Laminar Inflammation and the Equine Epidermal Epithelial Cell: Determining the Role in Laminar Failure

DISSERTATION

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By

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Abstract

Laminitis is a devastatingly painful, life-threatening disease of the equine digit and it is estimated that up to 15% of horses will be affected by it during their lifetime. Initiating causes include sepsis/systemic inflammatory response syndrome (SIRS) related conditions (such as colitis, enteritis, pleuropnuemonia, bowel ischemia, and metritis), metabolic syndrome/pituitary pars intermedia dysfunction, and severe contralateral limb lameness. The pathogenesis of laminitis has yet to be completely elucidated; however, several theories on the predominate mechanism exist and include metabolic/enzymatic dysfunction, mechanical, vascular dysfunction, and inflammation. Recent evidence shows a prominent proinflammatory response very early in the developmental phases of laminitis induced experimentally by administration of black walnut extract (BWE). As dysregulation of the inflammatory response is suspected to play an important role in sepsis/SIRS and subsequent multiple organ failure in humans, a similar mechanism has been proposed to occur in the horse, with the hoof being the predominate organ that fails. Laminar failure occurs when there is dysadhesion between the epidermal and dermal lamina at the basement membrane. This separation allows for rotation of the third phalanx in the hoof capsule or worse complete distal displacement of the third phalanx in the hoof capsule and possible sloughing of the hoof. Disruption of the basement membrane and decreased number of hemidesmosomes have been documented in the
lamina of horses affected with laminitis; however, epithelial cells are known to be
dynamic cells able to respond to microbial products and endogenously produced damage-
associated proteins by transmitting signals via pattern recognition receptors and
subsequently producing inflammatory mediators.

The studies conducted were performed 1) to determine if inflammation in the
front and hind limb lamina occur after administration of a carbohydrate overload (CHO),
which is a model that more closely resembles clinical cases of laminitis occurring from
sepsis, 2) to determine if phosphorylation of STAT proteins play a role in the
inflammatory response of the lamina, and 3) to determine if other organs have a similar
inflammatory response as the lamina after CHO administration. Additional studies were
performed to develop an understanding of how the equine epidermal cell respond to
bacterial ligands known to be produced in the cecum after CHO administration and to
develop a technique for isolation of RNA specifically from the equine laminar basal
epithelial cell (LBEC) using laser capture microdissection (LCM).

Results of these studies have proven that inflammation does occur in the front and
hind lamina after CHO administration; however, the response occurs at the onset of
lameness in these horses which is different from the BWE model where it occurs at the
early developmental phase. STAT3, but not STAT1, phosphorylation occurs in the
lamina and is most likely a direct result of the profound IL-6 response that has been
documented to occur in the lamina from horses receiving both BWE and CHO.
Inflammation, evidence by gene expression and leukocyte emigration, does occur in the
liver, lung and kidney after CHO administration; however, a different pattern of
inflammatory gene expression occurs in each tissue with the lamina overall having the greatest response of any organ evaluated. Equine epidermal epithelial cells in culture respond to the gram negative bacterial components, LPS and flagellin, by producing proinflammatory cytokines, but do not response to the bacterial component of gram positive organisms, which is similar to what is observed clinically where horses with sepsis resulting from a gram negative source are much more likely to develop laminitis.

Overall, inflammation appears to be involved in the development of laminitis. In addition, it is likely the laminar epithelial cell plays a much more active role, not only structurally but also producing and responding to inflammatory mediators. The methods developed to isolate RNA from the LCM technique will allow for extensive study of these cells in context of disease, thereby providing essential information from future studies.
DEDICATION

To my horse Mambo Skippie “Bo” and all the other horses that have suffered and lost their life from this devastating disease.
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Since my program has spanned many years and two universities, there are many people to thank for their support and help in completion of my PhD. I would first like to thank my advisor, Dr. Jim Belknap for taking on an unfamiliar transfer student part way through her PhD program – I am not sure if you knew what you were getting into. Your mentorship and guidance over the last three years has meant more than words can say. I must also thank Mauria Watts, our laboratory associate, to which without your friendship, laboratory assistance, and support, I know would not have been able to complete the work presented in this document. In addition, there were numerous people in the laboratory at OSU and elsewhere who contributed to sample collection, laboratory assistance and technical support. In particular I would like to thank to Dr. Phillip Johnson, Dr. Samuel Black, Dr. Rafael Falerios, Dr. Jarred Williams, Dr. Teresa Burns, Cailing Yin, Amanda Pettigrew, Ashley Gustafson, and Dr. Bhanu Chowdhary. My gratitude to Drs. Chandan Sen and Sashwati Roy and the members of the LCM Core laboratory (Muna Findley and Dr. Sabyasachi Biswas) for providing training and instruction on the techniques needed to learn laser capture microscopy. Special thanks to Dr. Rusty Moore for your mentorship and friendship over many years both at here at OSU and at LSU. I am not sure if you will ever know how much your help and support has meant to me. I must also thank Dr. Susan Eades, my co-advisor at LSU, for all her
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Vita

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Leise BS, Yin C, Pettigrew A, and Belknap JK. 2010. Proinflammatory cytokine responses of cultured


   evidence of the involvement of CXCL1, a keratinocyte-derived chemokine, in equine laminitis. J Vet


**Fields of Study**

Major Field:  Comparative and Veterinary Medicine
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Chapter 1: Introduction

LAMINITIS

History of Laminitis

Laminitis is a condition described in the horse as early as 300’s BC where Xenophon (380 BC) and Aristotle (330 BC) first mention barley disease and a resulting crippling disease of the horse’s feet. In 55AD, Collumella provided a more detailed description of laminitis suggesting that it resulted from blood descending to the feet and recommended bleeding from the middle of the leg. Apsyrtus discussed several possible causes of laminitis including traveling on hard surfaces, overeating, and drinking cold water when hot. Correlations between equine laminitis and conditions of the human foot were also made during this time in history with numerous recommendations and descriptions of bleeding at the coronary band as well as from the jugular vein were made to eliminate the “bad humors” from the horse’s body. The presence of an increased digital pulse was described by Youatt in 1831 saying that the artery at the pastern will throb violently and Zundel in 1886 discussed the laminar wedge and the sole being perforated by the tip of the third phalanx. Multiple sources during this time describe various trimming and shoeing techniques to treat laminitis. Zundel also described the treatment of hooves affected with by cooling with snow or crushed ice, which has been
supported recently experimentally by van Epps and Pollit.\textsuperscript{1,4,5} Experimental laminitis was reportedly induced by Akerblom in 1934 with carbohydrate overload and with repeated small intravenous doses of histamine.\textsuperscript{1} A grading system describing stance, weight bearing, and lameness associated with increasing severity of laminitis were well described by Obel in 1948 and is still used by veterinarians today. Obel also found histologically that laminar separation occurred at the secondary epidermal laminae.\textsuperscript{6} Throughout history, writings by numerous individuals have described the suspected causes, clinical signs and various approaches to treatment of laminitis in the horse. Of these writings, the majority of them have been published from 1949 to the present.\textsuperscript{1} Although there has been much research involving determination of the pathophysiology and ideal treatments and prevention of laminitis, the exact mechanisms involved and ideal treatment regimes remain to be elucidated.

\textit{Prevalence of laminitis}

Despite substantial research efforts over the last 40 years, laminitis continues to affect horses across the world. It continues to be ranked as the top priority in need for more research among equine practitioner as surveyed by the AAEP Foundation in 2009. A survey of horse owners conducted in the US by the USDA in 2000 found that approximately 13\% of horse owners/operators had horses that were affected by laminitis within the year surveyed, with 5\% of those horses dead due to the severity of their laminitis.\textsuperscript{7} A similar study in the UK found a prevalence of 7.1\% in their horse population.\textsuperscript{8} In a retrospective study, horses with laminitis had an extremely poor prognosis with only about 25\% of horse’s becoming sound, 25\% remaining somewhat
lame, and approximately 50% had not survived long-term. Risk factors for the development of laminitis in hospitalized patients have been associated with endotoxemia, diarrhea, abdominal surgery, and vascular abnormalities. Besides conditions that have a component of the systemic inflammatory response syndrome, laminitis also occurs in horses that have endocrinopathies and support limb lameness. Pasture-associated laminitis, which includes horses with metabolic syndrome/increased serum insulin concentrations, is estimated to account for 54% of all cases of laminitis.

**Structural anatomy and physiology of the equine foot**

The equine digit is a complex structure consisting of the outer hoof wall (epidermis), the third phalanx, the corium (dermis), and digital arteries, veins, capillaries and nerves. The outer hoof wall is comprised of three layers: the stratum externum, stratum medium, and stratum internum. The stratum medium is the thickest layer providing the majority of load support from the outer hoof wall through its tubular and intertubular horn structure. The stratum internum is the inner most layer of the hoof wall and includes the straum lamellatum, or layer of leaves (Figure 1.1). This layer is comprised of approximately 600 primary epidermal lamellae (PELs) that project from the surface in parallel rows. The center of each leaf is keratinized and attaches to the stratum medium on the outer most section. Interdigitating in between each leaf is the primary dermal lamellae. These leaves increases the surface area for attachment of the distal phalanx, providing the necessary strength needed to support the horse’s weight when standing.
The hoof wall continues to grow throughout the horse’s life and regeneration occurs at the coronet where germinal cells produce keratinocytes that mature and produce keratin as they migrate distally. Studies demonstrate that the majority of the hoof growth occurs proximally from the coronet germinal cells and proximal primary epidermal lamellae with little to no mitosis occurring distally.\textsuperscript{14} Exactly how the hoof wall grows distally is unknown; however, it has been hypothesized that remodeling of the epidermis and extracellular matrix to which it is attached involves the controlled release of the basal epithelial cells from the basement membranes through the activation of matrix metalloproteinases (MMPs) and inactivation by tissue inhibitors (TIMPs).\textsuperscript{12}

Underlying the hoof wall is the corium or dermis. This is a highly vascular layer containing a network of arteries, veins and capillaries. In addition, there are numerous sensory and vasomotor nerves located in this dense connective tissue layer which attaches to the third phalanx. The blood supply to the foot arises from the medial and lateral digital arteries and at the level of the proximal interphalangeal joint, sending major branches to the heels, which in turn supply the digital cushion, frog, and distal and palmar corium. At the middle of the second phalanx, the digital artery branches again and runs deep to the cartilages and extensor tendon with the medial and lateral arteries connecting and forming the coronary circumflex artery supplying the distal interphalangeal joint, coronary corium and the proximal lamellae of the toe and dorsal quarters. The terminal medial and lateral branches of the digital artery enter the solar canal of the third phalanx via foramina and unite with the artery of the opposite side to form the terminal arch with branches of these arteries radiating outward through dorsal foramina to form the
circumflex artery of the sole. There are three interconnected venous plexuses of the foot consisting of the dorsal venous plexus laying in the deep part of the lamellar corium, the palmar venous pleures in the deep part of the solar corium, and the coronary venous pleuxi laying in the coronary cushion. These three valveless plexi are drained by the medial and lateral digital veins. The lack of valves in the veins is suspected to play an important role in allowing for pressure changes within the hard encased shell of the hoof. The theory is that when the horse is weight bearing the internal deformation of the soft tissue structures collapse the veins, forcing evacuation of venous blood from the hoof very quickly. The horse also has numerous arteriovenous anastomoses (AVAs) within the dermal lamellae which form direct communications between the arteries and veins and when opened allows for a low resistance pathway to bypass the capillaries. There is an estimated 500 AVAs per cm² in the foot and they are believed to function in thermoregulation and pressure normalization of the equine digit. These AVAs are well innervated by autonomic vasomotor nerves and have thick walls of smooth muscle. Although initially it was believed that these AVAs opened allowing blood to be shunted away from the capillaries of the foot during laminitis, their exact function and possible role in laminitis has yet to be determined.

Histology of the equine lamina

Laminae are comprised of interdigiting epidermal and dermal components that are structured like two combs with intertwining teeth (Figure 1.1). This interdigitation occurs not only at eye level between the primary epidermal and dermal structures, but also microscopically between secondary epidermal and dermal lamellae. The addition of
150 to 200 secondary epidermal folds on each primary lamella (remembering that they are 550-600 PELs) allow for greatly increase surface area which is calculated to be 0.8 squared meters. Histologically the secondary epidermal lamella (SEL) are club-shape with rounded tips (Figure 1.2). The basal epithelial cells are like in skin, as they are a signal layer of columnar to cuboidal shaped cells with oval to round nuclei positioned away from the basement membrane at the apex of the cell (Figure 1.2). Electron microscopy has demonstrated epidermal basal cell attachment to the basal lamina is by hemidesmosome. Basal cell to basal cell and basal cell to suprabasal cell attachment is via desmosomes. These structures are similar to what has been reported in skin and provide the stability of the epidermis to the dermis. In early laminitis, decrease in hemidesmosome numbers as well as elongation of the SEL tips, and changes to the nuclear location have all been reported.

The secondary dermal lamellae (SDL) consist of tendon-like sheets of connective tissue emanating from the parietal surface of the third phalanx and connect to the SELs at the basement membrane (BM). The basement membrane is comprised of extracellular matrix components including collagen IV, laminin-1, fibronectin and heparin. The collagen IV filaments form cord-like structures that are ensheathed with the glycoproteins laminin-1, fibronectin, perlecan, and heparin which all together form the lamina densa. These cords are anchored by banded fibers of collagen VII that intermesh with the collagen type I fibrils of the lamellar dermis. Changes to the BM have been documented in 2 horses with acute laminitis, as immunostaining demonstrated loss of attachment of epidermal basal cells to the underlying membrane and disintegration of the
lamellar BM evidenced by extensive loss in type IV collagen, type VII collagen and laminin staining.\textsuperscript{20}

Attachment of the epidermal basal cell to the BM is via type 1 hemidesmosomes (Figure 1.3). Hemidesmosomes are comprised of six key components including a $\alpha_6\beta_4$ integrin heterodimer, CD151 (a tetraspanin), BP180 (type 17 collagen), BP230 and plectin (both plakin family proteins).\textsuperscript{21, 22} It is predominately the $\alpha_6\beta_4$ integrin heterodimer and partially BP180 that connect to laminin-332 (also known as laminin-5) of the BM.\textsuperscript{22} Plectin and BP230 are intracellular components of the hemidesmosome and attach to keratin filaments in the cytoplasm.\textsuperscript{22} It is the $\alpha_6\beta_4$ integrin heterodimer and plectin that form the key anchoring and stabilizing components of the basal cell to the BM.\textsuperscript{22} Plectin (at the actin binding domain) and $\beta_4$ (at the first fibronectin III region) association are essential in the initiating steps of hemidesmosome formation.\textsuperscript{23} It is believed that activation of a phophatase is required to induce $\beta_4$-plectin binding, as serine phosphorylation at residues 1356, 1360 and 1364 found in the connecting segment of $\beta_4$, will result in dysadhesion of the hemidesmosome.\textsuperscript{23, 24} After plectin and $\alpha_6\beta_4$ interact, BP180 and BP230 will be recruited to the hemidesmosome.\textsuperscript{23} CD151 is an early constituent of the hemidesmosomal structure and is bound to the $\alpha_6$ integrin early in formation.

Separation between the SEL and SDL is known as the key histological feature of laminitis and is often demonstrated by PAS staining of the BM. Decrease in $\alpha_6$ integrin, BP180 and laminin-5 immunohistochemical staining in the lamina from horses with oligofructose induced laminitis has been suggested by researchers to indicate BM
dysadhesion. Decreased number of hemidesmosomes have also been reported in laminar explants deprived of glucose and also in lamina from horse with experimentally induced laminitis; therefore, the loss of these critical structures are suspected to play a role in laminar failure. More information is needed on the steps that occur in hemidesmosome disassembly. Phosphorylation of the serine residues on the β4 integrin (S1356, S1360, S1364), stimulated by epidermal growth factor, is believed to result in a conformation change that will not allow for plectin binding. Factors such as macrophage stimulating protein (found in wound fluid) have been found to induce serine phosphorylation of the β4 integrin connecting segment via PKCα, leading to hemidesmosome disassembly. Another possible mechanism that can block hemidesmosome formation is through calmodulin-plectin interaction, which occurs when increased calcium stimulates calmodulin to bind to the actin binding domain of the plectin, blocking binding of the β4 integrin.

Clinical presentation of laminitis

Clinical history of horses presenting with laminitis can be variable, depending on the initiating cause. Horses with acute laminitis usually have either had a recent illness, recent non-weight bearing lameness, or recent changes in pasture grazing. Horses with chronic laminitis often present with an acute exacerbation of their chronic condition.

