Microbial ecology of the equine hindgut during oligofructose-induced laminitis. *The ISME Journal*, *2*(11), 1089–1100. http://doi.org/10.1038/ismej.2008.67

Minamizaki, T., Yoshiko, Y., Kozai, K., Aubin, J. E., & Maeda, N. (2009). EP2 and EP4 receptors differentially mediate MAPK pathways underlying anabolic actions of prostaglandin E2 on bone formation in rat calvaria cell cultures. *Bone*, *44*(6), 1177–1185. http://doi.org/10.1016/j.bone.2009.02.010

Miossec, P., Korn, T., & Kuchroo, V. K. (2009). Interleukin-17 and type 17 helper T cells. *The New England Journal of Medicine*, *361*(9), 888–898. http://doi.org/10.1056/NEJMra0707449

Mittal, M., Siddiqui, M. R., Tran, K., Reddy, S. P., & Malik, A. B. (2014). Reactive Oxygen Species in Inflammation and Tissue Injury. *Antioxidants & Redox Signaling*, *20*(7), 1126–1167. http://doi.org/10.1089/ars.2012.5149

Miwa, M. E. A. (1999). Induction of apoptosis in bovine articular chondrocyte by prostaglandin E2 through cAMP-dependent pathway, 1–8.

Miyaura, C. (2000). Impaired Bone Resorption to Prostaglandin E2 in Prostaglandin E

Receptor EP4-knockout Mice. *Journal of Biological Chemistry*, *275*(26), 19819–19823. http://doi.org/10.1074/jbc.M002079200

Mohajer, B., & Ma, T. Y. (2000). Eicosanoids and the small intestine. *Prostaglandins and Other Lipid Mediators*, *61*(3-4), 125–143.

Mohan, S., Nakao, Y., Honda, Y., Landale, E., Leser, U., Dony, C., et al. (1995). Studies on the mechanisms by which insulin-like growth factor (IGF) binding protein-4 (IGFBP-4) and IGFBP-5 modulate IGF actions in bone cells. *Journal of Biological Chemistry*, *270*(35), 20424–20431.

Monreal, L., Sabaté, D., Segura, D., Mayós, I., & Homedes, J. (2004). Lower gastric ulcerogenic effect of suxibuzone compared to phenylbutazone when administered orally to horses. *Research in Veterinary Science*, *76*(2), 145–149. http://doi.org/10.1016/j.rvsc.2003.10.004

Moore, R. M., Muir, W. W., & Granger, D. N. (1995). Mechanisms of gastrointestinal ischemia-reperfusion injury and potential therapeutic interventions: a review and its implications in the horse. *Journal of Veterinary Internal Medicine / American College of Veterinary Internal Medicine*, *9*(3), 115–132.

Moraes, B. M., do Amaral, B. C., Morimoto, M. S. S., Vieira, L. G. C., Perazzo, F. F., & Carvalho, J. C. T. (2007). Anti-inflammatory and analgesic actions of etoricoxib (an NSAID) combined with misoprostol. *Inflammopharmacology*, *15*(4), 175–178. http://doi.org/10.1007/s10787-006-1581-x

Moreno-Rubio, J., Herrero-Beaumont, G., Tardı o, L., álvarez-Soria, M. Á., & Largo, R. (2010). Nonsteroidal antiinflammatory drugs and prostaglandin E 2modulate the synthesis of osteoprotegerin and RANKL in the cartilage of patients with severe knee osteoarthritis. *Arthritis & Rheumatism*, *62*(2), 478–488. http://doi.org/10.1002/art.27204

Morgan, M. J., & Liu, Z.-G. (2010). Crosstalk of reactive oxygen species and NF-κB signaling. *Nature Publishing Group*, *21*(1), 103–115. http://doi.org/10.1038/cr.2010.178

Morimoto, K., Shirata, N., Taketomi, Y., Tsuchiya, S., Segi-Nishida, E., Inazumi, T., et al. (2014). Prostaglandin E2-EP3 Signaling Induces Inflammatory Swelling by Mast Cell Activation. *The Journal of Immunology*, *192*(3), 1130–1137. http://doi.org/10.4049/jimmunol.1300290

Morisset, S., Patry, C., Lora, M., & de Brum-Fernandes, A. J. (1998). Regulation of cyclooxygenase-2 expression in bovine chondrocytes in culture by interleukin 1alpha, tumor necrosis factor-alpha, glucocorticoids, and 17beta-estradiol. *The Journal of Rheumatology*, *25*(6), 1146–1153.

Morita, I. (2002). Distinct functions of COX-1 and COX-2. *Prostaglandins and Other Lipid Mediators*, *68-69*, 165–175.

Morita, I., Schindler, M., Regier, M. K., Otto, J. C., Hori, T., DeWitt, D. L., & Smith, W. L. (1995). Different intracellular locations for prostaglandin endoperoxide H synthase-1 and -2. *Journal of Biological Chemistry*, *270*(18), 10902–10908.

Moriyama, T., Higashi, T., Togashi, K., Iida, T., Segi, E., Sugimoto, Y., Tominaga, T., Narumiya, S., & Tominaga, M. (2005). Molecular Pain. *Molecular Pain*, *1*(1), 3–13. http://doi.org/10.1186/1744-8069-1-3

Morris, D. D., Crowe, N., & Moore, J. N. (1990). Correlation of clinical and laboratory data with serum tumor necrosis factor activity in horses with experimentally induced endotoxemia. *American Journal of Veterinary Research*, *51*(12), 1935–1940.

Morton, A. J. (2005). Diagnosis and Treatment of Septic Arthritis. *Veterinary Clinics of North America: Equine Practice*, *21*(3), 627–649. http://doi.org/10.1016/j.cveq.2005.08.001

Morton, A. J., Campbell, N. B., Gayle, J. M., Redding, W. R., & Blikslager, A. T. (2005). Preferential and non-selective cyclooxygenase inhibitors reduce inflammation during lipopolysaccharide-induced synovitis. *Research in Veterinary Science*, *78*(2), 189–192. http://doi.org/10.1016/j.rvsc.2004.07.006

Mosca, M., Polentarutti, N., Mangano, G., Apicella, C., Doni, A., Mancini, F., et al. (2007). Regulation of the microsomal prostaglandin E synthase-1 in polarized mononuclear phagocytes and its constitutive expression in neutrophils. *Journal of Leukocyte Biology*, *82*(2), 320–326. http://doi.org/10.1189/jlb.0906576

Moses, V. S., & Bertone, A. L. (2002). Nonsteroidal anti-inflammatory drugs. *Veterinary Clinics of NA: Equine Practice*, *18*(1), 21–37– v.

Moses, V. S., Hardy, J., Bertone, A. L., & Weisbrode, S. E. (2001). Effects of anti-inflammatory drugs on lipopolysaccharide-challenged and -unchallenged equine synovial explants. *American Journal of Veterinary Research*, *62*(1), 54–60.

Mócsai, A., Walzog, B., & Lowell, C. A. (2015). Intracellular signalling during neutrophil recruitment. *Cardiovascular Research*, *107*(3), 373–385. http://doi.org/10.1093/cvr/cvv159

Mukherjee, D., Nissen, S. E., & Topol, E. J. (2001). Risk of cardiovascular events associated with selective COX-2 inhibitors. *Jama*, *286*(8), 954–959.

Murakami, M., Nakashima, K., Kamei, D., Masuda, S., Ishikawa, Y., Ishii, T., et al. (2003). Cellular Prostaglandin E2 Production by Membrane-bound Prostaglandin E Synthase-2 via Both Cyclooxygenases-1 and -2. *Journal of Biological Chemistry*, *278*(39), 37937–37947. http://doi.org/10.1074/jbc.M305108200

Murakami, M., Naraba, H., Tanioka, T., Semmyo, N., Nakatani, Y., Kojima, F., et al. (2000). Regulation of Prostaglandin E2 Biosynthesis by Inducible Membrane-associated Prostaglandin E2 Synthase That Acts in Concert with Cyclooxygenase-2. *Journal of Biological Chemistry*, *275*(42), 32783–32792. http://doi.org/10.1074/jbc.M003505200

Murray, J. J., Tonnel, A. B., Brash, A. R., Roberts, L. J. 2., Gosset, P., Workman, R., et al. (1986). Release of prostaglandin D2 into human airways during acute antigen challenge. *The New England Journal of Medicine*, *315*(13), 800–804. http://doi.org/10.1056/NEJM198609253151304

Murrell, G. A., Jang, D., & Williams, R. J. (1995). Nitric oxide activates metalloprotease enzymes in articular cartilage. *Biochemical and Biophysical Research Communications*, *206*(1), 15–21. http://doi.org/10.1006/bbrc.1995.1003

Muthuswamy, R., Mueller-Berghaus, J., Haberkorn, U., Reinhart, T. A., Schadendorf, D., & Kalinski, P. (2010). PGE2 transiently enhances DC expression of CCR7 but inhibits the ability of DCs to produce CCL19 and attract naive T cells. *Blood*, *116*(9), 1454–1459. http://doi.org/10.1182/blood-2009-12-258038

Műzes, G. (2012). Changes of the cytokine profile in inflammatory bowel diseases. *World Journal of Gastroenterology*, *18*(41), 5848–14. http://doi.org/10.3748/wjg.v18.i41.5848

Nakamura, C., Nelson, S., Lippton, H., Bagby, G., & Summer, W. (1992). Contrasting effects of misoprostol on systemic and intrapulmonary lipopolysaccharide-induced tumor necrosis factor-alpha. *Life Sciences*, *50*(24), 1869–1876.