Clinical signs of laminitis include increased digital pulses, increased temperature of the affected hooves, weight shifting, lameness, reluctance to lift feet, and positive response to hoof tester applied to the toe region of the foot. The characteristic saw horse stance of a laminitic horse is described as the horse having increased weight placed
on the hind limbs, with these limbs placed up under the horse and the forelimbs placed in front of the chest as if appearing to decrease the weight off the more commonly affected front feet.\textsuperscript{29} The severity of the lameness can be quite variable, but a stiff, short strided gait is often noted and appears worse when the horse is asked to turn. In horses with hind limb laminitis, the gait and stance will be different than when the front feet alone are affected with the horse having an exaggerated lift of the hind limb and rapid return to stance in order to relieve pain in the opposite hind limb. It is not uncommon for horses to be reluctant to move and may even spend long periods of time in sternal or lateral recumbency. A grading system based on the severity of pain associated with laminitis was developed by Obel in 1948.\textsuperscript{6} Obel grade 1 laminitis is present when the horse alternately and incessantly lifts their feet, but lameness is only noted as a short, stilted gait at the trot.\textsuperscript{6} Obel Grade 2 is described as the horse, although willing to walk, displays a short, stilted gait while walking and is reluctant to have a foot lifted off the ground.\textsuperscript{6} Obel grade 3 lameness is when the horse is reluctant to move and strongly resists having the foot lifted, and Obel grade 4 is present when the horse refuses to move, only doing so if forced.\textsuperscript{6}

Changes to hoof shape or structure may occur gradually or rapidly depending on severity of the laminitic episode. Horses with acute laminitis, having little to no rotation, will have normal appearing hoof. As the third phalanx (P3) rotates (Figure 1.4), prolapsing or bulging of the solar surface may occur as the distal tip of the P3 points down and the distance between P3 and the solar surface decreases. Horses with severe acute laminitis that involve displacement of P3 (also known as sinking) will often have
bulging of the solar corium and cavitation of the coronary band. In cases where complete
laminar failure has occurred, oozing of serum or blood from the coronary band can occur
just prior to sloughing of the hoof capsule. Horses with chronic laminitis can have
variable shape to the hoof, but characteristically known to have a dished appearance to
the dorsal hoof surface and increased heel height. Horizontal growth rings on the dorsal
surface may be present indicating variation in growth between the heel and toe regions.
The white line on the solar surface may be wider than normal and bruising of the solar
surface can also be present.

Other diagnostic tests that may assist with the clinical diagnosis of laminitis
includes perineural anesthesia and radiography. Local analgesia with lidocaine of the
medial and lateral palmar or plantar digital nerve at distal aspect of the proximal
sesamoids (abaxial sesamoid block) will result in significant to complete analgesia of the
foot and resolution of the pain/lameness. Radiography is used to diagnosis laminitis
(Figure 1.4) as well as for monitoring progression and assessing treatment.²⁸ Lateral and
dorsal palmar (or plantar) projections allow for the assessment of the position of the third
phalanx in reference to the hoof wall.²⁸ Rotation of P3 in acute cases is measured by
comparing the angle between the dorsal hoof wall and ground to the angle of the dorsal
aspect of P3 and the ground. Displacement of P3 (or sinking) is best evident in serial
radiographs by having increasing distance between the coronary band and the extensor
process of P3 and/or decreasing distance between the dorsal distal tip of P3 and the solar
surface. Other changes seen radiographically in chronic cases include remodeling or
resorption of the distal tip of P3 and increased concavity of the dorsal hoof wall.
Venography, which involves application of a distal limb tourniquet, injection of an iodinated contrast agent into the palmar/plantar digital vein, and acquisition of lateral radiograph of P3, has also been used more recently in both acute and chronic cases to determine if adequate blood flow is present throughout the foot. Lack of contrast in certain areas of the foot can be suggestive of disease severity and serial venograms may be used to assess progression of disease and response to treatment.

Pathophysiology of laminitis - Overview

The development of laminitis in horses has been associated with several conditions including sepsis/systemic inflammatory response syndrome (SIRS) occurring with strangulating gastrointestinal disorders, enterocolitis, pneumonia, metritis and grain overload, equine metabolic disease, pituitary pars intermedia dysfunction (PPID), and those with mechanical overload due to severe contralateral lameness. With many diverse conditions associated with the development of laminitis, the pathogenesis must be complex. Over the years, researchers have attempted to find one theory that will explain how this devastating disease occurs in the horse; however, with increasing knowledge it is now becoming accepted that the numerous proposed mechanisms are interconnected with each other. Four main mechanisms are believed to play a role in laminitis and include inflammation and extracellular matrix (ECM) degradation, endothelial and vascular dysfunction, endocrine/metabolic derangements, and mechanical overload.

Pathophysiology of laminitis – Inflammation and ECM degradation

As the term implies, laminitis or laminar inflammation, has been documented to occur early during the developmental stages of black walnut extract (BWE) induced
laminitis. Increased laminar mRNA concentrations of IL-1β, IL-6, CXCL1, IL-8, MAIL, COX-2, ICAM-1, and E-Selectin have been reported in the developmental (1.5h and 3h) and onset of lameness phases of laminitis after BWE administration. Leukocyte emigration from the vasculature into the lamina has also been documented in both the BWE and carbohydrate overload models (CHO) of laminitis. In addition, an equine-specific microarray that was used to evaluate lamina from horses receiving BWE supported earlier reports with genes associated with leukocyte activation and emigration up-regulated, as well as other genes involved in the inflammatory process, such as antioxidants and antimicrobial proteins. This early and aggressive inflammatory response in the lamina is believed to be similar to that of human sepsis/SIRS in which over expression of proinflammatory mediators results in damage or complete failure of organs, such as the lamina in the horse. Despite similarities between human and equine SIRS in regard to inflammatory mediators, the affects of oxidant injury on organ failure appears to be a less important factor in equine lamina. Although horses possess endogenous xanthine oxidase and catalase in their lamina, no difference after BWE administration was observed, suggesting that there is no significant involvement of reactive oxygen species in the development of laminitis. However, increases of 4-hydroxyl-2-nonenal (4-HNE), which is a lipid aldehyde that forms as a result of lipid peroxidation, has been found in the lamina of horses receiving BWE to induce laminitis. Enzymatic degradation of the extracellular matrix has been proposed to result in detachment of the basal epithelial cells from the basement membrane and underlying
dermis. Normal tissue maintenance requires that the extracellular matrix undergo degradation and re-synthesization. In laminitis, enzymatic dysfunction occurs where the balance is shifted towards degradation resulting from either an increased production of pro-matrix metalloproteases (MMPs) zymogens via gene transcription, activation of pro-MMP, and/or changes in production of tissue inhibitors of metalloproteases (TIMPs). The gelatinases, MMP-2 and MMP-9, were the first enzymes found to be up-regulated in the lamina from horses with naturally occurring laminitis when compared with non-laminitic horses. Increase in MMP-2 gene expression during the developmental phase of laminitis has been found to occur in the basal epithelial cells and activity is believed to be increased due to decreases in TIMP-2. Decrease laminar immunostaining of the membrane bound MMP-14 has been reported along with an increase in MMP-14 gene expression after oligofructose administration. As MMP-14 has been shown to activate pro-MMP-2, the authors suggested that the decrease in MMP-14 protein in acute laminitis resulted in MMP-2 activation and this decrease stimulated increased gene production to replenish the membrane loss of MMP-14; however, another study reported no change in MMP-14 gene expression in laminar tissue from horses receiving a starch based carbohydrate overload. Dramatic increases in another metalloprotease, ADAMTS-4 (a disintegrin and metalloprotease containing thrombospondin repeats) have been reported in both naturally occurring and experimentally induced laminitis. ADAMTS-4, an aggrecanase, can cleave proteoglycan aggrecan, which functions to provide mechanical resistance to compressive
loads and has been found to be increased in normal lamina compared with other tissues such as lung and liver of the horse.\textsuperscript{62}

Inflammation and metalloprotease expression and activation are closely intertwined. MMP and ADAMTS expression can be up-regulated by inflammatory mediators, such as IL-1\textbeta and IL-6, and some MMPs, such as MMP-2 and -9, can function as negative feedback mediators by degrading cytokines like IL-1\textbeta.\textsuperscript{57} Production of the neutrophil chemoattractant molecules IL-8 and CXCL1 have been reported to be increased in the lamina of horses with experimentally induced laminitis.\textsuperscript{41, 44} Neutrophil accumulation and MMP-9 gene expression has been reported to be increased in the lamina of horses with experimentally induced laminitis and MMP-9 concentrations were directly correlated with myeloperoxidase content, indicating that neutrophils serve as the primary source for this protease.\textsuperscript{50, 61} Neutrophils may also be important in the cleavage of proMMP-2 as neutrophil-derived proteases, such as elastase and protease-3, can work in a MMP-14 independent manner.\textsuperscript{61}

\textit{Pathophysiology of laminitis – Endothelial and vascular dysfunction}

Platelet activation, evidenced by microthrombi and the occurrence of platelet-neutrophil aggregates, has been reported to occur in horses with laminitis.\textsuperscript{63-65} Treatment with a platelet activator inhibitor prior to induction of laminitis by CHO attenuated the increase in platelet-neutrophil aggregates and prevented the development of laminitis in all ponies.\textsuperscript{66} In addition to enhancing an inflammatory response, platelets may also have vascular affects. Increased plasma concentrations of serotonin (5-HT) and thromboxane A\textsubscript{2} (TXA\textsubscript{2}) have been reported in horses given oligofructose to induce laminitis.\textsuperscript{67}
Production of 5-HT and TXA₂ was suspected to result from endotoxin stimulation of the platelets. Both 5-HT and TXA₂ are potent vasoconstrictors and have been demonstrated to cause constriction of equine digital/laminar vessels in vitro and to play a role in mediating LPS induced digital hypoperfusion in horses.

Both increased and decreased blood flow have been reported to occur in the equine digit affected by laminitis. The particular response is most likely dependent upon several factors including model examined, period of time evaluated, technique used to assess blood flow and/or vasoconstriction, and location of vessels studied. Although increased blood flow has been reported with laminitis, the presence of increased laminar concentrations of endothelin-1, a potent vasoconstrictor, has also been reported in horses with laminitis and administration of an endothelin-1 antagonist has been reported to reduce vascular resistance in the digit after CHO administration. In studies using an extracorporeal model to assess digital perfusion in the horse, specific hemodynamic forces of the equine laminar microcirculation have been defined in both the BWE and CHO models of laminitis. Increased venous resistance secondary to venoconstriction has been reported to result in increased hydrostatic forces in the capillaries, thereby promoting the movement of fluid across the capillary bed into the laminar interstitium, resulting in lamina edema. Interestingly, the degree of venoconstriction was less pronounced in horse receiving BWE, compared with horses receiving CHO, and it has been suggested that perhaps it is the increased venoconstriction that causes more severe laminar damage in these horses. Since it appears that laminar veins are more sensitive
to select vasoconstrictors than arteries, vеноконstrictия может быть ключом в технических изменениях, связанных с развитием ламинит.

Pathophysiology of laminitis – Endocrine and metabolic derangements

Endocrinopathic laminitis is used to describe laminitis that is associated with either obesity, insulin resistance, pituitary dysfunction (PPID), or glucocorticoid administration. Horses that are obese and horses with pituitary dysfunction are commonly diagnosed with insulin resistance. Ponies with laminitis or at risk of developing laminitis have been found to have increased serum insulin concentrations and found to be relatively insulin resistance compared with non-laminitic controls. Explanations on how insulin resistance may contribute to laminar failure include impaired glucose uptake of the laminar epithelial cells, insulin effects on vascular dysfunction, and obesity related inflammation. Administration of high concentrations of insulin intravenously has been used experimentally to induce laminitis in both ponies and horses; however, glucose was controlled in this study and remained at normal physiological concentrations. The lack of GLUT-4 in laminar tissue and insulin-independent glucose uptake by laminar explants suggest that insulin itself and not glucose intolerance may be primarily involved in the development of laminitis in insulin-resistant horses.

Insulin resistance may also predispose horses to laminitis by its effects on the endothelium and digital vasculature. Insulin receptors are present on the endothelial cells of horses, and in other species insulin has been shown to stimulate both vasodilatory (through nitric oxide and PI-3K) and vasoconstrictive (through endothelin-1 and MAPK)
pathways. Stimulation of the MAPK pathway and inhibition of PI-K3 by insulin results in an imbalance between nitric oxide and endothelin-1, resulting in vasoconstriction, platelet activation, and leukocyte adhesion all which has been proposed to play a role in laminitis. Hypertension has been documented in laminitis-prone ponies with insulin resistance during the summer when they are at an increase risk to develop laminitis while grazing on rich pasture.

Although obesity may be a risk factor for laminitis, obesity itself is not a cause of laminitis. It is much more likely that factors associated with obesity, such as insulin resistance and inflammation, play a role in the development of laminitis in these horses. Adipose tissue is now known to be a producer of inflammatory mediators, including leptin, adiponectin, TNFα, IL-1β, IL-6, monocytes chemoattractant protein 1 (MCP-1) and plasminogen activator-inhibitor-1, which can act both locally and remotely throughout the body. In addition to inflammatory mediators produced by adipose tissue, increases in circulating free fatty acids can also contribute to insulin resistance and are known to be involved in proinflammatory signaling through toll-like receptors (TLR4). Increased TNFα and IL-1β mRNA concentrations have been reported in an obese cohort of horses and found to be a risk factor for insulin resistance. Another report in laminitic prone ponies found increases in serum TNFα concentrations and suggested that inflammation but not oxidative stress may play a role in the development of laminitis in these ponies. Increased mRNA concentrations of the proinflammatory cytokines IL-1β and IL-6 have also been reported in the nuchal crest of horses compared with other fat depots in the horse. The nuchal crest of obese horses can become particularly thickened and this
phenotype is considered by veterinarians to increases risk of a horse developing laminitis and/or having insulin resistance; however, no difference in proinflammatory cytokine gene expression was found between horses with insulin resistance and those that were insulin sensitive, still posing the question can adipose production of inflammatory mediators cause insulin induced laminitis.$^{90}$

**Pathophysiology of laminitis – Mechanical**

Laminitis resulting from a severe non-weight bearing lameness in the contralateral limb is an example of mechanical overload and is suspected to result from an alteration in the biomechanical forces of the normal digit.$^{33,91,92}$ Biomechanical forces acting on the normal equine digit include the tensile forces of the lamellar attachments between the P3 and hoof wall, the compressive forces of the downward vertical load through the bony column of the limb, the tensile forces from the proximal palmar traction of the deep digital flexor tendon (DDFT) on P3, the tensile forces of the proximal dorsal pull of the common digital extensor tendon, and the compressive forces generated against the digital cushion/sole (Figure 1.5).$^{9,92,93}$ When these forces are in balance the horse is able to support its weight on four small feet; however, when damage to the lamellar attachment occurs an imbalance of forces results and the downward vertical load and palmar traction of the DDFT allows for P3 to pull away from the hoof wall.$^{9}$ Weight of the horse therefore obviously plays a role in the development of laminitis.$^{33,94,95}$ It has been reported that horses bear 58% of their body weight in the forelimbs and 42% of their body weight on the hindlimbs.$^{29}$ This increase in weight bearing of the front limbs is the speculated reason for the increased incidence of laminitis occurring in the front feet vs.
the hind feet. Furthermore, decreased weight on one limb due to an injury or painful condition would obviously result in a shift in body weight to the opposite limb. Prolonged weight bearing shifted to the contralateral limb in these conditions will often result in severe laminar breakdown that is often life-threatening. Prevention of contralateral limb laminitis is therefore aimed towards attempting to neutralize forces on the digit and includes placing frog support and/or bedding the horse on deep soft footing to minimize compressive forces on the digital cushion and decrease the tensile forces of the DDFT, placement of a cast or putting the horse in a sling to decrease weight bearing loads through the digit, and placement of a heel wedge to decrease tensile forces of the DDFT.

**Experimental models of laminitis**

Experimental models to induce laminitis in horses are used to study the developmental stages of this disease and include nasogastric administration of either a carbohydrate overload or an extract from the heartwood of the black walnut tree. The classical carbohydrate overload model, also known as the starch induction model, has been well described and involves the administration of 85% corn starch and 15% wood flour at 17.6 g/kg of bodyweight. This model closely resembles grain overload in the horse. Microbial changes within the cecum occurs after administration of CHO and results in numerous alterations within the cecum, including a decrease in pH, decrease in gram negative bacteria, increase in endotoxin production, increase in gram positive bacteria, significant mucosal disruption, and an increase in paracellular permeability. This cecal disruption causes systemic alterations, such as an increase in
plasma endotoxin concentrations, a metabolic acidosis secondary to increased serum lactate, a decreased plasma volume due to fluid shifts into the cecum, and cardiovascular effects that vary with the stage of development.