Nakao, K., Murase, A., Ohshiro, H., Okumura, T., Taniguchi, K., Murata, Y., et al. (2007). CJ-023,423, a Novel, Potent and Selective Prostaglandin EP4 Receptor Antagonist with Antihyperalgesic Properties. *Journal of Pharmacology and Experimental Therapeutics*, *322*(2), 686–694. http://doi.org/10.1124/jpet.107.122010

Nakashima, T., Hayashi, M., Fukunaga, T., Kurata, K., Oh-hora, M., Feng, J. Q., et al. (2011). Evidence for osteocyte regulation of bone homeostasis through RANKL expression. *Nature Medicine*, *17*(10), 1231–1234. http://doi.org/10.1038/nm.2452

Nakatani, Y., Hokonohara, Y., Kakuta, S., Sudo, K., Iwakura, Y., & Kudo, I. (2007). Knockout mice lacking cPGES/p23, a constitutively expressed PGE2 synthetic enzyme,

are peri-natally lethal. *Biochemical and Biophysical Research Communications*, *362*(2), 387–392. http://doi.org/10.1016/j.bbrc.2007.07.180

Nakayama, T. (2005). Prostaglandin E2 promotes degranulation-independent release of MCP-1 from mast cells. *Journal of Leukocyte Biology*, *79*(1), 95–104. http://doi.org/10.1189/jlb.0405226

Natori, S., Fujii, Y., Kurosawa, H., Nakano, A., & Shimada, H. (1997). Prostaglandin E1 protects against ischemia-reperfusion injury of the liver by inhibition of neutrophil adherence to endothelial cells. *Transplantation*, *64*(11), 1514–1520.

Netea, M. G., Nold-Petry, C. A., Nold, M. F., Joosten, L. A. B., Opitz, B., van der Meer, J. H. M., et al. (2009). Differential requirement for the activation of the inflammasome for processing and release of IL-1beta in monocytes and macrophages. *Blood*, *113*(10), 2324–2335. http://doi.org/10.1182/blood-2008-03-146720

Neuder, L. E., Keener, J. M., Eckert, R. E., Trujillo, J. C., & Jones, S. L. (2009). Role of p38 MAPK in LPS induced pro-inflammatory cytokine and chemokine gene expression in equine leukocytes. *Veterinary Immunology and Immunopathology*, *129*(3-4), 192–199. http://doi.org/10.1016/j.vetimm.2008.11.006

Nguyen, D. N., Sangild, P. T., Ostergaard, M. V., Bering, S. B., & Chatterton, D. E. W. (2014). Transforming growth factor- 2 and endotoxin interact to regulate homeostasis via interleukin-8 levels in the immature intestine. *AJP: Gastrointestinal and Liver Physiology*, *307*(7), G689–G699. http://doi.org/10.1152/ajpgi.00193.2014

Nishigaki, N., Negishi, M., & Ichikawa, A. (1996). Two Gs-coupled prostaglandin E receptor subtypes, EP2 and EP4, differ in desensitization and sensitivity to the metabolic inactivation of the agonist. *Molecular Pharmacology*, *50*(4), 1031–1037.

Nobes, C. D., Hawkins, P., Stephens, L., & Hall, A. (1995). Activation of the small GTP-binding proteins rho and rac by growth factor receptors. *Journal of Cell Science*, *108 (Pt 1)*, 225–233.

Noguchi, K., Iwasaki, K., Endo, H., Kondo, H., Shitashige, M., & Ishikawa, I. (2000). Prostaglandins E2 and I2 downregulate tumor necrosis factor alpha-induced intercellular adhesion molecule-1 expression in human oral gingival epithelial cells. *Oral Microbiology and Immunology*, *15*(5), 299–304.

Norberg, J. K., Sells, E., Chang, H.-H., Alla, S. R., Zhang, S., & Meuillet, E. J. (2013). Targeting inflammation: multiple innovative ways to reduce prostaglandin E 2. *Pharmaceutical Patent Analyst*, *2*(2), 265–288. http://doi.org/10.4155/ppa.12.90

North, T. E., Goessling, W., Walkley, C. R., Lengerke, C., Kopani, K. R., Lord, A. M., et al. (2007). Prostaglandin E2 regulates vertebrate haematopoietic stem cell homeostasis. *Nature*, *447*(7147), 1007–1011. http://doi.org/10.1038/nature05883

Notoya, K., Jovanovic, D. V., Reboul, P., Martel-Pelletier, J., Mineau, F., & Pelletier, J. P. (2000). The Induction of Cell Death in Human Osteoarthritis Chondrocytes by Nitric Oxide Is Related to the Production of Prostaglandin E2 Via the Induction of Cyclooxygenase-2. *The Journal of Immunology*, *165*(6), 3402–3410. http://doi.org/10.4049/jimmunol.165.6.3402

Okumura, T., Murata, Y., Taniguchi, K., Murase, A., & Nii, A. (2010). Effects of the selective EP 4antagonist, CJ-023,423 on chronic inflammation and bone destruction in rat adjuvant-induced arthritis. *Journal of Pharmacy and Pharmacology*, *60*(6), 723–730. http://doi.org/10.1211/jpp.60.6.0007

Olstad, K., Ekman, S., & Carlson, C. S. (2015). An Update on the Pathogenesis of Osteochondrosis. *Veterinary Pathology*, *52*(5), 785–802. http://doi.org/10.1177/0300985815588778

Olstad, K., Hendrickson, E. H. S., Carlson, C. S., Ekman, S., & Dolvik, N. I. (2013). Transection of vessels in epiphyseal cartilage canals leads to osteochondrosis and osteochondrosis dissecans in the femoro-patellar joint of foals; a potential model of juvenile osteochondritis dissecans. *Osteoarthritis and Cartilage*, *21*(5), 730–738. http://doi.org/10.1016/j.joca.2013.02.005

Omori, K., Kida, T., Hori, M., Ozaki, H., & Murata, T. (2014). Multiple roles of the PGE 2-EP receptor signal in vascular permeability. *British Journal of Pharmacology*, *171*(21), 4879–4889. http://doi.org/10.1111/bph.12815

Orsini, J. A., Ryan, W. G., Carithers, D. S., & Boston, R. C. (2012). Evaluation of oral administration of firocoxib for the management of musculoskeletal pain and lameness associated with osteoarthritis in horses. *American Journal of Veterinary Research*, *73*(5), 664–671. http://doi.org/10.2460/ajvr.73.5.664

Osiri, M., Ruxrungtham, K., Nookhai, S., Ohmoto, Y., & Deesomchok, U. (1998). IL-1beta, IL-6 and TNF-alpha in synovial fluid of patients with non-gonococcal septic arthritis. *Asian Pacific Journal of Allergy and Immunology / Launched by the Allergy and Immunology Society of Thailand*, *16*(4), 155–160.

Ottonello, L., Morone, M. P., Dapino, P., & Dallegri, F. (1995). Cyclic AMP-elevating agents down-regulate the oxidative burst induced by granulocyte-macrophage colony-stimulating factor (GM-CSF) in adherent neutrophils. *Clinical and Experimental Immunology*, *101*(3), 502–506.

Owens, J. G., Kamerling, S. G., Stanton, S. R., Keowen, M. L., & Prescott-Mathews, J. S. (1996). Effects of pretreatment with ketoprofen and phenylbutazone on experimentally induced synovitis in horses. *American Journal of Veterinary Research*, *57*(6), 866–874.

Page, T. H., Turner, J. J. O., Brown, A. C., Timms, E. M., Inglis, J. J., Brennan, F. M., et al. (2010). Nonsteroidal Anti-Inflammatory Drugs Increase TNF Production in Rheumatoid Synovial Membrane Cultures and Whole Blood. *The Journal of Immunology*, *185*(6), 3694–3701. http://doi.org/10.4049/jimmunol.1000906

Palazzo, A. J., Jones, S. P., Anderson, D. C., Granger, D. N., & Lefer, D. J. (1998). Coronary endothelial P-selectin in pathogenesis of myocardial ischemia-reperfusion injury. *The American Journal of Physiology*, *275*(5 Pt 2), H1865–72.

Palmer, J. L., Bertone, A. L., Malemud, C. J., & Mansour, J. (1996). Biochemical and biomechanical alterations in equine articular cartilage following an experimentally-induced synovitis. *Osteoarthritis and Cartilage*, *4*(2), 127–137.

Parazzoli, S., Harmon, J. S., Vallerie, S. N., Zhang, T., Zhou, H., & Robertson, R. P. (2012). Cyclooxygenase-2, Not Microsomal Prostaglandin E Synthase-1, Is the Mechanism for Interleukin-1 -induced Prostaglandin E2 Production and Inhibition of Insulin Secretion in Pancreatic Islets. *Journal of Biological Chemistry*, *287*(38), 32246–32253. http://doi.org/10.1074/jbc.M112.364612

Park, J. Y., Pillinger, M. H., & Abramson, S. B. (2006). Prostaglandin E2 synthesis and secretion: The role of PGE2 synthases. *Clinical Immunology*, *119*(3), 229–240. http://doi.org/10.1016/j.clim.2006.01.016

Park, Y.-G., Kang, S.-K., Kim, W.-J., Lee, Y.-C., & Kim, C.-H. (2004). Effects of TGF-β, TNF-α, IL-β and IL-6 alone or in combination, and tyrosine kinase inhibitor on cyclooxygenase expression, prostaglandin E2 production and bone resorption in mouse calvarial bone cells. *The International Journal of Biochemistry & Cell Biology*, *36*(11), 2270–2280. http://doi.org/10.1016/j.biocel.2004.04.019

Parry, G. C., & Mackman, N. (1997). Role of cyclic AMP response element-binding protein in cyclic AMP inhibition of NF-kappaB-mediated transcription. *The Journal of Immunology*, *159*(11), 5450–5456.

Peat, F. J., & Kawcak, C. E. (2015). Musculoskeletal Pathology. *Veterinary Clinics of North America: Equine Practice*, *31*(2), 407–424. http://doi.org/10.1016/j.cveq.2015.04.003

Pelletier, J. P., Jovanovic, D. V., Lascau-Coman, V., Fernandes, J. C., Manning, P. T., Connor, J. R., et al. (2000). Selective inhibition of inducible nitric oxide synthase

reduces progression of experimental osteoarthritis in vivo: possible link with the reduction in chondrocyte apoptosis and caspase 3 level. *Arthritis & Rheumatism*, *43*(6), 1290–1299. http://doi.org/10.1002/1529-0131(200006)43:6<1290::AID-ANR11>3.0.CO;2-R

Penfield, A., & Dale, M. M. (1985). Prostaglandins E1 and E2 enhance the stimulation of superoxide release by 1-oleoyl-2-acetylglycerol from human neutrophils. *FEBS Letters*, *181*(2), 335–338.

Peroni, J. F. (2005). Predisposition for venoconstriction in the equine laminar dermis: implications in equine laminitis. *Journal of Applied Physiology*, *100*(3), 759–763. http://doi.org/10.1152/japplphysiol.00794.2005

Perryman, L. E. (2004). Molecular pathology of severe combined immunodeficiency in mice, horses, and dogs. *Veterinary Pathology*, *41*(2), 95–100.

Pessler, F., Chen, L. X., Dai, L., Gomez-Vaquero, C., Diaz-Torne, C., Paessler, M. E., et al. (2008). A histomorphometric analysis of synovial biopsies from individuals with Gulf War Veterans' Illness and joint pain compared to normal and osteoarthritis synovium. *Clinical Rheumatology*, *27*(9), 1127–1134. http://doi.org/10.1007/s10067-008-0878-0

Pick, E. (2014). Role of the Rho GTPase Rac in the activation of the phagocyte NADPH oxidase. *Small GTPases*, *5*(1), e27952–23. http://doi.org/10.4161/sgtp.27952

Pick, R., Brechtefeld, D., & Walzog, B. (2013). Intraluminal crawling versus interstitial neutrophil migration during inflammation. *Molecular Immunology*, *55*(1), 70–75. http://doi.org/10.1016/j.molimm.2012.12.008

Platt, D., & Bayliss, M. T. (1994). An investigation of the proteoglycan metabolism of mature equine articular cartilage and its regulation by interleukin-1. *Equine Veterinary Journal*, *26*(4), 297–303.

Poree, B., Kypriotou, M., Chadjichristos, C., Beauchef, G., Renard, E., Legendre, F., et al. (2008). Interleukin-6 (IL-6) and/or soluble IL-6 receptor down-regulation of human type II collagen gene expression in articular chondrocytes requires a decrease of Sp1.Sp3 ratio and of the binding activity of both factors to the COL2A1 promoter. *Journal of Biological Chemistry*, *283*(8), 4850–4865. http://doi.org/10.1074/jbc.M706387200

Prisk, V., & Huard, J. (2003). Muscle injuries and repair: the role of prostaglandins and inflammation. *Histology and Histopathology*, *18*(4), 1243–1256.

Radi, Z. A., Kehrli, M. E. J., & Ackermann, M. R. (2001). Cell adhesion molecules, leukocyte trafficking, and strategies to reduce leukocyte infiltration. *Journal of*

Veterinary Internal Medicine / American College of Veterinary Internal Medicine, *15*(6), 516–529.

Radonić, A., Thulke, S., Mackay, I. M., Landt, O., Siegert, W., & Nitsche, A. (2004). Guideline to reference gene selection for quantitative real-time PCR. *Biochemical and Biophysical Research Communications*, *313*(4), 856–862. http://doi.org/10.1016/j.bbrc.2003.11.177

Raisz, L. G. (1999). Prostaglandins and bone: physiology and pathophysiology. *Osteoarthritis and Cartilage*, *7*(4), 419–421.

Rao, P., & Knaus, E. E. (2008). Evolution of nonsteroidal anti-inflammatory drugs (NSAIDs): cyclooxygenase (COX) inhibition and beyond. *Journal of Pharmacy & Pharmaceutical Sciences : a Publication of the Canadian Society for Pharmaceutical Sciences, Societe Canadienne Des Sciences Pharmaceutiques*, *11*(2), 81s–110s.

Raud, J. (1990). Vasodilatation and inhibition of mediator release represent two distinct mechanisms for prostaglandin modulation of acute mast cell-dependent inflammation. *British Journal of Pharmacology*, *99*(3), 449–454.

Rausch-Derra, L. C., Huebner, M., & Rhodes, L. (2015). Evaluation of the safety of long-term, daily oral administration of grapiprant, a novel drug for treatment of osteoarthritic pain and inflammation, in healthy dogs. *American Journal of Veterinary Research*, *76*(10), 853–859. http://doi.org/10.2460/ajvr.76.10.853

Rausch-Derra, L., Huebner, M., Wofford, J., & Rhodes, L. (2016). A Prospective, Randomized, Masked, Placebo-Controlled Multisite Clinical Study of Grapiprant, an EP4 Prostaglandin Receptor Antagonist (PRA), in Dogs with Osteoarthritis. *Journal of Veterinary Internal Medicine*, *30*(3), 756–763. http://doi.org/10.1111/jvim.13948

Read, J. R., Boston, R. C., Abraham, G., Bauquier, S. H., Soma, L. R., & Nolen-Walston, R. D. (2012). Effect of prolonged administration of clenbuterol on airway reactivity and sweating in horses with inflammatory airway disease. *American Journal of Veterinary Research*, *73*(1), 140–145. http://doi.org/10.2460/ajvr.73.1.140

Rechenberg, von, B., Guenther, H., McIlwraith, C. W., Leutenegger, C., Frisbie, D. D., Akens, M. K., & Auer, J. A. (2000a). Fibrous tissue of subchondral cystic lesions in horses produce local mediators and neutral metalloproteinases and cause bone resorption in vitro. *Veterinary Surgery : vs*, *29*(5), 420–429.