In this starch model, the developmental phase is typically characterized by hypotension evidenced by a drop in central venous pressure 12 to 16 hours post administration followed by hypertension and the onset of clinical lameness (Obel Grade 1) occurring between 24-40 hours after CHO administration, progressing to severe lameness (Obel Grade 3) between 40-72 hours. In addition, fever, diarrhea, colic, dehydration (evidence by increases in packed cell volume and total protein), lactic acidosis and leukocytosis have been reported to occur to varying degrees and frequency.

A more recent carbohydrate model has been developed to mimic laminitis resulting from ingestion of lush pasture. A link between laminitis and the nonstructural storage carbohydrate, fructan, has been suggested by some researchers. This model consists of the administration of oligofructose (OF), in which Obel Grade 1 and 3 lamenesses are detected at approximately 24-36 hours and 48 hours, respectively. Oligofructose is extracted commercially from the roots of chicory and in this model is administered to horses at 10 g/kg of body weight dissolved in 4 liters of tap water via nasogastic intubation. A low-dose OF model has also been used where horses receive OF at only 5 g/kg of body weight. Both dosages can result in horses developing laminitis; however, with the low dose model there is an increase in the number of horses that do not develop laminitis. A combined endotoxin and low dose OF model has also been reported where horses administered endotoxin did not develop laminitis, 2/8
horses administered OF developed laminitis, and 5/8 horse administered endotoxin followed by OF developed laminitis.\textsuperscript{112} An initial osmotic compartmental fluid shift occurs in horses administered OF, causing mild hemoconcentration, hyperproteinaemia, hypernatraemia and hyperchloraemia.\textsuperscript{110} These biochemical changes were further altered with the onset of profuse watery diarrhea, resulting in electrolyte and fluid losses typical of moderate to severe colitis.\textsuperscript{110} Diarrhea is less severe with lower doses and even with the 10 g/kg dose it typically resolves by 24-32 hours after OF administration.\textsuperscript{110} Like the starch model, administration of OF results in endotoxin production, decrease in cecal gram negative bacteria, increase in cecal gram positive bacteria, and the development of lactic acidosis.\textsuperscript{67, 110, 113} In addition, there is evidence of laminar destruction documented by histopathology and/or radiography in both starch and oligofructose CHO overload models.\textsuperscript{110, 114, 115}

The black walnut extract (BWE) model was developed from the clinical observation that horses bedded and believed to have ingested shavings from the black walnut tree commonly develop laminitis.\textsuperscript{116, 117} The BWE model involves soaking 2 g/kg of body weight of black walnut shavings from the heartwood of a tree harvested in the fall of the year into 7 to 8 liters of water for 12 hours followed by filtering the aqueous solution and administering it via nasogastric tube.\textsuperscript{118} It is expected with administration of the BWE, 80\% of horses will respond by developing clinical signs of lameness within 10-12 hours.\textsuperscript{38, 40, 73, 118} In addition, a developmental stage, in which leukopenia occurs, is described to occur at approximately 3 to 4 hours after BWE administration.\textsuperscript{119} The rapid onset of lameness, well defined developmental stage, and milder side effects have made
this model desirable to use in the study of laminitis. However, histological or gross indications of laminar destruction/failure rarely occur in the BWE model, especially compared with the CHO models.\textsuperscript{28, 115}

Insulin resistance occurs in horses with pituitary pars intermedia dysfunction (PPID)\textsuperscript{120}, metabolic syndrome\textsuperscript{84, 121}, and endotoxemia\textsuperscript{122} and an association between insulin resistance and laminitis has been proposed.\textsuperscript{123} Most recently a hyperinsulinaemic, euglycemic clamp model has been developed to mimic laminitis secondary to insulin resistance.\textsuperscript{85, 86} In this model, a constant rate insulin infusion of 6 mIU/kg of body weight is given intravenously for 48 to 72 hours while maintaining blood glucose values at 5±1 mM with a variable rate glucose infusion.\textsuperscript{85, 86, 124} During this model mean serum insulin concentrations were greater than 1000 μIU/ml in both ponies\textsuperscript{85} and horses\textsuperscript{86}; however, horses developed laminitis within 48 hours and ponies within 72 hours. Laminar ultrastructural changes in ponies exposed to prolonged hyperinsulinaemia resulted in decreased number of hemidesmosomes and sporadic separation of epidermal basal cells at the basement membrane with infiltrating leukocytes.\textsuperscript{125} Although the mechanism resulting from insulin administration at these rates are unknown, it has been hypothesized that activation of proinflammatory chemokines and cytokines, alterations of glucose metabolism in the lamina, and disturbances in vascular function may all play a role.\textsuperscript{85, 86, 124}

\textit{Treatment of laminitis}

Developing successful treatments for laminitis has been as elusive as determining the mechanisms of development, and therefore are mostly aimed at prevention with the
aim of limiting or blocking the suspected causes. Preventive therapies include treatment of the primary disease, anti-inflammatories,\textsuperscript{126} analgesics,\textsuperscript{127} anti-thrombotics,\textsuperscript{126, 128, 129} vasodilators,\textsuperscript{78, 130, 131} anti-oxidants,\textsuperscript{132} anti-endotoxic therapies,\textsuperscript{126, 133} cyrotherapy,\textsuperscript{5, 134} and mechanical support.\textsuperscript{135} In an epidemiological study on laminitis the most common therapies used consisted of phenylbutazone (68\% of cases), acepromazine (34\% of cases), dimethyl sulphoxide (DMSO, 27\% of cases), and flunixin meglumine (19\% of cases).\textsuperscript{132} Since laminitis is severely painful for many affected horses, analgesia (including phenylbutazone, flunixin meglumine and intravenous CRI of lidocaine) is considered by many to be one of the most important treatments. Of the various therapies proposed for prevention, only cyrotherapy has been proven experimentally to be successful in preventing or decreasing the severity of laminitis.\textsuperscript{5, 134} With the development of novel anti-inflammatory therapy\textsuperscript{136} and the evidence of inflammation being present early in the developmental stages\textsuperscript{36, 38, 39, 44, 47, 48, 61} further research evaluating these therapies may lead to better therapeutics in the future.

**SEPSIS**

*Sepsis and SIRS in humans*

In 1995, there were approximately 750,000 cases of sepsis in the US and with an increase of approximately 1\% each year it was suspected that by 2020 there would be an estimated 1 million cases per year.\textsuperscript{137} Sepsis is involved in approximately 2\% of all hospitalizations and although mortality rates have decreased over the years, the total number of cases has increased, resulting in an increased number of sepsis-related deaths.
with an estimated 9.3% of all deaths in the US related to sepsis.\textsuperscript{138} Care for patients with sepsis requires long hospitalization stays in ICU and/or in intermediate care and costs are estimated to exceed $16 billion per year in the US.\textsuperscript{138} Therefore, determining the causes and risk factors of sepsis, developing an understanding of the pathophysiology of sepsis, and critically evaluating possible therapeutic measures for sepsis has become a priority in critical care medicine. In order to help guide physicians in diagnosing and treating sepsis and help standardize research protocols for improved understanding, the American College of Chest Physicians and Society of Critical Care Medicine formed a consensus on the definitions of the systemic inflammatory response syndrome (SIRS), sepsis, severe sepsis and septic shock (Table 1.1).\textsuperscript{138, 139}

Systemic inflammatory response syndrome (SIRS) is the current phrase used to define the inflammatory response independent of its cause. While SIRS can occur secondary to infection (which is then termed as sepsis), it can also occur from other insults such as severe tissue trauma, hemorrhagic shock, tissue ischemia, burns, pancreatitis, and immune-mediated organ injury.\textsuperscript{139} Sepsis includes the conditions that can occur with SIRS, however the initiating cause is either documented or suspected to be due to an infection within the patient.\textsuperscript{139} The most common sites for infection resulting in sepsis have been associated with either the respiratory tract or within the abdomen.\textsuperscript{137} Although gram negative infections once predominated, gram positive infections are now more common with polymicrobial conditions also occurring.\textsuperscript{140} Fungal infections as a cause of sepsis have also been increasing and are estimated to be
the cause in up to 15% of cases. However, in some cases no causative organism can be found. These cases still may be truly septic or may simply suffer from SIRS.

A sequela to SIRS and/or sepsis is the development of organ dysfunction. Multiple organ dysfunction syndrome (MODS) is defined to occur when there is the presence of altered organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention. MODS has been further categorized into primary and secondary MODS where primary involve the organ(s) of initial insult that then result in SIRS whereas secondary MODS is a result of SIRS and the host response to the overwhelming inflammatory response. Typical organs involved in MODS include, but are not limited to the lungs, kidney, and liver. Involvement of these organs represents severe illness, significantly complicating treatment and decreasing prognosis of the patient.

**Pathogenesis of sepsis**

The pathogenesis of sepsis is complex and incompletely understood. Dysregulation of the inflammatory response, dysregulated coagulation, increase in cellular death, and energy loss through mitochondrial dysfunction have all been proposed to play a role in sepsis. Which one predominates may depend on the initiating cause. Regardless, current treatment and preventions are aimed at these theories.

A prevailing theory is that sepsis is a direct result of an uncontrolled inflammatory response and it is from that theory that the term SIRS was derived, as it appears that it is not simply the organism causing damage but the host response to the presence of an organism or direct tissue injury. This theory has come from many
studies involving animal models of sepsis, including endotoxin administration and the well known cecal ligation and puncture (CLP) model in rodents. A predominate finding in these studies was that increases in circulating proinflammatory cytokines were present, particularly TNFα, IL-1β, and IL-6. The presence of a pathogen (bacteria or tissue debris) will trigger the resident inflammatory cells, such as macrophages, at the local site to phagocytosis and produce proinflammatory cytokines. These proinflammatory cytokines can then result in production of adhesion molecules to recruit more inflammatory cells into the environment. Thus, it is clear to see how this response could become out of control and result in an overproduction of inflammatory mediators that can result in significant tissue damage. However, despite the increased production of proinflammatory cytokines, such as TNFα and IL-1β, in sepsis the use of antibodies to block their receptors has not improved outcome in septic patients. Leukocyte migration into tissues during sepsis/SIRS is known to occur throughout multiple organs and is suspected to play a role in the development of organ failure. Although neutrophils are essential for the eradication of pathogens and injured cells, release of reactive oxygen species and protease can damage surrounding tissue.

In attempts to limit this overwhelming inflammatory response, a compensatory anti-inflammatory response occurs and the term CARS has been coined to describe the overcorrection/correction of the inflammatory response. It has been proposed that this anti-inflammatory response modifies or adapts the immune status of the patient. Classical mediators involved in CARS include IL-10, TGFβ, PGE2, heat shock molecules, and glucocorticoids. The production and secretion of these mediators result in
inhibition of cytokine, chemokine and free radical production, macrophage cytotoxic activity, LPS-induced tissue factor expression and procoagulant activity, and monocyte adhesion to endothelial cells.\textsuperscript{148, 149} Despite the ability for the anti-inflammatory mediator to resolve SIRS, it has been suggested by some that the CARS response may enhance the patients susceptibility to nosocomial infections.\textsuperscript{148, 150, 151} Apoptosis of CD4 T-cells, B-cell and dendritic cells and anergy within the immune system may play a role.\textsuperscript{141} have also been reported. The two-hit or polymicrobial models of sepsis using CLP followed by peritoneal administration of bacteria 48 hours later demonstrated severe impairment in the ability to clear the infection and absence of an early inflammatory response at the site.\textsuperscript{152}

Cytokine production due to sepsis/SIRS can also have a procoagulant effect on patients. It is estimated that up to 30-50\% of patients develop disseminated intravascular coagulation.\textsuperscript{151} One key component activating this cascade is tissue factor (TF) production from endothelial and mononuclear cells. This cascade results in the conversion of prothrombin to thrombin followed by the generation of fibrin from fibrinogen. Breakdown of fibrin by plasmin is impaired due to high concentrations of plasmingen-activator inhibitor-1, resulting in the distribution of fibrin clots in small vessels and impaired blood flow to tissues and potential organ damage.\textsuperscript{151}

Cellular hibernation, cytopathic hypoxia and mitochondrial dysfunction are terms used to describe a condition where oxidative phosphorylation or impaired ATP production results in cellular damage and may play a role in the development of organ damage during sepsis/SIRS.\textsuperscript{141, 153} Decreased oxygen consumption due to a depletion of
nicotinamide adenine dinucleotide has been observed in enterocytes\textsuperscript{141} and myocardial damage during sepsis where coronary blood flow and oxygen tensions are normal\textsuperscript{153} suggest that cellular hypoxia can occur without changes to oxygen delivery to tissues. Further evaluation into mitochondrial function and ATP-ADP production is needed to determine what role if any these mechanisms play in the development of organ failure.

**INFLAMMATORY SIGNALING PATHWAYS**

**Toll-like receptor (TLRs) signaling**

Cells of the innate immune system as well as epithelial cells possess pattern recognition receptors (PRRs) which bind conserved molecular structures of pathogens known as pathogen-associated molecular patterns (PAMPs). Of these PRRs, the best known and most important are the toll-like receptors (TLRs).\textsuperscript{154} The first TLR was identified in the *Drosophila* and was implicated in patterning during development.\textsuperscript{155} It was noted that the intracellular domain had close similarities to the intracellular domain of the mammalian interleukin-1 receptor.\textsuperscript{156} Further studies demonstrated that this TLR played an essential role in LPS recognition and response in mammals.\textsuperscript{157}

To date, there are 10 TLRs known in mammals.\textsuperscript{154, 158} Each TLR recognizes different pathogens based off their PAMS; however, some TLRs can recognize multiple different types of pathogens and can respond to many structurally unrelated ligands, which may be derived from different groups of pathogens.\textsuperscript{154} For example, both TLR2 and TLR4 can recognize gram positive organisms.\textsuperscript{154} Classically, TLR2 forms heterodimers with either TLR1 or TLR6 to recognize lipoproteins and gram positive
lipoteichoic acid, TLR3 recognizes double stranded viral RNA, TLR4 recognizes gram negative lipopolysaccharide (LPS) and respiratory syncytial virus fusion protein, TLR5 recognizes bacterial flagellin, TLR7 recognizes synthetic imidazoquinolines (anti-viral compounds), and TLR9 recognizes bacterial unmethylated CpG DNA (Figure 1.6). As of yet, there is no known ligand for TLR8 and 10.

The first and probably best described TLR is TLR4. The discovery of this TLR helped to describe how mammals respond to LPS, as previous explanations relied on interaction with CD14, which had no cytoplasmic signaling domain. TLR4, like all TLRs have 2 domains, an extracellular domain which has leucine rich repeats that composes the variable portion of the receptor allowing for recognition of PAMPs, and the conserved intracellular/cytoplasmic domain which is known as the TIR domain (toll-like/interleukin-1 receptor domain). The exact method of how TLR4 binds LPS is unknown; however, several associated binding factors are necessary as without them TLR4 cannot directly bind LPS. Initial exposure to LPS, which is a component of the outer cell wall membrane of gram negative bacteria, results in binding of lipid A portion of LPS molecule to a lipopolysaccharide binding protein (LBP). This facilitates binding to the TLR4 receptor when LBP-LPS complex interacts with CD14, which in turn interacts with TLR4 and the associated protein MD-2. This all occurs on the extracellular surface and it is believed that MD-2 may actually be the binding site for the LPS molecule, but must interact with TLR4 to transduce its signal in the cell. Once LPS is bound to the LBP-CD14-MD2-TLR4 complex several different signal cascades can occur, including MyD88 dependent pathway (Figure 1.7) and the TRIF dependent
With the MyD88 pathway, ligand binding results in MyD88 and its associated adaptor-like protein MAL recruitment to the TIR domain of TLR4. This is followed by IRAK-4 and IRAK-1 phosphorylation which results in TRAF-6 recruitment. TRAF-6 subsequently activates TAK and associated proteins TAP1-3. Together this activates IκK, a kinase that phosphorylates IκB, allowing for NFκB (typically p50 and p65 components) to become activated and translocated to the nucleus where they bind to the cis-promotor region and stimulate transcription of over 40 different proinflammatory genes. TAK can also activate JNK which will result in AP-1 migrating to nucleus and as a transcription factor stimulate proinflammatory gene transcription.

The TRIF dependent pathway uses different adaptor proteins once the LPS-CD14-MD2-TLR4 complex is formed. Instead of MyD88 and MAL, TRIF and TRAM bind to the TIR domain of TLR4. This results in a signaling cascade that can still result in NFκB activation through IκK inhibition removal. Additionally, activation of IRF-3 and IRF-7 can be triggered, which will result in increased gene production of IFNβ and IFNα.

Many of the proinflammatory genes that are up-regulated help the host defend against and limit infection. NFκB will result in the production of the proinflammatory cytokines such as TNFα, IL-1β, IL-6 and CXCL-8. CXCL-8 along with other chemokines, such as CXCL1, are chemotaxic to leukocytes and will recruit them towards the epithelial cells to help remove the microorganisms. TNFα and IL-1β will help activate macrophages to promote the production of ROS within leukocytes to destroy phagocytosis organisms. Adhesion molecules such as ICAM-1 and E-selectin are also
upregulated through NFκB and these molecules allow for the attachment and subsequent diapdesis of leukocytes through the endothelial cell barrier into the tissues. All of these responses and more result from the antigen binding of LPS to the TLR4 receptor and are used by the host to destroy microorganisms and limit infection.

**Signal transducing activators of transcription factors (STATs) signaling**

The signal transducing activators of transcription factors (STATs) are latent intracytoplasmic proteins that once activated via phosphorylation are translocated to the nucleus and serve as transcription factors that result in the production of various inflammatory genes as well as those regulating cell growth and development. Seven different STATs are known including STAT 1, 2, 3, 4, 5a, 5b, and 6. STAT 1 is inducible by type I and II interferons (IFNs) and is involved in the innate immune response as well as inhibiting growth, suppressing tumor formation, and promoting apoptosis. STAT2 can form heterodimers with STAT1 when induced by interferons. STAT3 is activated by cytokines (most commonly IL-6 and IL-10) and growth factors (such as epidermal growth factor (EGF)) and results in a wide range of functions including cell growth and proliferation, the prevention of apoptosis, and production of cytokines. STAT4 is stimulated by IL-12 and is involved in the development of a Th1 response, whereas STAT6 is stimulated by IL-4 and is involved in the development of a Th2 response. STAT5a and b are involved in prolactin and growth hormone signaling and hematopoietic development, including erythropoiesis, granulopoiesis and lymphopoiesis. Although they vary in their functions, the STATs share a fairly well-conserved structure consisting of a series of six domains, including a N-terminal
region that stabilizes STAT dimers, a coiled coil region, a DNA binding domain, a linker
domain, Src-homology 2 domain (SH2) domain which is responsible for receptor
binding, and a C-terminal transcriptional activation (TAD) domain which also serves as
the location of tyrosine and serine phosphorylation. Of these six domains, the N-
terminal domain is the least conserved among the STATs, suggesting that this part of the
protein mediates the cellular response.

Phosphorylation or activation of STATs can occur by several different pathways.
The most studied and first described pathway is JAK-STAT pathway activated by
cytokine binding to a cell surface receptor. Transmembrane cytokine (such as IFN or
IL-6) receptor dimerization and ligand binding allows for two non-covalently bound
Janus kinase (JAK) proteins to be apposed, thereby allowing subsequent phosphorylation
of the JAK protein. JAK phosphorylation on tyrosine residues leads to release of the
JAK catalytic site which allows for binding of the SH2 domain of the STAT protein.
STATs are then phosphorylated at a tyrosine residue where they then form dimers
(Figure 1.8) and are translocated to the nucleus through associations with importins to
serve as transcription factors. STATs appear to constantly cycle between
cytoplasm and nucleus and stimulation of the STAT pathway via cytokines appear
to have a stronger effect on nuclear retention then their effect on nuclear import.

Besides activation by cytokines through cell surface receptors, JAKs can also be
activated by ligands that bind to G-protein coupled receptors (which include the CCL and
CXCL chemokines receptors) and subsequently results in STAT tyrosine
phosphorylation. STATs can also be phosphorylated without JAK activation either
directly by growth factor binding (such as epidermal or platelet derived growth factors) and activation via receptor tyrosine kinases or through non-receptor onogentic tyrosine kinases which are often from viral origin.\textsuperscript{169}

In addition to tyrosine phosphorylation, serine phosphorylation of STAT1, 3, 4 and 5a/b has also been reported to occur.\textsuperscript{170} Initially it was reported that serine phosphorylation was necessary for full transcriptional activation,\textsuperscript{171,172} and although it is still believed that full activation of STAT1 requires both tyrosine and serine phosphorylation, recent reports suggest that requirement of serine phosphorylation varies depending on type of cell and source of extracellular stimulation.\textsuperscript{173} For example, serine 727 STAT1 phosphorylation has been reported to occur secondary to cellular stress (from UV radiation) and inflammatory signals (LPS and TNF\textalpha) through p38MAPK activation,\textsuperscript{170,174} therefore, it is possible to have serine STAT1 phosphorylation but not tyrosine STAT1 phosphorylation. Effect of STAT3 serine phosphorylation are much more variable where in some cases serine phosphorylation may inhibit tyrosine phosphorylation instead of enhancing it.\textsuperscript{170} For example, basal levels of serine phosphorylation of STAT3 in the absence of tyrosine phosphorylation is essential for osteoclastogenesis and when increases in STAT3 tyrosine705 phosphorylation occurs macrophages will be formed instead of osteoclasts.\textsuperscript{175}

Inactivation of STAT occurs through the production of inhibitors directed toward various steps in the pathway, through the production of phosphatases, or by protein degradation via the ubiquitin-proteosome pathway.\textsuperscript{161,176} Multiple inhibitors are known, including suppressor of cytokine signaling (SOCS), cytokine-inducible SH2-containing
protein (CIS), and protein inhibitor of activated STAT (PIAS). SOCS1 and SOCS3 are known to play important roles in controlling the inflammatory response secondary to ischemia-reperfusion injury, wound healing, and endotoxic shock.

STAT1 and STAT3, despite being very similar in structure, have reportedly many opposing actions of each other. STAT1 activation promotes apoptosis and cell cycle arrest while STAT3 activation inhibits apoptosis, and stimulates cell growth and migration. In addition, it has been reported that STAT1 activation results in a proinflammatory response, while STAT3 activation is more anti-inflammatory. However, as with many signaling mediators these facts are not so black and white. STAT3 in particular, appears to have both pro- and anti-inflammatory effects secondary to either tyrosine or serine phosphorylation alone or in combination.

Since a significant inflammatory response resulting from cascade of cytokine and chemokine production occurs in patients with sepsis/SIRS is it not unexpected that both STAT1 and STAT3 would have important cell signaling roles in these conditions. While STAT1 activation via IFNγ results in activation of macrophages and enhanced killing of bacteria, it also can result in the production of deleterious inflammatory mediators such as TNFα or HMGB1, which may increase morbidity and mortality in septic patients. STAT3 also appears to have conflicting roles in sepsis/SIRS with some reports stating a protective effect of STAT3 activation and others reports suggesting that STAT3 may support continued inflammation in these patients. Substantial increases in multiple cytokines (IL-1β, TNFα, IL-6, IL-12 and IFNγ) lead to uncontrolled local and systemic inflammation and increased lethality in a group of conditional STAT3 knockout mice (no
STAT3 in neutrophils or macrophages) with septic peritonitis secondary to CLP when compared with the wild type. Neutrophil clearance in mice has also been reported to be increased with hyperactivation of STAT3 activation through the gp130 receptor in combination with a decreased production in CXCL1 and no change in neutrophil apoptosis. However, tyrosine phosphorylation of STAT3 secondary to MCP-1 induced macrophage activation and STAT3 tyrosine phosphorylation is critical for IL-1β and IL-6 production in response to LPS and live bacteria. Inhibitors of STAT3 also protected mice against CLP induced multiple organ damage and death when compared with an untreated group.

EPITHELIAL CELL FUNCTION AND STRUCTURE IN DISEASE

Keratinocytes and the innate immune system

The keratinocyte, an epidermal epithelial cell, is known to be an important part of the innate immune system, not only in barrier function but also in its ability to respond to bacterial products through toll-like receptors and subsequent production of inflammatory mediators. Keratinocytes have been reported to produce a wide range of chemokines which will in turn result in the stimulation of leukocyte migration. RANTES (CCL5), MCP-1 (CCL2), interferon gamma induced protein of 10 kd (IP-10;CXCL10), CXCL8, and Gro-α (CXCL1) have all been reported to be produced by keratinocytes and are often found to be up-regulated during inflammatory disease states such as psoriasis and atopic dermitis. In addition, chemokines receptors, such as CCR3 the receptor for RANTES, have also been found to be up-regulated in
keratinocytes in culture suggesting that these cells can not only produce chemokines but respond to them as well.  

Keratinocytes are also known to produce proinflammatory cytokines, including TNFα, IL-1β, IL-6, IL-10, IL-12, IL-15, IL-19, and IL-20, and increased expression has been documented in many skin-related disease states, such as psoriasis. Some of these cytokines, like IL-20 and IL-19, are reported to have a role in proliferation and differentiation resulting in hyperkeratosis in patients with psoriasis. Keratinocytes in culture are known to produce proinflammatory cytokines, including TNFα, IL-1β, IL-6, IL-12, and IL-15. Human keratinocytes do not produce TNFR2 (binds more to membrane bound TNFα - no death domain), indicating that the keratinocyte response to TNFα is mediated through TNFR1 (binds more to soluble TNFα - death domain and NF-KB). IL-20 and IL-19, members of the Il-10 family, have increased expression in keratinocytes in disease states such as psoriasis and are suspected to have a role in proliferation and differentiation resulting in hyperkeratosis with this disease. IL-18 has also been described to be increased in inflammatory skin diseases such as psoriasis and atopic dermatitis. IL-18 receptor alpha has also been reported to be expressed in keratinocytes and upregulation occurs with IFNγ alone and IFNγ - TNF combined. Other receptors reported to be on keratinocytes are IL-4Rα and IL-13R1α and compared with normal skin, increased expression of IL-4Rα have been reported in psoriasis and atopic dermatitis. IL-13R1α localization occurs in all layers with psoriasis and in upper layer of epidermis with atopic dermatitis compared to normal skin where localization of the receptor is typically in the lower layers of keratinocytes only.
IL-12 production by keratinocytes have been reported with allergic contact dermatitis and is suggested to stimulate IFNγ production by keratinocytes to further promote a TH1 response in this disease.\textsuperscript{190}

Complement is an important component of the innate immune system and keratinocytes have the ability to produce certain elements of the complement cascade including constitutive secretion of C3, factor B, factor I, and factor H.\textsuperscript{191-194} Increased C3 secretion has also been reported after stimulation with proinflammatory cytokines and expression of the C3a receptor has been detected on human keratinocytes with IFNγ reported to strongly up-regulate this receptor.\textsuperscript{195} Furthermore, the up-regulation of C3aR secondary to IFNγ stimulation resulted in induction of CCL2, CCL5, CXCL8, CXCL10 and C3 expression in keratinocytes.\textsuperscript{195} Human keratinocytes have also been found to constitutively express the terminal complement components C5, C7, C8γ and C9; however, only C7 and C9 were found to be released by the keratinocyte, and the release of C9 was found to be induced by TNFα in a dose dependent manner.\textsuperscript{196}

Due to the importance of the inflammatory response of the keratinocyte to bacterial toxins both in physiologic and pathologic skin conditions, numerous studies have been performed on the response of human keratinocytes to different bacterial components. Researchers have characterized the response of the human keratinocyte to multiple PAMPs including lipopolysaccharide, lipoteichoic acid, peptidoglycan, flagellin, and bacterial DNA.\textsuperscript{197-200} These PAMPs are now known to be recognized by different PRRs including TLRs and nucleotide-binding oligomerization domain (NOD) receptors. TLR2 has been widely described as a PRR for proteins from gram positive bacteria.
including lipoproteins and lipoteichoic acid. Although PGN was originally reported to also induce signaling via binding to TLR2, recent research has indicated that this toxin mainly binds to (and induces inflammatory signaling through) the NOD proteins in the cytoplasm. TLR4, the first-discovered and best-described TLR, is the primary PRR for LPS from gram-negative bacteria. Two additional TLRs reported to be present in the human epithelium but have received less attention are TLR5, which recognizes flagellin from gram positive and negative organisms, and TLR9, which recognizes bacterial DNA (unmethylated CpG DNA) from diverse bacterial organisms.

Keratinocytes are known to express and respond to several antimicrobial proteins and damage associated proteins which can serve to protect against pathogens (antimicrobial proteins), stimulate proliferation and differentiation of keratinocytes, stimulate leukocyte migration, and stimulate production of cytokines and chemokines. Human beta-defensin 2, 3 and 4 has been reported to increase gene expression and protein production of IL-6, IL-10, IP-10, MCP-1, MIP-3α and RANTES. Furthermore the human beta-defensins induced phosphorylation of EGFR, STAT1 and STAT3, all which play an important role in keratinocyte migration and proliferation. Calprotectin, a member of the S100 antimicrobial proteins, has been found in equine laminar epithelium as well as human skin keratinocytes with inflammatory disorders. Production of calprotectin in human keratinocytes results from stimulation by proinflammatory cytokines and can result in proliferation and migration of keratinocytes and as a chemotaxic agent for neutrophils. Secretory leukocyte protease inhibitor (SLPI), a small cationic protein, not only serves to inhibit proteinases from white cells,
but also has antibacterial and anti-fungal properties. Production of SLPI has been found to be increased in keratinocytes after cutaneous injury.\textsuperscript{209} In addition to anti-protease activity, SLPI has been found to inhibit NF-κB production in the lung\textsuperscript{210} as well as TLR2 and TLR4 receptor response in monocytes to LTA and LPS, respectively.\textsuperscript{211} The endogenous molecule HMGB1 (high mobility group box protein 1) known as a DAMP protein, can be released actively or passively by necrosis from epithelial cells in response to both infectious and non-infectious inflammation (such as with ischemia-reperfusion, autoimmunity, and trauma).\textsuperscript{212} HMGB1 results in increased production of other cytokines, activates adhesion and migratory functions of leukocytes, and stimulates production of reactive oxygen species by neutrophils.\textsuperscript{212} It can also promote tissue repair and regeneration, and in a diabetic wound mouse model, HMGB1 accelerates healing compared with inhibition of this compound.\textsuperscript{212}

**Keratinocytes, epidermal growth factor and inflammation**

Epidermal growth factor receptor, which is a predominate receptor on keratinocytes and has been found to be up-regulated in plasma membrane laminar preparation from horses with chronic laminitis, has important roles in controlling both inflammation and epithelial proliferation and migration.\textsuperscript{213, 214} Cytokines can stimulate production of EGFR ligands by keratinocytes which upon activation of their receptors can result in up-regulation or down-regulation of various factors involved in immune/inflammatory disorders. Up-regulation of antimicrobial proteins (such as HBD2, HBD3, and SLPI), toll-like receptors (TLR5 and 9) and IL-8 have all been reported upon EGFR activation\textsuperscript{213} and tend to be increased in chronic inflammatory
conditions of the skin. However, down-regulation of many chemokines (including RANTES, MCP-1, and IP-10) and cytokines (TNFα, IFNγ, and ILβ) is also observed after stimulation of the EGFR and blockade of this receptor will result in worsening of inflammatory conditions with an increased influx of neutrophils and production of proinflammatory cytokines. Activation of the EGFR is also important in keratinocyte survival and migration. The EGFR is closely associated with the hemidesmosome component α6β4 integrin and phosphorylation of EGFR is required for normal disassembly and keratinocyte migration.

**Epithelial cells and sepsis/SIRS**

The epithelial cell is known to play an important role in sepsis/SIRS and MODS, particularly involving the lung, gut and kidney. These conditions are associated with an overwhelming production of numerous cytokines and chemokines, many of which can come from the epithelial cell itself. They are able to respond to microbial products via toll-like receptors and with the production of antimicrobial proteins. It has been suggested that stimulation of TLR4 in the renal tubular epithelial cell by endotoxin can result in a spectrum of injury including mild tubular swelling and vacuolization to severe inflammation, oxidative stress and widespread apoptosis. TLR4 has also been reported to be up-regulated after ischemia-reperfusion injury in the kidney and TLR4 deficiency blunted the chemokine/cytokine response by tubular epithelial cells inhibiting macrophage and neutrophil accumulation. Complement receptor C5a has been found on renal tubular epithelial cells and alveolar lung epithelial cells and activation of this receptor is known to enhance the production of the CXC chemokines and
proinflammatory cytokines. C5aR antagonists were able to abolish the up-regulation of chemokines and attenuate neutrophilic inflammation in a model of acute kidney disease.