Rechenberg, von, B., McIlwraith, C. W., Akens, M. K., Frisbie, D. D., Leutenegger, C., & Auer, J. A. (2000b). Spontaneous production of nitric oxide (NO), prostaglandin (PGE2) and neutral metalloproteinases (NMPs) in media of explant cultures of equine synovial

membrane and articular cartilage from normal and osteoarthritic joints. *Equine Veterinary Journal*, *32*(2), 140–150.

Reid, G., Wielinga, P., Zelcer, N., van der Heijden, I., Kuil, A., de Haas, M., et al. (2003). The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs. *Proceedings of the National Academy of Sciences*, *100*(16), 9244–9249.

Ren, K., & Torres, R. (2009). Role of interleukin-1β during pain and inflammation. *Brain Research Reviews*, *60*(1), 57–64. http://doi.org/10.1016/j.brainresrev.2008.12.020

Renema, P., Housley, N. A., Audia, J. P., & Alvarez, D. F. (2016). cAMP Potentiates Caspase-1 Activation in Pulmonary Microvascular Enfothelial Cells During Pseudomonas aeruginosa Infection. *The FASEB Journal*, *30*(1 supplement).

Ricciotti, E., & FitzGerald, G. A. (2011). Prostaglandins and Inflammation. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *31*(5), 986–1000. http://doi.org/10.1161/ATVBAHA.110.207449

Richard, E. A., Depecker, M., Defontis, M., Leleu, C., Fortier, G., Pitel, P. H., & Couroucé-Malblanc, A. (2014). Cytokine Concentrations in Bronchoalveolar Lavage Fluid from Horses with Neutrophilic Inflammatory Airway Disease. *Journal of Veterinary Internal Medicine*, *28*(6), 1838–1844. http://doi.org/10.1111/jvim.12464

Richard, V. J., Murry, C. E., Jennings, R. B., & Reimer, K. A. (1988). Therapy to reduce free radicals during early reperfusion does not limit the size of myocardial infarcts caused by 90 minutes of ischemia in dogs. *Circulation*, *78*(2), 473–480.

Richardson, D. W., & Dodge, G. R. (2000). Effects of interleukin-1beta and tumor necrosis factor-alpha on expression of matrix-related genes by cultured equine articular chondrocytes. *American Journal of Veterinary Research*, *61*(6), 624–630.

Rieser, C., Bock, G., Klocker, H., Bartsch, G., & Thurnher, M. (1997). Prostaglandin E2 and tumor necrosis factor alpha cooperate to activate human dendritic cells: synergistic activation of interleukin 12 production. *The Journal of Experimental Medicine*, *186*(9), 1603–1608.

Riggs, L. M., Franck, T., Moore, J. N., Krunkosky, T. M., Hurley, D. J., Peroni, J. F., et al. (2007). Neutrophil myeloperoxidase measurements in plasma, laminar tissue, and skin of horses given black walnut extract. *American Journal of Veterinary Research*, *68*(1), 81–86. http://doi.org/10.2460/ajvr.68.1.81

Robinson, J., Watson, F., Bucknall, R. C., & Edwards, S. W. (1992). Activation of neutrophil

reactive-oxidant production by synovial fluid from patients with inflammatory joint disease. Soluble and insoluble immunoglobulin aggregates activate different pathways in primed and unprimed cells. *Biochemical Journal*, *286 (Pt 2)*, 345–351.

Roemer, F. W., Javaid, M. K., Guermazi, A., Thomas, M., Kiran, A., Keen, R., et al. (2010). Anatomical distribution of synovitis in knee osteoarthritis and its association with joint effusion assessed on non-enhanced and contrast-enhanced MRI. *Osteoarthritis and Cartilage*, *18*(10), 1269–1274. http://doi.org/10.1016/j.joca.2010.07.008

Ross, T. N., Kisiday, J. D., Hess, T., & McIlwraith, C. W. (2012). Evaluation of the inflammatory response in experimentally induced synovitis in the horse: a comparison of recombinant equine interleukin 1 beta and lipopolysaccharide. *Osteoarthritis and Cartilage*, *20*(12), 1583–1590. http://doi.org/10.1016/j.joca.2012.08.008

Ross-Jones, T. N., McIlwraith, C. W., Kisiday, J. D., Hess, T. M., Hansen, D. K., & Black, J. (2015). Influence of an n-3long-chain polyunsaturated fatty acid-enriched diet on experimentally induced synovitis in horses. *Journal of Animal Physiology and Animal Nutrition*, *100*(3), 565–577. http://doi.org/10.1111/jpn.12359

Rossetti, R. G., Seiler, C. M., Brathwaite, K., & Zurier, R. B. (1995). Effect of Misoprostol on Acute and Chronic Inflammation. *American Journal of Therapeutics*, *2*(9), 600–606.

Rossitto, M., Ujjan, S., Poulat, F., & Boizet-Bonhoure, B. (2014). Multiple roles of the prostaglandin D2 signaling pathway in reproduction. *Reproduction*, *149*(1), R49–R58. http://doi.org/10.1530/REP-14-0381

Rowan, A. D., Koshy, P. J., Shingleton, W. D., Degnan, B. A., Heath, J. K., Vernallis, A. B., et al. (2001). Synergistic effects of glycoprotein 130 binding cytokines in combination with interleukin-1 on cartilage collagen breakdown. *Arthritis & Rheumatism*, *44*(7), 1620–1632. http://doi.org/10.1002/1529-0131(200107)44:7<1620::AID-ART285>3.0.CO;2-B

Ryo, A., Suizu, F., Yoshida, Y., Perrem, K., Liou, Y.-C., Wulf, G., et al. (2003). Regulation of NF-kappaB signaling by Pin1-dependent prolyl isomerization and ubiquitin-mediated proteolysis of p65/RelA. *Molecular Cell*, *12*(6), 1413–1426.

Sabeh, F., Fox, D., & Weiss, S. J. (2010). Membrane-Type I Matrix Metalloproteinase-Dependent Regulation of Rheumatoid Arthritis Synoviocyte Function. *The Journal of Immunology*, *184*(11), 6396–6406. http://doi.org/10.4049/jimmunol.0904068

Sachs, D., Villarreal, C. F., Cunha, F. Q., Parada, C. A., & Ferreira, S. H. (2009). The role of PKA and PKCε pathways in prostaglandin E2-mediated hypernociception. *British Journal of Pharmacology*, *156*(5), 826–834. http://doi.org/10.1111/j.1476-

5381.2008.00093.x

Sadik, C. D., & Luster, A. D. (2012). Lipid-cytokine-chemokine cascades orchestrate leukocyte recruitment in inflammation. *Journal of Leukocyte Biology*, *91*(2), 207–215. http://doi.org/10.1189/jlb.0811402

Sadik, C. D., Kim, N. D., & Luster, A. D. (2011). Neutrophils cascading their way to inflammation. *Trends in Immunology*, *32*(10), 452–460. http://doi.org/10.1016/j.it.2011.06.008

Saegusa, M., Murakami, M., Nakatani, Y., Yamakawa, K., Katagiri, M., Matsuda, K., et al. (2003). Contribution of membrane-associated prostaglandin E2 synthase to bone resorption. *Journal of Cellular Physiology*, *197*(3), 348–356. http://doi.org/10.1002/jcp.10356

Saklatvala, J. (1986). Tumour necrosis factor alpha stimulates resorption and inhibits synthesis of proteoglycan in cartilage. *Nature*, *322*(6079), 547–549. http://doi.org/10.1038/322547a0

Saklatvala, J. (2004). The p38 MAP kinase pathway as a therapeutic target in inflammatory disease. *Current Opinion in Pharmacology*, *4*(4), 372–377. http://doi.org/10.1016/j.coph.2004.03.009

Sakuma, Y., Li, Z., Pilbeam, C. C., Alander, C. B., Chikazu, D., Kawaguchi, H., & Raisz, L. G. (2004). Stimulation of cAMP production and cyclooxygenase-2 by prostaglandin E2 and selective prostaglandin receptor agonists in murine osteoblastic cells. *Bone*, *34*(5), 827–834. http://doi.org/10.1016/j.bone.2003.12.007

Samuelsson, B., Morgenstern, R., & Jakobsson, P. J. (2007). Membrane Prostaglandin E Synthase-1: A Novel Therapeutic Target. *Pharmacological Reviews*, *59*(3), 207–224. http://doi.org/10.1124/pr.59.3.1

Sanchavanakit, N., Saengtong, W., Manokawinchoke, J., & Pavasant, P. (2015). TNF-α stimulates MMP-3 production via PGE2 signalling through the NF-kB and p38 MAPK pathway in a murine cementoblast cell line. *Archives of Oral Biology*, *60*(7), 1066–1074. http://doi.org/10.1016/j.archoralbio.2015.04.001

Sands, W. A., Woolson, H. D., Milne, G. R., Rutherford, C., & Palmer, T. M. (2006). Exchange Protein Activated by Cyclic AMP (Epac)-Mediated Induction of Suppressor of Cytokine Signaling 3 (SOCS-3) in Vascular Endothelial Cells. *Molecular and Cellular Biology*, *26*(17), 6333–6346. http://doi.org/10.1128/MCB.00207-06

Sangiah, S., MacAllister, C. C., & Amouzadeh, H. R. (1989). Effects of misoprostol and

omeprazole on basal gastric pH and free acid content in horses. *Research in Veterinary Science*, *47*(3), 350–354.

Saper, C. B., Romanovsky, A. A., & Scammell, T. E. (2012). Neural circuitry engaged by prostaglandins during the sickness syndrome. *Nature Neuroscience*, *15*(8), 1088–1095. http://doi.org/10.1038/nn.3159

Scandella, E. (2002). Prostaglandin E2 is a key factor for CCR7 surface expression and migration of monocyte-derived dendritic cells. *Blood*, *100*(4), 1354–1361. http://doi.org/10.1182/blood-2001-11-0017

Scanzello, C. R., & Goldring, S. R. (2012). The role of synovitis in osteoarthritis pathogenesis. *Bone*, *51*(2), 249–257. http://doi.org/10.1016/j.bone.2012.02.012

Schaefer, L., Babelova, A., Kiss, E., Hausser, H.-J., Baliova, M., Krzyzankova, M., et al. (2005). The matrix component biglycan is proinflammatory and signals through Toll-like receptors 4 and 2 in macrophages. *Journal of Clinical Investigation*, *115*(8), 2223–2233. http://doi.org/10.1172/JCI23755

Schlueter, A. E., & Orth, M. W. (2004). Equine osteoarthritis: a brief review of the disease and its causes. *Equine and Comparative Exercise Physiology*, *1*(4), 221–231. http://doi.org/10.1079/ECEP200428

Schofield, Z. V., Woodruff, T. M., Halai, R., Wu, M. C.-L., & Cooper, M. A. (2013). Neutrophils--a key component of ischemia-reperfusion injury. *Shock (Augusta, Ga.)*, *40*(6), 463–470. http://doi.org/10.1097/SHK.0000000000000044

Schuster, V. L. (2002). Prostaglandin transport. *Prostaglandins and Other Lipid Mediators*, *68-69*, 633–647.

Scott, J., Harris, G.J., Pinder E.M., Macfarlane J.G., Hellyer T.P., Rostron.A.J. et al. (2016). Exchange protein directly activated by cyclic AMP (EPAC) activation reverses neutrophil dysfunction induced by β2-agonists, corticosteroids, and critical illness. *Journal of Allergy and Clinical Immunology*, *137*(2), 535–544. http://doi.org/10.1016/j.jaci.2015.07.036

Sedger, L. M., & McDermott, M. F. (2014). TNF and TNF-receptors: From mediators of cell death and inflammation to therapeutic giants - past, present and future. *Cytokine & Growth Factor Reviews*, *25*(4), 453–472. http://doi.org/10.1016/j.cytogfr.2014.07.016

Sedgwick, A. D., Dawson, J., & LEES, P. (1987). Influence of chemotactic agents on the locomotion of equine polymorphonuclear and mononuclear leucocytes. *Research in Veterinary Science*, *43*(1), 55–58.

Seibert, K., Zhang, Y., Leahy, K., Hauser, S., Masferrer, J., & Isakson, P. (1997). Distribution of COX-1 and COX-2 in normal and inflamed tissues. *Advances in Experimental Medicine and Biology*, *400A*, 167–170.

Seifert, U., Bialy, L. P., Ebstein, F., Bech-Otschir, D., Voigt, A., Schröter, F., et al. (2010). Immunoproteasomes Preserve Protein Homeostasis upon Interferon-Induced Oxidative Stress. *Cell*, *142*(4), 613–624. http://doi.org/10.1016/j.cell.2010.07.036

Sellam, J., & Berenbaum, F. (2010). The role of synovitis in pathophysiology and clinical symptoms of osteoarthritis. *Nature Reviews. Rheumatology*, *6*(11), 625–635. http://doi.org/10.1038/nrrheum.2010.159

Sellers, R. S., Radi, Z. A., & Khan, N. K. (2010). Pathophysiology of Cyclooxygenases in Cardiovascular Homeostasis. *Veterinary Pathology*, *47*(4), 601–613. http://doi.org/10.1177/0300985810364389

Serezani, C. H., Ballinger, M. N., Aronoff, D. M., & Peters-Golden, M. (2008). Cyclic AMP. *American Journal of Respiratory Cell and Molecular Biology*, *39*(2), 127–132. http://doi.org/10.1165/rcmb.2008-0091TR

Shalom-Barak, T., Quach, J., & Lotz, M. (1998). Interleukin-17-induced gene expression in articular chondrocytes is associated with activation of mitogen-activated protein kinases and NF-kappaB. *Journal of Biological Chemistry*, *273*(42), 27467–27473.

Sharma, J. N., & Jawad, N. M. (2005). Adverse Effects of COX-2 Inhibitors. *The Scientific World JOURNAL*, *5*, 629–645. http://doi.org/10.1100/tsw.2005.82

Sheats, M. K., Pescosolido, K. C., Hefner, E. M., Sung, E. J., Adler, K. B., & Jones, S. L. (2014). Myristoylated Alanine Rich C Kinase Substrate (MARCKS) is essential to β2-integrin dependent responses of equine neutrophils. *Veterinary Immunology and Immunopathology*, *160*(3-4), 167–176. http://doi.org/10.1016/j.vetimm.2014.04.009

Sheibanie, A. F., Yen, J. H., Khayrullina, T., Emig, F., Zhang, M., Tuma, R., & Ganea, D. (2007). The Proinflammatory Effect of Prostaglandin E2 in Experimental Inflammatory Bowel Disease Is Mediated through the IL-23->IL-17 Axis. *The Journal of Immunology*, *178*(12), 8138–8147. http://doi.org/10.4049/jimmunol.178.12.8138

Shi, L., Reid, L. H., Jones, W. D., Shippy, R., Warrington, J. A., Baker, S. C., et al. (2006). The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements. *Nature Biotechnology*, *24*(9), 1151–1161. http://doi.org/10.1038/nbt1239

Shibakawa, A., Aoki, H., Masuko-Hongo, K., Kato, T., Tanaka, M., Nishioka, K., & Nakamura, H. (2003). Presence of pannus-like tissue on osteoarthritic cartilage and its histological character. *Osteoarthritis and Cartilage*, *11*(2), 133–140. http://doi.org/10.1053/joca.2002.0871

Shimpo, H., Sakai, T., Kondo, S., Mishima, S., Yoda, M., Hiraiwa, H., & Ishiguro, N. (2009). Regulation of prostaglandin E2 synthesis in cells derived from chondrocytes of patients with osteoarthritis. *Journal of Orthopaedic Science*, *14*(5), 611–617. http://doi.org/10.1007/s00776-009-1370-7

Shiro, T., Kakiguchi, K., Takahashi, H., Nagata, H., & Tobe, M. (2013). 7-Phenyl-imidazoquinolin-4(5H)-one derivatives as selective and orally available mPGES-1 inhibitors. *Bioorganic & Medicinal Chemistry*, *21*(11), 2868–2878. http://doi.org/10.1016/j.bmc.2013.03.069

Shirtliff, M. E., & Mader, J. T. (2002). Acute Septic Arthritis. *Clinical Microbiology Reviews*, *15*(4), 527–544. http://doi.org/10.1128/CMR.15.4.527-544.2002

Silva, E., Gaivão, M., Leitão, S., Amaro, A., Costa, L. L. D., & Mateus, L. (2008). Blood COX-2 and PGES gene transcription during the peripartum period of dairy cows with normal puerperium or with uterine infection. *Domestic Animal Endocrinology*, *35*(3), 314–323. http://doi.org/10.1016/j.domaniend.2008.07.001

Singhal, P. C., Sankaran, R. T., Nahar, N., Shah, N., & Patel, P. (2000). Vasoactive agents modulate migration of monocytes across glomerular endothelial cells. *Journal of Investigative Medicine : the Official Publication of the American Federation for Clinical Research*, *48*(2), 110–117.

Slater, S.J., Seiz, J.L., Stagliano, B.A., Stubbs, C.D. (2001) Interaction of protein kinase C isoenzymes with Rho GTPases. *Biochemistry. 40*(14). 4437-45.