*Epithelial cells, sepsis/SIRS and structural failure*

Cell structure components such as E-cadherins, actin cytoskeletal filaments, tight junctions, gap junctions and hemidesmosomes also can be affected by the inflammation and injury associated with sepsis and may perhaps lead to further dysfunction and subsequent failure of these epithelial organs. E-cadherins are localized just beneath the tight junctions. Reactive oxygen species, such as hydrogen peroxide, will induce E-cadherin redistribution resulting in disruption of tight junctions and increased paracellular permeability. This disruption of tight junctions from ROS has also been reported to occur through actin cytoskeleton rearrangement which is regulated by Rho and Rac1 GTPase proteins. Hypoxia in renal epithelial cells has been reported to result in actin cytoskeleton rearrangement altering the interactions between integrins and adhesion molecules, leading to disruption of tubular integrity, epithelial sloughing and breakdown of the basement membrane. Coxxin 43, a channel component of the gap junction, has been reported to be up-regulated in the dermis and lung after acute injury. Up-regulation increases the number of channels that may be allowed to open to the extracellular space resulting in edema, the spread of inflammatory mediators and subsequent cell injury. The principle component of the hemidesmosome that allows for epithelial cell attachment to the basement membrane is the α6β4 integrin heterodimer which serves as transmembrane receptor for the extracellular molecule laminin-5.
Reports of decreased α6β4 integrin and poor preservation of laminin-5 in the basement membrane has been observed in chronic inflammatory conditions of the periodontal epithelium and may play an important role in the development of laminitis as this is the location of ultimate failure between the epidermis and dermis.

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Figure 1.1. Dermal lamellae covering the third phalanx and epidermal lamellae on the internal side of the hoof (stratum internum). These “layers of leaves” interdigitate with each other to increase the surface area and increase the strength within the hoof.
Figure 1.2. Histology of lamina. Cross section of lamina at 10X stained with cresyl violet. PEL=primary epidermal lamina (with dark keratinized center), PDL=primary dermal lamina, DDL=deep dermal lamina/corium which attaches to the third phalanx. Insert demonstrates the SEL=secondary epidermal lamina which contain the LBECs (laminar basal epithelial cells) and the suprabasal epithelial cells (not labeled) and the SDL=secondary dermal lamina.
Figure 1.3. Structure of type I hemidesmosome
Figure 1.4. Lateral radiographs of the equine distal limb from a normal horse and a horse with laminitis. Note the red lines on the normal radiograph (left) are parallel to each other whereas there is evidence of rotation of the third phalanx (P3) in the laminitic foot (right) as the tip of P3 is pointing down and the dorsal aspect of P3 is not parallel to the hoof wall.
Figure 1.5. Biomechanical forces acting on the static weight bearing digit.

W=weight bearing forces through the center of the hoof. G=compressive ground reaction forces in response to weight bearing. L=tensile forces acting across the digital lamina.

T=tensile forces from the pull of the deep digital flexor tendon.
Table 1.1 Definitions of sepsis and SIRS

<table>
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<th>Syndrome</th>
<th>Definition</th>
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| Systemic inflammatory response syndrome (SIRS) | 2 or more of the following  
|                                 | Temperature >100.4°F or <96.8°F  
|                                 | Pulse >90 beats per minute  
|                                 | Respiratory rate >20 breaths per minute  
|                                 | or PaCO₂ <32mmHg  
|                                 | White blood cells >12,000/mm³ or  
|                                 | <4000/mm³ or >10% immature bands |
| Sepsis                          | SIRS due to suspected or confirmed infection                             |
| Severe sepsis                   | Sepsis associated with organ dysfunction, hypoperfusion or hypotension   |
| Septic shock                    | Sepsis induce hypotension despite adequate fluid resuscitation along with the presence of perfusion abnormalities |

* Table from O’Brien et al, 2007
Figure 1.6. TLRs and their ligands
Figure 1.7. MyD88 dependent pathway signaling of TLR4
Figure 1.8. Tyrosine phosphorylation of STAT proteins
Chapter 2. Laminar Inflammatory Gene Expression in the Carbohydrate Overload Model of Equine Laminitis

ABSTRACT

Objectives: To determine if a similar pattern of laminar inflammation, characterized by proinflammatory mediator expression, occurs in the carbohydrate overload (CHO) model of laminitis as has been previously reported for the black walnut extract (BWE) model.

Methods: Sixteen horses administered 17.6 g of starch (85% corn starch/15% wood flour)/kg body weight via nasogastric tube were anesthetized either after developing a temperature >102°F (DEV group, n=8) or at the onset of Obel grade 1 lameness (OG1 group, n=8). Control horses (CON group, n=8) were anesthetized 24 hours after NG administration of 6 liters of deionized water. Laminar tissue was collected from horses while under anesthesia, followed by humane euthanasia. Real time-quantitative PCR was used to assess laminar mRNA concentrations of genes involved in inflammatory signaling.

Results: Increased mRNA concentrations (P<0.05) for IL-1β, IL-6, IL-12p35, COX-2, E-selectin, and ICAM-1 were present in laminae from horses with OG1 lameness, but not at the DEV time, when compared with the CON horses. No differences between the
groups were found for IL-2, IL-4, IL-10, TNF-α, IFN-γ, or COX-1 at either the DEV or OG1 time points.

**Conclusions:** There is a notable difference in the temporal pattern of inflammatory events between the BWE and CHO models, with the majority of laminar inflammatory events appearing to occur at or near the onset of lameness in the CHO model, whereas many of these events peak earlier in the developmental stages in the BWE model. This suggests that, in addition to circulating inflammatory molecules, there may be a local phenomenon in the CHO model resulting in the simultaneous onset of multiple laminar events including endothelial activation, leukocyte emigration, and proinflammatory cytokine expression.

**INTRODUCTION**

Extensive studies of equine laminitis using the black walnut extract (BWE) model indicate that equine laminitis, a common clinical sequela to many systemic diseases associated with gram-negative sepsis, has similar pathophysiological events as organ injury/failure in human sepsis, including leukocyte extravasation and inflammatory mediator production. Increases in inflammatory gene expression reported with the BWE model include increases in proinflammatory cytokines, chemokines, cyclooxygenase-2, and endothelial adhesion molecules important in leukocyte adhesion and extravasation.¹⁻⁷ In these reports, the laminar inflammatory response was observed early in the course of development, occurring as early as 1.5h post-administration of BWE.⁵ Whereas some of these laminar inflammatory events peak in the early developmental/prodromal period in
the BWE model\textsuperscript{2,5}, others continue to increase through the onset of lameness phase which occurs approximately 10-13 hours post BWE administration.\textsuperscript{1}

The CHO model of equine laminitis appears to more closely mimic clinical cases of laminitis that occur secondary to sepsis-related diseases (i.e. enterocolitis, large intestinal strangulation, acute metritis) than the BWE model due to: 1) the documented presence of endotoxemia, 2) the extended time frame of systemic signs of sepsis prior to onset of lameness, and 3) a similar high incidence of structural failure of the laminae (which rarely occurs in the BWE model).\textsuperscript{8-12} Administration of an overload of CHOs results in a decrease in cecal pH, an increase in lactic acid producing bacteria, death of gram negative bacteria and substantial damage to the cecal mucosal barrier resulting in absorption of bacterial pathogen associated molecular pattern molecules (PAMPs) including endotoxin.\textsuperscript{8,12-15} In the oligofructose model, plasma endotoxin concentrations were found to peak at 8h with increases in plasma TNF-\textgreek{a} concentrations being present between 12 and 24 hours.\textsuperscript{8} The temporal pattern of these events presumably reflects the time necessary for the cecal disruption to result in mucosal barrier dysfunction allowing bacterial products to enter the systemic circulation and cause a systemic inflammatory response that is similar to sepsis in humans.

Experimental models of human sepsis have been used to help develop treatments to improve morbidity and mortality for clinical cases. The most common models used (most performed in laboratory rodents) are the intravenous infusion of endotoxin and the cecal ligation and puncture (CLP) model.\textsuperscript{16,17} Like the experimental models used to induce laminitis, the different models can produce variable results making it difficult to
determine effective treatments for sepsis. Although endotoxin infusion models have yielded valuable information regarding the response of animals/humans to sepsis, the patterns of inflammatory responses do not usually accurately reflect the responses observed in humans with sepsis.\textsuperscript{17} Similar to the BWE model of equine laminitis, LPS administration generally results in a more rapid but more transient cytokine response than observed in clinical sepsis cases, with cytokines peaking between 1.5 to 4 hours and declining by 8 hours post LPS administration.\textsuperscript{16}

The presence of laminar inflammation has been well documented in the early stages of BWE-induced laminitis and has been proposed to play a prominent role in laminar injury and failure.\textsuperscript{1-6, 18-20} Although the pattern of the laminar inflammatory response has not been well described in the CHO model, the reported systemic response is more similar to the clinical case of laminitis in the septic horse, including a sustained response regarding immunological, cardiovascular and metabolic alterations, and the development of lameness.\textsuperscript{8, 11, 12, 21, 22} Thus, it is critical to detail the laminar inflammatory response in the CHO model both to determine if these inflammatory events occur in multiple models of laminitis, and to establish the temporal pattern of inflammatory signaling in this clinically relevant model in order to design future anti-inflammatory treatment regimens.

**MATERIALS AND METHODS**

*Animals*
The experimental protocol was approved by the Institutional Animal Care and Use Committee. Twenty-four adult horses with a median body weight of 421 kg (ranging from 341 kg to 524 kg) and a median age of 5 years (ranging from 3 to 12 years old) were used in this study. Each horse was determined to be healthy and free of digital pathology determined by physical and lameness examinations and radiographic evaluation of the distal phalanx. Horses were divided into three groups: a control group (CON, n=8), a developmental period group (DEV, n=8), and an Obel Grade I lameness group (OG1, n=8). Prior to the experiment, the horses were quarantined for two weeks at the University of Missouri, housed in stalls and fed a regular ration of free choice hay.

Carbohydrate overload model

A CHO gruel consisting of 85% cornstarch and 15% wood flour (17.6 g/kg body weight) was administered to each horse in the DEV and OG1 groups by nasogastric tube as previously described.\textsuperscript{12, 23} Six liters of deionized water were administered via nasogastric tube to each horse in the control group. All horses received complete physical examinations, consisting of rectal temperature, heart rate, respiratory rate, abdominal sounds, digital pulses, evaluation with hoof testers, and gait evaluation immediately prior to nasogastric tube passage, and at 2-hour intervals following administration of either CHO or water. Blood was taken from an intravenous jugular catheter at 0, 2, 4, 8, 12, 16 hours in all horses, and every 4 hours after 20 hours in the OG1 group until onset of Obel grade 1 lameness (horse lifts feet incessantly, short, stilted gait at trot). Anesthesia was induced in the DEV group within 2 hours of developing a temperature greater than 102°F (occurring between 12 and 22 hours) and in the OG1
group at the onset of lameness (occurring between 20 and 48 hours). Although classically a 20% drop in central venous pressure is often used to determine the DEV time point in the CHO model\textsuperscript{22}, fever was used in this study as it occurs at approximately the same time as the drop in CVP and was a much more repeatable and accurate variable to measure. Due to the facts that 1) it was anticipated from previous reports that up to 30% of the horses may not respond (not exhibit any signs) to the CHO administration and 2) the investigators were also interested in assessing laminar inflammatory signaling in these “non-responders”, it was established prior to performing the animal protocols that animals in the DEV group which did not exhibit fever would be anesthetized for sampling at 24 hours after CHO administration, and horses in the OG1 group that did not exhibit lameness would be anesthetized at 48 hours after CHO administration. Two horses in each of DEV (no fever) and OG1 groups did not respond (no fever or lameness) to the CHO administration and were anesthetized for sample harvesting at the appropriate times. Control horses were anesthetized at 24 hours after administration of water. Anesthesia was induced with a combination of xylazine (1 mg/kg of body weight IV) and ketamine (2.2 mg/kg of body weight IV). Horses were placed in lateral recumbency and anesthesia was maintained with isoflurane. While under general anesthesia, a tourniquet was placed which was followed by rapid removal of all four feet by disarticulation of the metacarpophalangeal joint. Laminar tissue was rapidly harvested after 1cm thick sagittal sections of the digit were cut with a band saw. These tissues were immediately snap frozen in liquid nitrogen and later transferred to -80°C for storage until used for isolation.
of total RNA. Once the tissues were collected, each horse was subjected to euthanasia with pentobarbital sodium and phenytoin sodium (20 mg/kg of body weight IV).

**RNA isolation and cDNA synthesis**

Total RNA was extracted from three separate sections of dorsal lamina from each horse in the CON, DEV, and OG1 groups using a kit (Absolutely RNA Miniprep; Stratagene, LaJolla, CA) which includes a DNase treatment to remove genomic DNA contamination. PolyA mRNA was then isolated (mRNA extraction kit; Roche, Indianapolis, IN) and used to make complementary DNA (cDNA) for each sample via reverse transcription (Retroscript; Ambion Inc; Austin, TX) using at total of 400 ng of mRNA. The cDNA was frozen at -20°C and stored until used for real-time quantitative PCR (RT-qPCR) analysis.

**Real-time qPCR procedure**

Real-time quantitative PCR was performed using a thermocycler (LightCyler, Roche Molecular Biochemical, Indianapolis, IN) and quantified with external standards with the fluorescent format for SYBR Green I dye as previously described. Primers were designed from equine-specific sequences for IL-1β, IL-2, IL-4, IL-6, TNF-α, IFN-γ, COX-1, COX-2, E-Selectin and the housekeeping genes (β-actin, β-2 microglobulin, glyceraldehyde-3 phosphate dehydrogenase, and TATA-box binding protein) have been previously reported. New primers for IL-12p35 (f 5’-CGTTTTAGCCCGTCTCAA-3’ and r 5’-AACTCCACCTGGTACATCTTCA-3’), ICAM-1 (f 5’-GACGCCCAAGCCACCTTTTCA-3’ and r 5’-GACCCCTGCACTTTCTTTACTCA-3’), and IL-10 (f 5’-
GAGGCTGCGGCGCTGTCATC-3’ and r 5’-
TTTTTCATCTTCGTTGTCATATAGGCTTCT-3’) also were designed from equine-
specific sequences. Concentrations of IL-4 mRNA were also determined using an
additional set of previously reported primers (f 5’-AAGGGCAAGAATTCGTGCAT-3’
and r 5’-CGCTCAGGCATTCTTTGATCA-3’) designed from equine-specific
sequences. All primers were screened using gel electrophoresis and melt curve analysis
(LightCycler, Roche Molecular Biochemical, Indianapolis, IN) to confirm amplification of
a single cDNA fragment of the correct melting temperature and size. Amplified cDNA
fragments of each gene were ligated into a vector (TOPO 010 E.Coli; Invitrogen,
Carlsbad, CA) and the vectors linearized with Hind III restriction enzyme (Invitrogen,
Carlsbad, CA) for the use of templates to generate a standard curve for the RT-qPCR
reaction. Amplified cDNA fragments were sequenced after cloning to confirm correct
DNA sequence for the products of each primer.

Each cDNA sample was diluted 1:5 and 1:500 with 1X TE buffer to be used in the
cytokine and housekeeping PCR reactions, respectively. PCR reactions were performed
in glass capillaries containing 5 μl of diluted sample and 15 μl of PCR master mixture.
Master mix included the following: 1 unit of Taq polymerase (Invitrogen, Carlsbad, CA),
0.2 units of uracil-N-glycosylase (Roche Molecular Biochemical, Indianapolis, IN),
1:10,000 dilution of SYBR Green stock solution, forward and reverse primers, PCR
nucleotide plus (Roche Molecular Biochemical, Indianapolis, IN) and PCR buffer. The
PCR buffer (20mmol/l Tris-HCL) contained 0.05% each of Tween 20 and nonionic
detergent. Primers for ICAM-1, IL-12p35, and IL-10 were used at a concentration of 2.5 μmol/L. All remaining primers were used at a concentration of 5 μmol/L.