Smallwood, J. I., & Malawista, S. E. (1995). Misoprostol Stimulates cAMP Generation in Human Leukocytes: Synergy with Colchicine Suggests a New Potential for Established Drugs. *American Journal of Therapeutics*, *2*(9), 725–729.

Smith, B.P. (2015). Large Animal Internal Medicine. Fifth Edition. Elsevier Mosby. St. Louis, MO.

Smith, W. G., Thompson, J. M., Kowalski, D. L., & McKearn, J. P. (1996). Inhaled misoprostol blocks guinea pig antigen-induced bronchoconstriction and airway inflammation. *American Journal of Respiratory and Critical Care Medicine*, *154*(2 Pt 1), 295–299. http://doi.org/10.1164/ajrccm.154.2.8756797

Sokolowska, M., Chen, L. Y., Liu, Y., Martinez-Anton, A., Qi, H. Y., Logun, C., et al. (2015). Prostaglandin E2 Inhibits NLRP3 Inflammasome Activation through EP4 Receptor and Intracellular Cyclic AMP in Human Macrophages. *The Journal of Immunology*, *194*(11), 5472–5487. http://doi.org/10.4049/jimmunol.1401343

Sommerfelt, R. M., Feuerherm, A. J., Skuland, T., & Johansen, B. (2015). Cytosolic Phospholipase A2 Modulates TLR2 Signaling in Synoviocytes. *Plos One*, *10*(4), e0119088–15. http://doi.org/10.1371/journal.pone.0119088

Song, X.-Y. R., Torphy, T. J., Griswold, D. E., & Shealy, D. (2002). Coming of age: anti-cytokine therapies. *Molecular Interventions*, *2*(1), 36–46.

Sreeramkumar, V., Fresno, M., & Cuesta, N. (2011). Prostaglandin E2 and T cells: friends or foes&quest. *Immunology and Cell Biology*, *90*(6), 579–586. http://doi.org/10.1038/icb.2011.75

St-Onge, M., Flamand, N., Biarc, J., Picard, S., Bouchard, L., Dussault, A.-A., et al. (2007). Characterization of prostaglandin E2 generation through the cyclooxygenase (COX)-2 pathway in human neutrophils. *Biochimica Et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, *1771*(9), 1235–1245. http://doi.org/10.1016/j.bbalip.2007.06.002

Steeve, K. T., Marc, P., Sandrine, T., Dominique, H., & Yannick, F. (2004). IL-6, RANKL, TNF-alpha/IL-1: interrelations in bone resorption pathophysiology. *Cytokine & Growth Factor Reviews*, *15*(1), 49–60. http://doi.org/10.1016/j.cytogfr.2003.10.005

Stichtenoth, D. O., Thoren, S., Bian, H., Peters-Golden, M., Jakobsson, P. J., & Crofford, L. J. (2001). Microsomal prostaglandin E synthase is regulated by proinflammatory cytokines and glucocorticoids in primary rheumatoid synovial cells. *The Journal of Immunology*, *167*(1), 469–474.

Strassheim, D., Park, J. S., & Abraham, E. (2002). Sepsis: current concepts in intracellular signaling. *International Journal of Biochemistry and Cell Biology*, *34*(12), 1527–1533.

Suda, K., Udagawa, N., Sato, N., Takami, M., Itoh, K., Woo, J. T., et al. (2004). Suppression of Osteoprotegerin Expression by Prostaglandin E2 Is Crucially Involved in Lipopolysaccharide-Induced Osteoclast Formation. *The Journal of Immunology*, *172*(4), 2504–2510. http://doi.org/10.4049/jimmunol.172.4.2504

Sugimoto, Y., & Narumiya, S. (2007). Prostaglandin E Receptors. *Journal of Biological Chemistry*, *282*(16), 11613–11617. http://doi.org/10.1074/jbc.R600038200

Sun, W.-C., Moore, J. N., Hurley, D. J., Vandenplas, M. L., & Murray, T. F. (2007). Effects of stimulation of adenosine A2A receptors on lipopolysaccharide-induced production of

reactive oxygen species by equine neutrophils. *American Journal of Veterinary Research*, *68*(6), 649–656. http://doi.org/10.2460/ajvr.68.6.649

Sun, W.-C., Moore, J. N., Hurley, D. J., Vandenplas, M. L., Fortes, B., Thompson, R., & Linden, J. (2010). Differential modulation of lipopolysaccharide-induced expression of inflammatory genes in equine monocytes through activation of adenosine A2A receptors. *Veterinary Immunology and Immunopathology*, *134*(3-4), 169–177. http://doi.org/10.1016/j.vetimm.2009.08.018

Sun, W.-C., Moore, J. N., Hurley, D. J., Vandenplas, M. L., Linden, J., Cao, Z., & Murray, T. F. (2008). Adenosine A2A receptor agonists inhibit lipopolysaccharide-induced production of tumor necrosis factor-α by equine monocytes. *Veterinary Immunology and Immunopathology*, *121*(1-2), 91–100. http://doi.org/10.1016/j.vetimm.2007.08.011

Sung, S. S., & Walters, J. A. (1991). Increased cyclic AMP levels enhance IL-1 alpha and IL-1 beta mRNA expression and protein production in human myelomonocytic cell lines and monocytes. *Journal of Clinical Investigation*, *88*(6), 1915–1923. http://doi.org/10.1172/JCI115515

Suzawa, T., Miyaura, C., Inada, M., Maruyama, T., Sugimoto, Y., Ushikubi, F., et al. (2000). The role of prostaglandin E receptor subtypes (EP1, EP2, EP3, and EP4) in bone resorption: an analysis using specific agonists for the respective EPs. *Endocrinology*, *141*(4), 1554–1559. http://doi.org/10.1210/endo.141.4.7405

Swan, C. E., & Breyer, R. M. (2011). Prostaglandin E2 modulation of blood pressure homeostasis: studies in rodent models. *Prostaglandins and Other Lipid Mediators*, *96*(1-4), 10–13. http://doi.org/10.1016/j.prostaglandins.2011.07.001

Takafuji, V. A., McIlwraith, C. W., & Howard, R. D. (2002). Effects of equine recombinant interleukin-1alpha and interleukin-1beta on proteoglycan metabolism and prostaglandin E2 synthesis in equine articular cartilage explants. *American Journal of Veterinary Research*, *63*(4), 551–558.

Takahashi, S., Takeuchi, K., & Okabe, S. (1999). EP4 receptor mediation of prostaglandin E2-stimulated mucus secretion by rabbit gastric epithelial cells. *Biochemical Pharmacology*, *58*(12), 1997–2002.

Talpain, E., Armstrong, R. A., Coleman, R. A., & Vardey, C. J. (1995). Characterization of the PGE receptor subtype mediating inhibition of superoxide production in human neutrophils. *British Journal of Pharmacology*, *114*(7), 1459–1465.

Tamura, K., Sakurai, T., & Kogo, H. (2006). Relationship between prostaglandin E2 and vascular endothelial growth factor (VEGF) in angiogenesis in human vascular

endothelial cells. *Vascular Pharmacology*, *44*(6), 411–416. http://doi.org/10.1016/j.vph.2006.02.009

Tanabe, T., & Tohnai, N. (2002). Cyclooxygenase isozymes and their gene structures and expression. *Prostaglandins and Other Lipid Mediators*, *68-69*, 95–114.

Tang, C. H., Yang, R. S., & Fu, W. M. (2005). Prostaglandin E2 Stimulates Fibronectin Expression through EP1 Receptor, Phospholipase C, Protein Kinase C , and c-Src Pathway in Primary Cultured Rat Osteoblasts. *Journal of Biological Chemistry*, *280*(24), 22907–22916. http://doi.org/10.1074/jbc.M500130200

Tang, O. S., Gemzell-Danielsson, K., & Ho, P. C. (2007). Misoprostol: Pharmacokinetic profiles, effects on the uterus and side-effects. *International Journal of Gynecology & Obstetrics*, *99*, S160–S167. http://doi.org/10.1016/j.ijgo.2007.09.004

Tang, O. S., Schweer, H., Lee, S. W. H., & Ho, P. C. (2009). Pharmacokinetics of repeated doses of misoprostol. *Human Reproduction*, *24*(8), 1862–1869. http://doi.org/10.1093/humrep/dep108

Tang, O. S., Schweer, H., Seyberth, H. W., Lee, S. W. H., & Ho, P. C. (2002). Pharmacokinetics of different routes of administration of misoprostol. *Human Reproduction*, *17*(2), 332–336.