Uracil-N-glycosylase activation, to prevent PCR product carryover, was conducted at 50°C for 2 minutes and was followed by denaturation at 95°C for 2 minutes. Amplification occurred for 40 to 45 cycles, with the annealing temperature set at 1-5°C below the melting temperature for each specific set of primers, extension was set at 72°C for 5 seconds, and fluorescence acquisition for 10 seconds in the SYBR Green format. Single fluorescence acquisition in each cycle was set at either 80 or 82°C, depending on the melting temperature of the cDNA product of interest as previously described.6,7 After amplification cycling, melting curves of the PCR product were acquired through a stepwise increase in temperature from 65-95°C.

Standard and target samples were prepared in separate capillaries. Standard curves and water for negative control were performed for each gene of interest and amplified with each series of reactions. Standards were made up of 10-fold serial dilutions of linearized plasmids containing the different gene-specific cDNA inserts. All samples were run in duplicate.

**Data analysis**

Average copy number from each sample was determined for each gene (housekeeping and inflammatory marker). As previously reported, RT-qPCR data from the four housekeeping genes for each sample was evaluated by the computer software program geNorm (Ghent University; Ghent, Belgium) to determine which two genes received the best acceptable score to be used for normalization.2 β-actin and
glyceraldehyde-3 phosphate dehydrogenase were selected and then used by the geNorm software to create a normalization factor for each sample. To determine the corrected copy number value for each sample the amplification data obtained by RT-qPCR for each gene was divided by the normalization factor of the selected housekeeping gene for the same sample. After normalization, the fold change from the average control value was calculated for each sample. Data were analyzed non-parametrically using the Kruskal-Wallis and Dunn’s multiple comparisons test to compare groups. Although RT-qPCR data were obtained for the two non-responders (horses without developing fever i.e. >101.5°F) in the DEV group and two non-responders (horses without displaying signs of fever or Obel grade 1 lameness) in the OG1 group, the data from these horses were not included in statistical analyses. However, the data from non-responders are included on the graphs to demonstrate the pattern of inflammatory gene expression in these animals. Only six control horses were used in the analysis (two randomly removed) to allow for equal groups. Statistical significance was set at P<0.05 to maintain type I error for all tests.

RESULTS

Six of eight horses in the DEV group developed fevers with an average high temperature of 103.6°F (range 103.2°F to 105.1°F) which occurred between 12 and 22 hours post-CHO administration (median 17 hours). Six of eight horses in the OG1 group developed signs of Obel grade 1 lameness (lifting feet incessantly and demonstrating a short, stilted gait at trot) which occurred between 20 and 48 hours post-CHO
administration (median 25 hours). Two horses in the DEV group and two in the OG1 group did not develop a fever (≥101.5°F) or lameness, respectively (non-responders in OG1 group also did not develop a fever at any point). Laminar mRNA concentrations of IL-1β, IL-6, IL-12p35, COX-2, E-Selectin, and ICAM-1 were significantly increased in the OG1 horses when compared to control horses; however, no significant changes were present in the DEV horses for these inflammatory factors (Figure 2.1; Table 2.1-2.4). No change in laminar mRNA concentrations of TNF-α, IFN-γ, COX-1, IL-2, and IL-10 were present in either the DEV or OG1 groups (Table 2.1, 2.2, 2.4). Measureable concentrations of IL-4 mRNA were not present in any of the laminar tissue (CON, DEV and OG1) as the levels were below detection for our PCR. The PCR was validated with equine tissue from spleen, liver and kidney as well as sequenced plasmid standards that amplified with the use of the equine specific primers designed in our laboratory and from previous reports.24 Although laminar mRNA concentrations from the non-responders were not included in the statistical analysis, they are displayed on the graphs as open circles to demonstrate the absence of a laminar inflammatory response in these horses (Figure 2.1).

**DISCUSSION**

The studies of laminar inflammation in the BWE model of laminitis have supplied us with many inflammatory events to assess in a different laminitis model which more closely replicates clinical laminitis, the CHO model. To our knowledge, the current study is the first to document that a robust proinflammatory cytokine response also occurs in
the CHO model. However, the results also suggest a very different temporal pattern of cytokine expression than that reported in the BWE model.

Early increases in laminar mRNA concentrations of both IL-1β and IL-6 have been reported in the BWE model with a sustained increased in IL-6 mRNA concentration through the onset of lameness.\textsuperscript{1,4,5} In this study, laminar mRNA concentrations of IL-1β and IL-6 were only increased in the OG1 horses. Although the response was delayed compared with the BWE model, there was still a robust response with laminar IL-6 mRNA concentrations increasing >2000 fold from control values at the onset of lameness. These proinflammatory cytokines, which are an important part of the innate immune response, are also characteristic of the cytokine response reported with sepsis/SIRS in humans.\textsuperscript{25} Increased plasma concentrations of IL-1β and IL-6 have been negatively correlated with outcome in human patients with sepsis.\textsuperscript{26}

Increases in mRNA concentrations of TNF-α, a prominent inflammatory cytokine present in sepsis/SIRS in humans, have not been found in the laminar tissue of horses after BWE administration.\textsuperscript{1} Similar findings were also present in this study where no increase in TNF-α mRNA concentrations were present after CHO administration at either the DEV or OG1 time point. Previously, the absence of a laminar TNF-α response was purported to be due to the lack of mononuclear cells present in the laminar tissue; however, recent findings from our laboratory provide evidence of a population of macrophages within the primary and secondary dermis in both normal horses and those with laminitis.\textsuperscript{27} Although TNF-α is not present in laminar tissue during the development of laminitis, increases in serum concentrations of TNF-α have been reported in the
oligofructose model of laminitis. The source of the serum TNFα may be due to expression in visceral organs as increases in mRNA concentrations of TNF-α have been reported in the lung and liver from horses administered BWE; however, in pigs administered LPS no correlation between plasma cytokine concentrations and organ or peripheral blood cells mRNA concentrations were found. No increases in mRNA concentration of TNFα from peripheral blood mononuclear cells have also been reported after administration of oligofructose to horses. Therefore, it is possible to have a systemic increase in plasma cytokine response during the DEV and OG1 time without demonstrating a significant increase in other tissues such as the lamina. The lack of TNF-α response in the laminar tissue may be similar to reports in rats after endotoxin administration where systemic increases in TNF-α were present but local production of TNF-α in the interstitial fluid of the rat paw was not present. Compartmentalization of the inflammatory response in regard to expression of specific cytokines is another possible explanation for increases in TNF-α in some tissues and not in others. This is believed to occur in many models of sepsis/SIRS including a baboon model of lung sepsis where TNF-α was also not up-regulated whereas increases in IL-1β and IL-6 were found in the identical tissue samples.

Increases in mRNA concentration of IL-12p35 were present in laminar tissue at the onset of lameness and is similar to reports in the BWE model of laminitis where increases in IL-12 were only reported for the late (12h) time point. IL-12 is known to be important for stimulation of natural killer cells and T-cells to produce IFN-γ, which is part of the Th1/cell mediated response of adaptive immunity. However, similar to
previous reports in the BWE model, no change in IL-2 or IFN-γ mRNA concentrations was present at either time point in the current study. A possible explanation for these findings may be that a prominent Th₁ response does not occur in the laminar tissue of horses as seen with sepsis/SIRS in humans. Another possibility may be that the Th₁ response is slower in onset in this model, with only the up-regulation of IL-12p35 occurring in times evaluated. Perhaps later samples after OG1 may have had an increase in the production of IFN-γ or IL-2 mRNA. A delayed Th₁ response has been reported where the stimulation of monocytes, natural killer cells, and T-cells did not peak until after the innate inflammatory response occurred in a baboon model of pulmonary sepsis.

The results from this study also suggest that the lamina does not mount an anti-inflammatory response during the DEV or OG1 phases of laminitis, as no changes in IL-10 or IL-4 mRNA concentrations were found. These two cytokines are well known in the adaptive Th₂ response and IL-10 in particular is not only known to be up-regulated in human sepsis, but also believed to be prognostic for outcome in human patients with sepsis/SIRS. Laminar IL-10 mRNA concentrations were found to be decreased early in the BWE model at the 3h DEV time, but were back to control values by the time lameness occurred. The fact that IL-10 is not up-regulated in the CHO or BWE model of laminitis, when it is known to be a prominent component of human sepsis/SIRS, could be explained by the absence of a TNF-α response in these same horses. TNF-α is known to be a potent inducer of systemic IL-10 release and immunization of chimpanzees with an anti-monomclonal TNF-α antibody resulted in significant attenuation (by >60%) of the
plasma IL-10 response to endotoxin.\textsuperscript{36, 37} No change in laminar IL-10 mRNA concentrations may also be explained by compartmentalization of the inflammatory response similar to what occurs with sepsis/SIRS, where the pathophysiological events differ from organ to organ or from organ to peripheral blood.\textsuperscript{32} Therefore, although IL-10 does not change in the lamina in response to CHO overload, it is possible that other organs may have increased or decreased IL-10 expression in response to the CHO challenge. The lack of IL-4 response should not be due to a lack of lymphocytes, as both T- and B-lymphocytes are present in the laminar tissue.\textsuperscript{27} Finally, it is possible that, as expression of anti-inflammatory cytokines is a late event in very sick individuals in human sepsis studies\textsuperscript{36}, and IL-4 or IL-10 response does not occur until much later in the disease process or only occurs in a more severe disease process than that assessed in this model.

Increases in mRNA concentrations of the leukocyte adhesion molecules E-selectin and ICAM-1 were present in this study at the OG1 time point when compared to controls. In the BWE model, these adhesion molecules were markedly up-regulated very early, peaking at 1.5h after administration of BWE and decreasing precipitously in expression by the onset of lameness.\textsuperscript{1, 5} The endothelium is highly active and quite dynamic, responding rapidly to its local environment as indicated by the induction of ICAM-1 and E-Selectin by increased concentrations of proinflammatory cytokines IL-1β and IL-6.\textsuperscript{38, 39} Endothelial activation in the lamina, demonstrated by increased mRNA concentrations of adhesion molecules, supports additional preliminary findings from our laboratory, in which an influx of leukocytes are seen throughout the laminar dermis at the
developmental and OG1 time points after BWE administration. The delayed increases in mRNA concentrations of adhesion molecules observed in this study when compared to previous reports in the BWE model may be explained by the delayed production of local cytokines, such as IL-1β and IL-6, which have a positive effect on the transcriptional regulation of these adhesion molecules. Furthermore, presence of up-regulated adhesion molecules during the onset of lameness may be indicative of the severity of laminar injury in this model, as a similar study comparing non-survivors of sepsis to survivors found that continued elevation in ICAM-1 was found more commonly in non-survivors.

Similar to the difference in cytokine and adhesion molecule gene expression between the BWE and CHO models, laminar COX-2 mRNA concentration was only increased at the onset of lameness (vs. increases at both developmental and lameness time points in the BWE model). No change in COX-1 mRNA concentrations were present at any time point in this study which is consistent with other reports and is not unexpected considering COX-1 is constitutively expressed unlike COX-2 which is inducible in most tissues by inflammation. Although increases in mRNA expression of the COX isozymes may not directly correlate to protein concentrations and enzyme activity, increases in COX-2 protein concentrations have been documented in addition to increase in mRNA concentrations in the BWE model of laminitis. Additionally, the presence of cytokines (such as IL-1β), TLR ligands, and hypoxia (all of which are known or suspected to be occurring during the development of laminitis) can affect several post-transcriptional modulations in the stabilization of COX-2 mRNA. The importance of
COX-2 in models of sepsis is demonstrated by the use of COX-2 null mice exposed to endotoxin in which increased survival, decreased leukocyte influx into the kidneys and lungs, reduced NF-κB activation, and greatly increased IL-10 production occurred compared to the wild type mice. Further research is warranted in evaluating the use of the use of the new COX-2 equine specific inhibitor firocoxib to determine if it may be more beneficial in these cases. It will, however, be important to determine if the negative vascular effects observed in some human patients on coxibs (such as increase in thrombus formation or vasoconstriction) will adversely affect patients with laminitis.

It has been documented that not all horse are “responders” to the CHO model, with up to 25% of horses not exhibiting clinical signs of laminitis while the majority of animals in the same studies developed Obel grades 1 to 2 laminitis. The reason that some horses are non-responsive to CHO administration resulting in their protection from laminar injury is unknown. Although these non-responsive horses have usually been removed from previous studies, we were interested in comparing laminar inflammatory events in the non-responsive animals to those animals which responded to the clinical model. Similar to previous studies, approximately 25% (2 in each of the DEV and OG1 groups) of the horses did not respond to the administration of CHO. This lack of response was not only present in the laminar tissue, but was present at the systemic level with none of the four “non-responders” exhibiting a febrile response. Interestingly, none of the laminar inflammatory events present in the responsive horses in each group were present in these “non-responders” (see Figure 2.1). Although there were not enough “non-responders” for statistical analyses, the consistent lack of inflammatory signaling in the
four non-responsive horses suggests that the protection from CHO-induced laminar injury is upstream of a central event inducing the expression of diverse inflammatory molecules including a wide array of proinflammatory cytokines and chemokines, endothelial adhesion molecules, and COX-2. It is possible this lack of response may simply be explained by a different bacterial flora in the intestinal tract which does not result in the decrease in cecal pH, endotoxin production or the subsequent damage to the cecal mucosa observed in CHO overload; differences in bacterial flora have recently been demonstrated to affect the response to sepsis in models of human sepsis. Another explanation may be the non-responders have genetic polymorphism(s), as has been reported in human sepsis for such genes as TNF-α and TLR4, that result(s) in their protection and lack of laminar inflammation. Further research involving the protective mechanisms in these horses which keeps them from developing the clinical signs associated with CHO administration will be valuable in discovery of effective treatments for laminitis.

Overall, the CHO administration results in a laminar inflammatory response that is delayed in onset when compared to the early fulminant inflammatory response in the BWE model. Whereas animals in the BWE model undergo a rapid and aggressive inflammatory response by 1.5 h following BWE administration, the inflammatory gene expression in the majority of animals in the current study were no higher than control values at the DEV time point for most genes assessed. Interestingly, the same two animals in the DEV group were responsible for the two highest values for ICAM-1, COX-2, IL-1β and IL-6, indicating that the majority of variability in laminar
inflammatory gene results observed between animals is due to these two animals undergoing inflammatory signaling changes more rapidly than the other animals exposed to the same stimulus. As a more consistent inflammatory response is observed in the OG1 group, it is likely that a laminar inflammatory response is initiated in many of the animals in the time period between the onset of clinical signs of inflammation (fever in this case) and the onset of lameness. It is likely that the CHO model simply differs from the BWE model as demonstrated by the similarities and differences between the two models used to induce experimental sepsis in rodents, the CLP and endotoxin models.\textsuperscript{16, 17, 52} In the BWE model, the rapid absorption of a substance, which induces a profound systemic inflammation, is the likely cause of laminar inflammation in this model, similar to the rapid and marked systemic inflammatory response following the administration of LPS in experimental animals. In the CHO model, the absorption of an overwhelming amount of inflammatory trigger molecules most likely takes several hours, due to the time it takes for the intraluminal changes in flora and injury to the intestinal epithelium with resulting loss of the barrier function of the hindgut to occur. This is similar to the CLP rodent model of sepsis where there is a more gradual build-up but more sustained presence of bacterial products in the circulation than endotoxin models, leading to a similar organ injury as seen in clinical sepsis. It is unknown if the laminar inflammatory response is sustained for a longer period of time after CHO administration as no samples were collected after the OG1 time point. However, if sustained the inflammatory response is likely to play an important role in the protracted and increased severity of lameness and laminar injury that often occurs after grain overload.
In conclusion, results from this study suggest that, although delayed when compared to the BWE model, a marked laminar inflammatory response occurs in the CHO model of laminitis. Furthermore, with the numerous redundant inflammatory mediators produced in the lamina after CHO administration, targeting multiple inflammatory signaling pathways may be necessary to stop the development of laminitis. However, the fact that all laminar inflammatory events documented to occur in affected laminae do not occur in the horses which did not respond clinically to CHO overload leaves open the possibility that there still may be a central inflammatory signaling mechanism which, if blocked, may inhibit all deleterious inflammatory events likely to lead to severe laminar injury. Research evaluating new anti-inflammatory therapies, particularly those aimed at signal transduction and transcriptional control of inflammation will be essential to the search of finding successful treatments/preventions for this devastating disease.53

REFERENCES


30. Tadros L, Frank N, Horohov DW. Inflammatory cytokine blood messenger RNA expression during the development of oligofructose-induced laminitis in horses In: 2nd AAEP Equine Laminitis Research Workshop; 2009; West Palm Beach, FL; 2009.


Figure 2.1. Median fold changes in laminar mRNA concentrations after CHO administration in horses (DEV and OG1). Significantly increased inflammatory mediator when compared to control (CON) horses (*P<0.05; **P<0.01; ***P<0.001). Open circles and squares are the non-responders.
### Table 2.1  Cytokines of innate immunity

<table>
<thead>
<tr>
<th>Cytokines of Innate Immunity</th>
<th>CON</th>
<th>DEV</th>
<th>OG1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TNF-α</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mRNA* (25% - 75% percentile)</td>
<td>76,500</td>
<td>69,750</td>
<td>118,500</td>
</tr>
<tr>
<td>Fold increase*</td>
<td>0.08</td>
<td>1.5</td>
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</tr>
<tr>
<td>P-value</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td><strong>IL-1β</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mRNA* (25% - 75% percentile)</td>
<td>1345</td>
<td>3820</td>
<td>17,700</td>
</tr>
<tr>
<td>Fold increase*</td>
<td>2.4</td>
<td>11.2</td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>NS</td>
<td>P&lt;0.01</td>
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</tr>
<tr>
<td><strong>IL-6</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mRNA* (25% - 75% percentile)</td>
<td>141</td>
<td>5485</td>
<td>264,500</td>
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<td>Fold increase*</td>
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<tr>
<td>P-value</td>
<td>NS</td>
<td>P&lt;0.001</td>
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*mRNA values expressed as median cDNA copies per normalization factor.

*Fold increase expressed in medians from control. NS=non significant fold change from control.
Table 2.2. Cytokines of adaptive immunity

<table>
<thead>
<tr>
<th>Cytokines of Adaptive Immunity</th>
<th>CON</th>
<th>DEV</th>
<th>OG1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-2</strong></td>
<td>23.8</td>
<td>16.1</td>
<td>18.9</td>
</tr>
<tr>
<td>(25% - 75% percentile)</td>
<td>(7.1 – 55.9)</td>
<td>(7.4 – 30.4)</td>
<td>(10.4 – 122.2)</td>
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<tr>
<td>Fold Increase(^\d)</td>
<td>0.06</td>
<td>1.74</td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td><strong>IFN-γ</strong></td>
<td>87.7</td>
<td>43.5</td>
<td>68.3</td>
</tr>
<tr>
<td>(25% - 75% percentile)</td>
<td>(3.4 - 167)</td>
<td>(11.8 – 191)</td>
<td>(25.2 – 241)</td>
</tr>
<tr>
<td>Fold Increase(^\d)</td>
<td>0.42</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td><strong>IL-12p35</strong></td>
<td>125</td>
<td>293</td>
<td>405</td>
</tr>
<tr>
<td>(25% - 75% percentile)</td>
<td>(41 - 236)</td>
<td>(126 – 495)</td>
<td>(190 – 678)</td>
</tr>
<tr>
<td>Fold Increase(^\d)</td>
<td>3.8</td>
<td>5.3</td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>NS</td>
<td>P&lt;0.05</td>
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</tr>
<tr>
<td><strong>IL-10</strong></td>
<td>3430</td>
<td>3475</td>
<td>4565</td>
</tr>
<tr>
<td>(25% - 75% percentile)</td>
<td>(1530-5185)</td>
<td>(1635-6105)</td>
<td>(2335-9575)</td>
</tr>
<tr>
<td>Fold Increase(^\d)</td>
<td>1.02</td>
<td>1.35</td>
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</tr>
<tr>
<td>P-value</td>
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<td>NS</td>
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</tbody>
</table>

\(^\d\)Fold increase expressed in medians from control.  NS=non significant fold change from control.

\(^*\)mRNA values expressed as median cDNA copies per normalization factor.

IL-4 values below the level of detection and not included in this chart.
Table 2.3  Adhesion molecules

<table>
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<th>Adhesion molecule</th>
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<th>OG1</th>
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</thead>
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<tr>
<td><strong>E-Selectin</strong></td>
<td>103</td>
<td>117</td>
<td>1477</td>
</tr>
<tr>
<td>mRNA*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(25% - 75% percentile)</td>
<td>(32 - 309)</td>
<td>(54 - 574)</td>
<td>(767 - 3280)</td>
</tr>
<tr>
<td>Fold Increase^</td>
<td>0.9</td>
<td>10.4</td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>NS</td>
<td>P&lt;0.05</td>
<td></td>
</tr>
<tr>
<td><strong>ICAM-1</strong></td>
<td>6440</td>
<td>11,600</td>
<td>40,400</td>
</tr>
<tr>
<td>mRNA*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(25% - 75% percentile)</td>
<td>(3725 - 11,350)</td>
<td>(9200 - 65,100)</td>
<td>(19,180 - 54,250)</td>
</tr>
<tr>
<td>Fold Increase^</td>
<td>1.7</td>
<td>5.8</td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>NS</td>
<td>P&lt;0.05</td>
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</table>

*mRNA values expressed as median cDNA copies per normalization factor.

^Fold increase expressed in medians from control. NS=non significant fold change from control.
Table 2.4 COX enzymes

<table>
<thead>
<tr>
<th>COX enzyme</th>
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<th>DEV</th>
<th>OG1</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-1</td>
<td>mRNA*</td>
<td>7530</td>
<td>3960</td>
</tr>
<tr>
<td></td>
<td>(25% - 75% percentile)</td>
<td>(4230 – 14,150)</td>
<td>(1330 – 5555)</td>
</tr>
<tr>
<td>Fold Increase^</td>
<td>0.46</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>NS</td>
<td>NS</td>
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</tr>
<tr>
<td>COX-2</td>
<td>mRNA*</td>
<td>3615</td>
<td>4195</td>
</tr>
<tr>
<td></td>
<td>(25% - 75% percentile)</td>
<td>(1530 - 5660)</td>
<td>(2050 – 99,600)</td>
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<tr>
<td>Fold Increase^</td>
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<td>31.2</td>
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</tr>
<tr>
<td>P-value</td>
<td>NS</td>
<td>P&lt;0.05</td>
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</table>

*mRNA values expressed as median cDNA copies per normalization factor.

^Fold increase expressed in medians from control. NS=non significant fold change from control.
Chapter 3: Hindlimb Laminar Inflammatory Response is Similar to that Present in Forelimbs after Carbohydrate Overload in Horses

ABSTRACT

Objective: To determine if a similar proinflammatory response occurs in the hind lamina as previously reported for the forelimb.

Methods: Archived laminar samples from 12 horses administered 17.6 g of starch (85% corn starch/ 15% wood flour)/kg body weight via nasogastric tube that were anesthetized either after developing a temperature >102°F (DEV, n=6) or at the onset of Obel grade 1 lameness (OG1, n=6) were used in addition to 6 control horses (CON) that were anesthetized 24 hours after administration of water. RT-qPCR for selected proinflammatory mediators and MAC387 immunohistochemistry were performed. The data were analyzed non-parametrically to compare groups.

Results: Increases in laminar MAC387-positive leukocytes and laminar mRNA concentrations (P<0.05) for IL-1β, IL-6, COX-2, CXCL1 and CXCL8 were present in both fore- and hindlimb laminae from horses with OG1 lameness. CXCL1 and CXCL8 were also increased in forelimb and hindlimb laminae in the DEV horses.

Conclusions: Administration of a carbohydrate overload resulted in a similar inflammatory response in the hind lamina as previously reported for the forelimb lamina.
These findings suggest that other factors such as weight bearing may play an important role in the development of laminitis after a systemic inflammatory condition develops.

**INTRODUCTION**

The equine surgeon is commonly confronted with laminitis as a sequela to sepsis, either in the form of postoperative cases of acute abdomens with strangulating intestinal lesions,\(^1,2\) or as a sequela to other postoperative complications of abdominal and other clinical surgical cases such as anterior enteritis, enterocolitis, and pneumonia. Except in cases of support limb laminitis where the contralateral limb is at risk of laminitis regardless of whether it is a fore- or hindlimb,\(^3\) it is well known that laminitis in clinical patients occurs much more frequently in the forelimb than in the hindlimb.\(^1\) However, the reason for the much greater incidence of laminitis in the forelimb digits is not known. One compelling factor commonly discussed is that the horse carries more weight on the forelimbs, resulting in more physical stress on the forelimb laminae. Although the reported differences in weight bearing between the fore- and hindlimbs has been 58% and 42% respectively\(^4\), the question remains regarding whether or not this difference is enough to explain the much greater incidence of laminitis in the forelimbs vs. the hindlimbs.

A complete understanding of the pathophysiology of laminitis remains to be elucidated; however, numerous pathologic events have been reported to take place and likely play a role in laminar failure including vascular alterations,\(^5-8\) enzymatic and metabolic dysfunctions,\(^6,9-11\) and inflammatory injury.\(^12-14\) Interestingly, all of these
studies concentrated on the forelimb lamina, leaving a paucity of information regarding laminar events in the hind foot. Inflammatory signaling is reported to occur in most types of injury purported to result in laminitis, including ischemia-reperfusion, metabolic syndrome, and endotoxemia/sepsis. The presence of inflammation in the lamina of the front feet during the development of laminitis has been well documented in both the black walnut extract (BWE) and carbohydrate overload (CHO) experimental models of laminitis. Recently, our laboratory has found increases in the mRNA concentrations of cytokines (i.e. IL-1β, IL-6, IL-12p35), chemokines (i.e. CXCL1, CXCL8), COX-2, and endothelial adhesion molecules (i.e. ICAM-1, E-Selectin) at the onset of lameness in the front lamina from horses given CHO. Leukocyte emigration, evidence by immunostaining of calprotectin in neutrophils and macrophages, has also been reported at the onset of lameness in the dermal lamina of horses administered BWE and CHO.

Therefore, due to the fact that tissue inflammation is a central component of pathologic mechanisms reported to be involved in laminitis, and that the marked inflammatory signaling is reported to be present in the forelimb laminae in the two most widely used experimental models of equine laminitis, the purpose of this study was to determine if an inflammatory response occurs in the lamina of the hindlimb as previously reported in the forelimb lamina and if there were a difference in the magnitude of this response. Laminar tissue samples that were previously collected from control horses and horses administered CHO were used in order to assess mRNA concentrations of
proinflammatory mediators by RT-qPCR and to assess the presence of leukocyte emigration by immunohistochemical techniques.

**MATERIALS AND METHODS**

*Animals*

The experimental protocol was approved by the Institutional Animal Care and Use Committee. Archived samples from eighteen adult horses with a median body weight of 421 kg (ranging from 341 kg to 524 kg) and a median age of 5 years (ranging from 3 to 12 years old) were used in this study. Each horse was determined to be healthy and free of digital pathology determined by physical and lameness examinations and radiographic evaluation of the distal phalanx. Samples were obtained from three groups: a control group (CON, n=6), a developmental period group (DEV, n=6; determined by the onset of fever [$>102^\circ$F] which was used as an indication of the presence of systemic inflammation), and an Obel Grade I lameness group (OG1, n=6; defined by the horse lifting its feet incessantly and/or having a short, stilted gait at trot). Prior to the experiment, the horses were quarantined for two weeks at the University of Missouri, housed in stalls and fed a regular ration of free choice hay.

*Carbohydrate overload model*

A carbohydrate gruel consisting of 85% cornstarch and 15% wood flour (17.6 g/kg body weight) was administered to each horse in the DEV and OG1 groups by nasogastric tube as previously described.²⁸,³⁴,³⁵ Six liters of deionized water were administered via nasogastric tube to each horse in the control group. All horses received
complete physical examinations, consisting of rectal temperature, heart rate, respiratory rate, abdominal sounds, digital pulses, evaluation with hoof testers, and gait evaluation immediately prior to nasogastric tube passage, and at 2-hour intervals following administration of either carbohydrate or water. Anesthesia was induced in the DEV group within 2 hours of developing a temperature greater than 102°F (occurring between 12 and 22 hours) and in the OG1 group at the onset of lameness (occurring between 20 and 48 hours). Control horses were anesthetized at 24 hours after administration of water. Anesthesia was induced with a combination of xylazine (1 mg/kg of body weight IV) and ketamine (2.2 mg/kg of body weight IV). Horses were placed in lateral recumbency and anesthesia was maintained with isoflurane. While under general anesthesia the feet of all four limbs were rapidly removed (after placement of a tourniquet) by disarticulation of the metacarpophalangeal joint, and 1cm thick sagittal sections of the digit were cut with a band saw. The laminae were rapidly harvested and immediately snap frozen in liquid nitrogen and later transferred to -80°C for storage until used for isolation of total RNA. Additional laminar samples were cut (approx. 1cm x 1cm x 3 mm) and immediately immersed in neutral-buffered 10% formalin for 24 hours, followed by placement in 70% ethanol until paraffin embedding occurred. For each horse, three samples were embedded together in the same paraffin block. Once the tissues were collected, each horse was subjected to euthanasia with pentobarbital sodium and phenytoin sodium (20 mg/kg of body weight IV).

RNA isolation and cDNA synthesis
Total RNA was extracted from three separate sections of dorsal lamina of each horse in the CON, DEV, and OG1 groups using a kit (Absolutely RNA Miniprep; Stratagene, LaJolla, CA) which includes a DNase treatment to remove genomic DNA contamination. PolyA mRNA was then isolated (mRNA extraction kit; Roche, Indianapolis, IN) followed by the production of complementary DNA (cDNA) via reverse transcription (Retroscript; Ambion Inc; Austin, TX). A total of 400 ng of mRNA was used to make cDNA for each sample. The cDNA was frozen at -20°C and stored until used for real-time quantitative PCR (RT-qPCR) analysis.

**Real-time qPCR procedure**

Real-time quantitative PCR was performed using a thermocycler (LightCyler, Roche Molecular Biochemical, Indianapolis, IN) and quantified with external standards with the fluorescent format for SYBR Green I dye as previously described.\(^{25, 26}\) Previously reported primers were designed from equine-specific sequences for IL-1β, IL-6, COX-1, COX-2, CXCL1, and CXCL8 and the housekeeping genes (β-actin and glyceraldehyde-3 phosphate dehydrogenase).\(^{25, 26, 32}\) Amplified cDNA fragments of each gene were ligated into a vector (TOPO 010 E.Coli; Invitrogen, Carlsbad, CA) and the vectors linearized with Hind III restriction enzyme (Invitrogen, Carlsbad, CA) for the use of templates to generate a standard curve for the RT-qPCR reaction.\(^{25, 26}\)

Each cDNA sample was diluted 1:5 and 1:500 with 1X TE buffer to be used in the cytokine and housekeeping PCR reactions, respectively. PCR reactions were performed in glass capillaries containing 5 μl of diluted sample and 15 μl of PCR master mixture. Master mix included the following: 1 unit of *Taq* polymerase (Invitrogen, Carlsbad, CA),
0.2 units of uracil-N-glycosylase (Roche Molecular Biochemical, Indianapolis, IN), 1:10,000 dilution of SYBR Green stock solution, forward and reverse primers, PCR
nucleotide plus (Roche Molecular Biochemical, Indianapolis, IN) and PCR buffer. The
PCR buffer (20mmol/l Tris-HCL) contained 0.05% each of Tween 20 and nonionic
detergent. Primers for CXCL8 were used at a concentration of 2.5μmol/L and all
remaining primers were used at a concentration of 5μmol/L.

Uracil-N-glycosylase activation, to prevent PCR product carryover, was
conducted at 50°C for 2 minutes and was followed by denaturation at 95°C for 2 minutes.
Amplification occurred for 40 to 45 cycles, with the annealing temperature set at 1-5°C
below the melting temperature for each specific set of primers, extension was set at 72°C
for 5 seconds, and fluorescence acquisition for 10 seconds in the SYBR Green format.
Single fluorescence acquisition in each cycle was set at either 80 or 82°C, depending on
the melting temperature of the cDNA product of interest as previously described.25, 26
After amplification, melting curves of the PCR product were acquired through a stepwise
increase in temperature from 65-95°C.