Tanioka, T., Nakatani, Y., Semmyo, N., Murakami, M., & Kudo, I. (2000). Molecular Identification of Cytosolic Prostaglandin E2 Synthase That Is Functionally Coupled with Cyclooxygenase-1 in Immediate Prostaglandin E2Biosynthesis. *Journal of Biological Chemistry*, *275*(42), 32775–32782. http://doi.org/10.1074/jbc.M003504200

Teeling, J. L., Cunningham, C., Newman, T. A., & Perry, V. H. (2010). The effect of non-steroidal anti-inflammatory agents on behavioural changes and cytokine production following systemic inflammation: Implications for a role of COX-1. *Brain, Behavior, and Immunity*, *24*(3), 409–419. http://doi.org/10.1016/j.bbi.2009.11.006

Tetlow, L. C., Adlam, D. J., & Woolley, D. E. (2001). Matrix metalloproteinase and proinflammatory cytokine production by chondrocytes of human osteoarthritic cartilage: associations with degenerative changes. *Arthritis & Rheumatism*, *44*(3), 585–594. http://doi.org/10.1002/1529-0131(200103)44:3<585::AID-ANR107>3.0.CO;2-C

Thiéfin, G., & Beaugerie, L. (2005). Toxic effects of nonsteroidal antiinflammatory drugs on the small bowel, colon, and rectum. *Joint Bone Spine*, *72*(4), 286–294. http://doi.org/10.1016/j.jbspin.2004.10.004

Thompson, J. A., Mirza, M. H., Barker, S. A., Morgan, T. W., Bauer, R. W., & McConnico,

R. S. (2011). Clenbuterol toxicosis in three Quarter Horse racehorses after administration of a compounded product. *Journal of the American Veterinary Medical Association*, *239*(6), 842–849. http://doi.org/10.2460/javma.239.6.842

Tomlinson, J. E., & Blikslager, A. T. (2005). Effects of cyclooxygenase inhibitors flunixin and deracoxib on permeability of ischaemic-injured equine jejunum, 1–6.

Tomlinson, J. E., Wilder, B. O., Young, K. M., & Blikslager, A. T. (2004). Effects of flunixin meglumine or etodolac treatment on mucosal recovery of equine jejunum after ischemia. *American Journal of Veterinary Research*, *65*(6), 761–769.

Trebino, C. E., Stock, J. L., Gibbons, C. P., Naiman, B. M., Wachtmann, T. S., Umland, J. P., et al. (2003). Impaired inflammatory and pain responses in mice lacking an inducible prostaglandin E synthase. *Proceedings of the National Academy of Sciences*, *100*(15), 9044–9049.

Trumble, T. N., Billinghurst, R. C., & McIlwraith, C. W. (2004). Correlation of prostaglandin E2 concentrations in synovial fluid with ground reaction forces and clinical variables for pain or inflammation in dogs with osteoarthritis induced by transection of the cranial cruciate ligament. *American Journal of Veterinary Research*, *65*(9), 1269–1275.

Trumble, T. N., Trotter, G. W., Oxford, J. R., McIlwraith, C. W., Cammarata, S., Goodnight, J. L., et al. (2001). Synovial fluid gelatinase concentrations and matrix metalloproteinase and cytokine expression in naturally occurring joint disease in horses. *American Journal of Veterinary Research*, *62*(9), 1467–1477.

Tsujii, M., Kawano, S., Tsuji, S., Sawaoka, H., Hori, M., & DuBois, R. N. (1998). Cyclooxygenase regulates angiogenesis induced by colon cancer cells. *Cell*, *93*(5), 705–716.

Tung, J. T., Arnold, C. E., Alexander, L. H., Yuzbasiyan-Gurkan, V., Venta, P. J., Richardson, D. W., & Caron, J. P. (2002a). Evaluation of the influence of prostaglandin E2 on recombinant equine interleukin-1beta-stimulated matrix metalloproteinases 1, 3, and 13 and tissue inhibitor of matrix metalloproteinase 1 expression in equine chondrocyte cultures. *American Journal of Veterinary Research*, *63*(7), 987–993.

Tung, J. T., Fenton, J. I., Arnold, C., Alexander, L., Yuzbasiyan-Gurkan, V., Venta, P. J., et al. (2002b). Recombinant equine interleukin-1beta induces putative mediators of articular cartilage degradation in equine chondrocytes. *Canadian Journal of Veterinary Research = Revue Canadienne De Recherche Veterinaire*, *66*(1), 19–25.

Uematsu, S., Matsumoto, M., Takeda, K., & Akira, S. (2002). Lipopolysaccharide-dependent

prostaglandin E(2) production is regulated by the glutathione-dependent prostaglandin E(2) synthase gene induced by the Toll-like receptor 4/MyD88/NF-IL6 pathway. *The Journal of Immunology*, *168*(11), 5811–5816.

Umeki, S. (1990). Effects of non-steroidal anti-inflammatory drugs on human neutrophil NADPH oxidase in both whole cell and cell-free systems. *Biochemical Pharmacology*, *40*(3), 559–564.

Utomo, A., Cullere, X., Glogauer, M., Swat, W., & Mayadas, T. N. (2006). Vav Proteins in Neutrophils Are Required for Fc R-Mediated Signaling to Rac GTPases and Nicotinamide Adenine Dinucleotide Phosphate Oxidase Component p40(phox). *The Journal of Immunology*, *177*(9), 6388–6397. http://doi.org/10.4049/jimmunol.177.9.6388

Valitutti, S., Dessing, M., & Lanzavecchia, A. (1993). Role of cAMP in regulating cytotoxic T lymphocyte adhesion and motility. *European Journal of Immunology*, *23*(4), 790–795. http://doi.org/10.1002/eji.1830230403

van de Loo, F. A., Kuiper, S., van Enckevort, F. H., Arntz, O. J., & van den Berg, W. B. (1997). Interleukin-6 reduces cartilage destruction during experimental arthritis. A study in interleukin-6-deficient mice. *Ajpa*, *151*(1), 177–191.

van der Pouw Kraan, T. C., Boeije, L. C., Smeenk, R. J., Wijdenes, J., & Aarden, L. A. (1995). Prostaglandin-E2 is a potent inhibitor of human interleukin 12 production. *The Journal of Experimental Medicine*, *181*(2), 775–779.

Van Loon, J. P. A. M., de Grauw, J. C., Van DIERENDONCK, M., L'AMI, J. J., BACK, W., & van Weeren, P. R. (2010). Intra-articular opioid analgesia is effective in reducing pain and inflammation in an equine LPS induced synovitis model. *Equine Veterinary Journal*, *42*(5), 412–419. http://doi.org/10.1111/j.2042-3306.2010.00077.x

Vane, J. R., & Botting, R. M. (1996). Mechanism of action of anti-inflammatory drugs. *Scandinavian Journal of Rheumatology. Supplement*, *102*, 9–21.

Varani, J., & Ward, P. A. (1994). Mechanisms of endothelial cell injury in acute inflammation. *Shock (Augusta, Ga.)*, *2*(5), 311–319.

Venugopal, C., MARIAPPAN, N., HOLMES, E., KEARNEY, M., & BEADLE, R. (2012). Effect of potential therapeutic agents in reducing oxidative stress in pulmonary tissues of recurrent airway obstruction-affected and clinically healthy horses. *Equine Veterinary Journal*, *45*(1), 80–84. http://doi.org/10.1111/j.2042-3306.2012.00566.x

Verghese, M. W., McConnell, R. T., Strickland, A. B., Gooding, R. C., Stimpson, S. A., Yarnall, D. P., et al. (1995). Differential regulation of human monocyte-derived TNF

alpha and IL-1 beta by type IV cAMP-phosphodiesterase (cAMP-PDE) inhibitors. *Journal of Pharmacology and Experimental Therapeutics*, *272*(3), 1313–1320.

Visser, M. B., & Pollitt, C. C. (2011). The timeline of lamellar basement membrane changes during equine laminitis development. *Equine Veterinary Journal*, *43*(4), 471–477. http://doi.org/10.1111/j.2042-3306.2010.00292.x

Vogel, C., & Marcotte, E. M. (2012). Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nature Publishing Group*, *13*(4), 227–232. http://doi.org/10.1038/nrg3185

Waclawik, A. (2011). Novel insights into the mechanisms of pregnancy establishment: regulation of prostaglandin synthesis and signaling in the pig. *Reproduction*, *142*(3), 389–399. http://doi.org/10.1530/REP-11-0033

Waiser, J., Bohler, T., Stoll, J., Schumann, B., Budde, K., & Neumayer, H.-H. (2003). The immunosuppressive potential of misoprostol--efficacy and variability. *Clinical Immunology*, *109*(3), 288–294.

Wang, J.-P., Chang, L.-C., Lin, Y.-L., Hsu, M.-F., Chang, C.-Y., Huang, L.-J., & Kuo, S.-C. (2003). Investigation of the cellular mechanism of inhibition of formyl-methionyl-leucyl-phenylalanine-induced superoxide anion generation in rat neutrophils by 2-benzyloxybenzaldehyde. *Biochemical Pharmacology*, *65*(7), 1043–1051. http://doi.org/10.1016/S0006-2952(03)00006-6

Watanabe, K. (2002). Prostaglandin F synthase. *Prostaglandins and Other Lipid Mediators*, *68-69*, 401–407.

Weiss, D. J., Evanson, O. A., MacLeay, J., & Brown, D. R. (1998). Transient alteration in intestinal permeability to technetium Tc99m diethylenetriaminopentaacetate during the prodromal stages of alimentary laminitis in ponies. *American Journal of Veterinary Research*, *59*(11), 1431–1434.

Welch, H. C. E., Coadwell, W. J., Ellson, C. D., Ferguson, G. J., Andrews, S. R., Erdjument-Bromage, H., et al. (2002). P-Rex1, a PtdIns(3,4,5)P3- and Gbetagamma-regulated guanine-nucleotide exchange factor for Rac. *Cell*, *108*(6), 809–821.

Weller, C. L., Collington, S. J., Hartnell, A., Conroy, D. M., Kaise, T., Barker, J. E., et al. (2007). Chemotactic action of prostaglandin E2 on mouse mast cells acting via the PGE2 receptor 3. *Proceedings of the National Academy of Sciences*, *104*(28), 11712–11717. http://doi.org/10.1073/pnas.0701700104

Wen, A. Y., Sakamoto, K. M., & Miller, L. S. (2010). The Role of the Transcription Factor

CREB in Immune Function. *The Journal of Immunology*, *185*(11), 6413–6419. http://doi.org/10.4049/jimmunol.1001829

Westacott, C. I., Webb, G. R., Warnock, M. G., Sims, J. V., & Elson, C. J. (1997). Alteration of cartilage metabolism by cells from osteoarthritic bone. *Arthritis & Rheumatism*, *40*(7), 1282–1291. http://doi.org/10.1002/1529-0131(199707)40:7<1282::AID-ART13>3.0.CO;2-E

Westman, M., Korotkova, M., Klint, E. A., Stark, A., Audoly, L. P., Klareskog, L., et al. (2004). Expression of microsomal prostaglandin E synthase 1 in rheumatoid arthritis synovium. *Arthritis & Rheumatism*, *50*(6), 1774–1780. http://doi.org/10.1002/art.20286

Wheeldon, A., & Vardey, C. J. (1993). Characterization of the inhibitory prostanoid receptors on human neutrophils. *British Journal of Pharmacology*, *108*(4), 1051–1054.

Widomski, D., Fretland, D. J., Gasiecki, A. F., & Collins, P. W. (1997). The prostaglandin analogs, misoprostol and SC-46275, potently inhibit cytokine release from activated human monocytes. *Immunopharmacology and Immunotoxicology*, *19*(2), 165–174. http://doi.org/10.3109/08923979709007656

Williams, T. J. (1979). Prostaglandin E2, prostaglandin I2 and the vascular changes of inflammation. *British Journal of Pharmacology*, *65*(3), 517–524.

Wilson, R. W., Ballantyne, C. M., Smith, C. W., Montgomery, C., Bradley, A., O'Brien, W. E., & Beaudet, A. L. (1993). Gene targeting yields a CD18-mutant mouse for study of inflammation. *The Journal of Immunology*, *151*(3), 1571–1578.

Wise, H. (1996). The inhibitory effect of prostaglandin E2 on rat neutrophil aggregation. *Journal of Leukocyte Biology*, *60*(4), 480–486.

Wojdasiewicz, P., Poniatowski, X. U. A., & Szukiewicz, D. (2014). The Role of Inflammatory and Anti-Inflammatory Cytokines in the Pathogenesis of Osteoarthritis. *Mediators of Inflammation*, 1–19. http://doi.org/10.1155/2014/561459

Wong, D. M., Moore, R. M., & Brockus, C. W. (2012). Mechanisms of oxidative injury in equine disease. *Compendium (Yardley, PA)*, *34*(8), E6.

Wright, H. L., Moots, R. J., & Edwards, S. W. (2014). The multifactorial role of neutrophils in rheumatoid arthritis. *Nature Publishing Group*, *10*(10), 593–601. http://doi.org/10.1038/nrrheum.2014.80

Wright, H. L., Moots, R. J., Bucknall, R. C., & Edwards, S. W. (2010). Neutrophil function in inflammation and inflammatory diseases. *Rheumatology*, *49*(9), 1618–1631.

http://doi.org/10.1093/rheumatology/keq045

Wu, H., Wu, T., Hua, W., Dong, X., Gao, Y., Zhao, X., et al. (2015). PGE2 receptor agonist misoprostol protects brain against intracerebral hemorrhage in mice. *Neurobiology of Aging*, *36*(3), 1439–1450. http://doi.org/10.1016/j.neurobiolaging.2014.12.029

Xie, C., Ming, X., Wang, Q., Schwarz, E. M., Guldberg, R. E., O'Keefe, R. J., & Zhang, X. (2008). COX-2 from the injury milieu is critical for the initiation of periosteal progenitor cell mediated bone healing. *Bone*, *43*(6), 1075–1083. http://doi.org/10.1016/j.bone.2008.08.109

Xu, D., Rowland, S. E., Clark, P., Giroux, A., Cote, B., Guiral, S., et al. (2008). 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. *Journal of Pharmacology and Experimental Therapeutics*, *326*(3), 754–763. http://doi.org/10.1124/jpet.108.138776

Yamada, T., Specian, R. D., Granger, D. N., Gaginella, T. S., & Grisham, M. B. (1991). Misoprostol attenuates acetic acid-induced increases in mucosal permeability and inflammation: role of blood flow. *The American Journal of Physiology*, *261*(2 Pt 1), G332–9.

Yamamori, T., Inanami, O., Sumimoto, H., Akasaki, T., Nagahata, H., & Kuwabara, M. (2002). Relationship between p38 mitogen-activated protein kinase and small GTPase Rac for the activation of NADPH oxidase in bovine neutrophils. *Biochemical and Biophysical Research Communications*, *293*(5), 1571–1578.

Yen, J. H., Khayrullina, T., & Ganea, D. (2007). PGE2-induced metalloproteinase-9 is essential for dendritic cell migration. *Blood*, *111*(1), 260–270. http://doi.org/10.1182/blood-2007-05-090613

Yin, C., Pettigrew, A., Loftus, J. P., Black, S. J., & Belknap, J. K. (2009). Tissue concentrations of 4-HNE in the black walnut extract model of laminitis: Indication of oxidant stress in affected laminae. *Veterinary Immunology and Immunopathology*, *129*(3-4), 211–215. http://doi.org/10.1016/j.vetimm.2008.11.016

Yu, Y., & Chadee, K. (1998). Prostaglandin E2 stimulates IL-8 gene expression in human colonic epithelial cells by a posttranscriptional mechanism. *The Journal of Immunology*, *161*(7), 3746–3752.

Yuan, G. H., Tanaka, M., Masuko-Hongo, K., Shibakawa, A., Kato, T., Nishioka, K., & Nakamura, H. (2004). Characterization of cells from pannus-like tissue over articular cartilage of advanced osteoarthritis. *Osteoarthritis and Cartilage*, *12*(1), 38–45.

Zaccolo, M., Di Benedetto, G., Lissandron, V., Mancuso, L., Terrin, A., & Zamparo, I. (2006). Restricted diffusion of a freely diffusible second messenger: mechanisms underlying compartmentalized cAMP signalling. *Biochemical Society Transactions*, *34*(Pt 4), 495–497. http://doi.org/10.1042/BST0340495

Zhang, Y., Wang, J., Wheat, J., Chen, X., Jin, S., Sadrzadeh, H., et al. (2013). AML1-ETO mediates hematopoietic self-renewal and leukemogenesis through a COX/ -catenin signaling pathway. *Blood*, *121*(24), 4906–4916. http://doi.org/10.1182/blood-2012-08-447763

Zhou, L., & Nilsson, A. (2001). Sources of eicosanoid precursor fatty acid pools in tissues. *Journal of Lipid Research*, *42*(10), 1521–1542.