Standard and target samples were prepared in separate capillaries. Standard
curves and water for negative control were performed for each gene of interest and
amplified with each series of reactions. Standards were made up of 10-fold serial
dilutions of linearized plasmids containing the different gene-specific cDNA inserts. All
samples were run in duplicate. To further assess interassay variation between the front
and hind lamina, IL-6, CXCL8, and COX-2 RT-qPCR reactions were also performed
using the control and OG1 samples from both the front and hind feet of the same horses.
**Immunohistochemistry**

Laminar sections from control horses and sections from both the front and hind feet of the OG1 horses were used to determine leukocyte migration through the immunostaining of calprotectin, which will stain both activated macrophages and neutrophils, as well as stressed laminar epithelium. The paraffin-embedded samples were sectioned at 5-μm thickness, deparaffinized, and treated with a protease solution at 0.5 protease units/ml for 4 minutes (Proteinase K; Invitrogen, Carlsbad, CA). After blocking, sections were incubated with a mouse monoclonal anti-human calprotectin antibody using a 1:250 dilution at 37°C for 90 minutes (MAC387; Abcam, Cambridge, MA). A biotinylated anti-mouse antibody (VECTASTAIN ABC kit; Vector Laboratories, Burlingame, CA) was then applied using a 1:100 dilution at room temperature for 60 minutes. The immunoreactivity was observed using a peroxidase (DAB kit; Vector Laboratories, Burlingame, CA) following the instructions of the manufacturer and Harris hematoxylin (Fisher Scientific, Kalamazoo, MI) was used for the counterstain. The front lamina served as a positive control.

**Image analysis**

All slides were examined by light microscopy in order to identify the presence and location of stained cells and counting of cells for each image was blinded to remove bias from data collection. Whole slide digital images (WSIs) were obtained from stained sections using an automated scanning robot (T2 Scan Scope) with a 40× magnification at a spatial sampling period of 0.2 μm per pixel. WSIs were assessed using a software program (Aperio Image Scope, Vista CA) to randomly capture images from each section,
which were then transformed into jpg files. Ten random images were captured for each of the two areas of interest 1) deep dermal lamina (DDL; the area between the base of the laminae and the parietal surface of the distal phalanx, including the deep neurovascular structures) and 2) primary dermal lamina (PDL) for every horse. The number of immunoperoxidase-positive cells per high power field (40X) of each image was determined manually. The average number of immunoperoxidase-positive cells for each horse in the DDL and PDL were calculated and used for statistical analysis.

**Data analysis**

Average copy number from each sample was determined for each gene (housekeeping and inflammatory marker). As, previously reported, RT-qPCR data from the housekeeping genes for each sample were evaluated by the computer software program geNorm (Ghent University; Ghent, Belgium) and a normalization factor was determined.\(^{36}\) \(\beta\)-actin and glyceraldehyde-3 phosphate dehydrogenase were selected and then used by the geNorm software to create a normalization factor for each sample. To determine the corrected copy number value for each sample the amplification data obtained by RT-qPCR for each gene was divided by the normalization factor of the selected housekeeping gene for the same sample. After normalization, the fold change from the average control value was calculated for each sample. RT-qPCR and calprotectin immunohistochemistry data were analyzed non-parametrically using the Kruskal-Wallis and Dunn’s multiple comparisons test to compare groups. Statistical significance was set at \(P<0.05\) to maintain type I error for all tests.
RESULTS

Horses in the DEV group developed fevers with an average high temperature of 103.6°F (range 103.2°F to 105.1°F) occurring between 12 and 22 hours post-CHO administration (median 17 hours). Horses in the OG1 group developed signs of Obel grade 1 lameness (lifting feet incessantly and demonstrating a short, stilted gait at trot) occurring between 20 and 48 hours post-CHO administration (median 25 hours).

Laminar mRNA concentrations of CXCL1 and CXCL8 were significantly increased in the DEV (P<0.05) and in the OG1 (P<0.01) horses when compared to controls (Figure 3.1; Table 3.1). Laminar mRNA concentrations of IL-1β, IL-6 (P<0.001) and COX-2 (P<0.01) were significantly increased only in the OG1 horses when compared to controls (Figure 3.1; Table 3.1). No change in laminar mRNA concentrations of COX-1 was present in either the DEV or OG1 groups (Figure 3.1; Table 3.1). Both front and hind lamina at the OG1 time point demonstrated increases (P<0.05) in laminarIL-6, CXCL8, and COX-2 mRNA concentrations when compared to their respective controls (Figure 3.2). However, no significant difference between the front and hind lamina were noted in the OG1 horses (Figure 3.2). Comparisons between previously reported fold change values for inflammatory mediators of the front lamina and the current results for hind lamina are demonstrated in Table 3.1. The referenced results in Table 3.1 from front lamina were obtained from the same horses from which we used the hind lamina in the current study.
Leukocyte emigration occurred in the OG1 horses (front and hind lamina) in both the deep dermal lamina and the primary epidermal lamina evidence by significantly increased number of calprotectin positive cells per high power field (Figure 3.3). However, no significant difference in number of calprotectin positive cells between the front and hind feet were noted in the OG1 horses. Increased calprotectin staining was also present in the epithelial cells from the OG1 horses (Figure 3.4), similar to that previously reported for the forelimb lamina.33

**DISCUSSION**

Laminar failure secondary to a septic process or systemic inflammatory condition, occurring most commonly in forelimbs, is believed to be a result of multiple factors including both physiologic factors (e.g. inflammation, metabolic dysfunctions, enzymatic dysfunctions, and vascular changes) and mechanical factors, such as weight bearing. Obtaining a better understanding of which factors play a significant role in the development of laminar failure is essential to finding successful preventative and treatment regimes. The presence of inflammation in the lamina during the developmental and initial stages of lameness has been well documented in the front feet13, 14, 25, 26, 28-31, 36 and is believed to play an important role in laminar failure. However, inflammation has not been documented in the hind feet. The results of this study show that inflammation does indeed occur in the hind feet and appears to be of a similar magnitude as that reported in the fore feet.28, 32 Increases in hindlimb laminar gene expression of several proinflammatory mediators were present after the administration of CHO, including the
chemokines CXCL1 and CXCL8 in both the DEV and OG1 horses, and IL-1β, IL-6, and COX-2 in the OG1 horses. In order to remove interassay variation typically present when comparing results from different studies, samples from both the front and hind feet of each horse were performed in the same RT-qPCR reaction for IL-6, CXCL8, and COX-2 to directly compare their response. No significant difference was found between the OG1 hind feet and OG1 fore feet, determining that the degree of laminar inflammation was the same for both the front and hind feet. Comparisons between previously reported fold changes for the other genes of interest and the results from this study demonstrate similar magnitude of increase in inflammatory gene expression. Additionally, the magnitude of increase in leukocyte emigration into the laminar dermal tissue at the OG1 time after CHO administration was the same for the fore and hindlimbs as no significant differences were noted between the feet in the face of marked increases between CON and OG1 horses in both fore- and hindlimbs. Thus, the similar increases in laminar inflammatory mediator expression and leukocyte emigration indicate that comparable inflammatory processes occur in the forelimb and hindlimb laminae during the early stages of stages of laminitis. The extent of laminar injury from the inflammatory processes would be expected to be similar, regardless of whether initially resulting from vascular compromise, inflammatory injury from circulating pathogen products and mediators, enzymatic or metabolic dysregulation, or by a combination of events.

Little is known regarding the difference in the biomechanics between the front and hind feet of the horse; however, the most logical reason of why the forelimbs are
more affected in cases of laminitis is due to weight bearing loads. As discussed earlier, normal horses stand with the majority of their body weight (58%) on the forelimbs with equal distributions between the right and left limbs.\(^4\) Although reports are conflicting, weight appears to play a role in the development of laminitis and outcome in some patients.\(^{37-39}\) Horses that were reported to weigh greater than 550 kg were twice as likely to develop laminitis post enteritis\(^{38}\) and Baxter and Morrison reported that horses with lower body weights were more likely to survive distal displacement of the third phalanx.\(^{39}\) Although the forelimbs of horses are more commonly affected in laminitis, there are severe cases where all four limbs are involved. It is the authors’ observation that involvement of all limbs most commonly occurs in severe cases of sepsis, with distal displacement of P3 within the hoof capsule (indicative of complete laminar failure) occurring much more commonly than rotation. It is possible that these horses have initial changes in the fore feet, become painful and subsequently shift more weight to the rear resulting in increased stress on the hindlimb laminae. Although no significant difference in mean limb load was found after administration of CHO overload, increases in mean limb load in the hindlimbs has been found in chronic cases of laminitis\(^4\) Thus, it is possible that the combination of severe inflammation and increase weight on the hind feet (in attempts to remove weight from the more painful front feet) results in the condition where all four feet are affected. It is also possible that, due to the severity of the septic disease process in these animals, all laminae are so severely injured that they circumferentially fail with minimal stress thus resulting in distal displacement of all four distal phalanges.
Other factors in relation to weight may be more important than total body weight alone. For example, hoof size and/or shape may be additional factors to consider. Weight bearing with greater contact area, secondary to footing (soft vs. hard ground) or hoof size, presumably reduces mechanical stress acting on the laminar cell at or near the epidermal dermal interface. The overall shape of the hind foot varies from that of the front foot, often having a shorter toe and steeper hoof to ground angle. Altering the hoof size and shape by shorting the toe through trimming or beveling of the ground surface of the shoes, and increasing the hoof angle through the placement of heel wedges are common goals of treating the laminitic foot. Varying hoof angles may also produce areas of greater strain thereby more likely to result in failure during periods of inflammation. However, conflicting results in regards to dorsal laminar hoof wall strain have been reported, with some studies reporting decreased strain with increasing hoof angle through wedging of the heel vs. other studies that have found no difference in dorsal laminar hoof wall strain or weight bearing in the toe region following application of a heel wedge. Other studies have reported a change in distribution of forces on the hoof wall with changes in hoof angles insinuating increased stress on the dorsal hoof wall with a lower angle, but did not actually include data documenting decreased strain. Although many of the models are derived from ex vivo measurements, findings from these studies at least in part suggest that hoof shape and size may play a role in laminar function and failure. Future studies are necessary to determine how inflammation early in the development of laminitis may affect these models.
Another possibility for the increased frequency of forelimb laminitis vs. hindlimb, may be related to vascular changes secondary to weight bearing. Several researchers have documented decreased blood flow and/or changes in blood pressures during periods of increased weight bearing and increased blood flow under non-weight bearing conditions. Progressive increases in distal arterial resistance associated with the increased pressure exerted on the foot has been reported and is suspected to result from the compression of the soft tissue and vascular structure inside the foot resulting in both an increase in peripheral arterial resistance and an increase in digital venous pressure. Additionally it has been determined that digital venous pressures increase with increasing loads on the digit and decrease with decreasing loads, which was purported to provide a hydraulic shock-absorbing function of the digital vasculature. Although these data certainly provide support for the hypothesis that decreased digital blood flow due to greater weight bearing in the forelimbs may exacerbate the laminar injury occurring in laminitis and may therefore be the major factor putting the forelimbs at higher risk, the present data do not support this premise. Similar to the inflammatory response to sepsis, inflammation is a major component of the injury that occurs post ischemia, evidenced by increases in cytokine expression and increases in leukocyte emigration into affected tissue. Thus, the similar magnitude of inflammatory signaling in the fore- and hindlimbs indicates that a vascular event only occurring in the forelimbs is an unlikely cause of the predisposition of the forelimbs to undergo structural failure in the laminitic horse.
Overall, this study provides the first evidence that inflammation does occur with equal magnitude in the hindlimbs as previously reported for the forelimbs in the early stages of CHO induced laminitis. The presence of inflammation in this model and the infrequency of laminitis occurring in all four limbs secondary to grain overload or other systemic inflammatory diseases, suggest that inflammatory injury only plays a partial role in laminar failure. Further research is warranted to determine the importance of other possible factors involved, including mechanical factors such as weight bearing, and the shape of the foot.

REFERENCES


Figure 3.1. Laminar inflammatory mediators in hind lamina. Median fold changes in laminar inflammatory mediator mRNA concentrations after CHO administration in horses (DEV and OG1). Significantly increased inflammatory mediator when compared with control (CON) horses (*P<0.05; **P<0.01; ***P<0.001).
Figure 3.2. Comparison of front and hind laminar inflammatory response at OG1 lameness. Median fold change in laminar (both front and hind) IL-6, CXCL8 and COX-2 mRNA concentrations after CHO administration in OG1 horses were significantly increased when compared with control (CON) horses (*P<0.05). There was no significant difference between front and hind lamina of the OG1 horses.
Figure 3.3. Number of calprotectin (MAC387) positive cells in the deep dermal lamina and primary dermal lamina. Graphs demonstrate the average number of calprotectin (MAC387) positive cells per high power field (40X) in the deep dermal lamina and primary dermal lamina. Leukocyte migration was significantly increased (*P<0.05) in the OG1 horses (both front and hind) when compared with controls. There was no difference between the front and hind lamina. Images above graphs (captured at 20X magnification) demonstrate DDL and PDL regions, respectively.
Figure 3.4. Comparison of calprotectin immunostaining of the lamina between a CON horse and an OG1 horse. Note there is little to no staining of leukocytes in the CON horse vs. the OG1 (Insert shows leukocyte staining in the primary dermal lamina surrounding blood vessels). In addition, there is increased staining present in the stressed lamina epithelium in the OG1 horse compared with CON. Images captured at 20X magnification.
Table 3.1. Fold changes* in lamellar inflammatory mediator mRNA concentration after CHO administration

<table>
<thead>
<tr>
<th>Gene</th>
<th>Front**</th>
<th>Hind</th>
<th>Front**</th>
<th>Hind</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>NS</td>
<td>NS</td>
<td>11.2 ↑</td>
<td>27 ↑</td>
</tr>
<tr>
<td>IL-6</td>
<td>NS</td>
<td>NS</td>
<td>2089 ↑</td>
<td>2749 ↑</td>
</tr>
<tr>
<td>CXCL1</td>
<td>4.5 ↑</td>
<td>9.3 ↑</td>
<td>9.8 ↑</td>
<td>20 ↑</td>
</tr>
<tr>
<td>CXCL8</td>
<td>45.4 ↑</td>
<td>40 ↑</td>
<td>25.6 ↑</td>
<td>129 ↑</td>
</tr>
<tr>
<td>COX-1</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>COX-2</td>
<td>NS</td>
<td>NS</td>
<td>31.2 ↑</td>
<td>51.4 ↑</td>
</tr>
</tbody>
</table>

* Expressed as median fold change from respective controls (front vs. hind).
** Values from data of previous reports Leise et al (2010) for IL-1β, IL-6, COX-1 and COX-2 and Faleiros et al. (2010) for CXCL1 and CXCL8.
Values displayed are significant from controls (P<0.05); NS= non significant fold change from controls.
Chapter 4. Laminar Regulation of STAT1 and STAT3 in the Black Walnut Extract and Carbohydrate Overload Induced Models of Laminitis

ABSTRACT

Objective: To determine the regulation of STAT1 and STAT3 signaling in the laminae from horses administered black walnut extract (BWE) or carbohydrate overload (CHO) to induce laminitis.

Methods: Western hybridization analysis was performed to determine concentrations of tyrosine- and serine-phosphorylated STAT1 and STAT3 from archived laminar tissue samples from previous BWE and CHO protocols. Real time-quantitative PCR was also performed to assess mRNA concentrations of target genes of STAT1 (CIS, IRF-1 and ICE) and STAT3 (CIS, SOCS3) from lamina. Immunohistochemical analysis of STAT3 tyrosine phosphorylation was performed to determine the location of this phosphorylation.

Results: Increases in tyrosine and serine phosphorylation of STAT3 are present in lamina from both the BWE and CHO models at the developmental and onset of lameness time points. No change in tyrosine or serine STAT1 phosphorylation was present in the lamina from either the BWE or the CHO models. Only the BWE model demonstrated an increase in laminar mRNA concentrations of target genes CIS, IRF-1 and ICE (for
STAT1) at the developmental time. No change in STAT1 target gene mRNA concentrations was present at either the DEV or OG1 time in the CHO model. SOCS3 (for STAT3) mRNA concentrations were increased at the onset of lameness in both the CHO and BWE models.

**Conclusions:** Activation of the STAT3 signaling pathways is likely to play a role in equine laminitis, similar to its reported involvement in organ injury/failure in human sepsis. Regulation of the JAK-STAT pathway, through STAT3 inhibitors, may serve as potential therapeutic target for controlling the inflammatory response in the septic horse at risk for laminitis.

**INTRODUCTION**

As is well described in organ injury in human sepsis, sepsis-related laminitis in horses is associated with an intense local inflammatory response, most likely occurring due to circulating bacterial products, local flow disturbances (i.e. ischemia/reperfusion), or both. Dysregulation of inflammatory mediator expression, including large increases in proinflammatory cytokine expression, is well documented both in experimental models of sepsis related organ injury⁴⁻⁷ and models of sepsis-related laminitis.⁸⁻⁷ One of the major inflammatory signaling pathways believed to be involved in sepsis is the JAK-STAT pathway.⁸,⁹

The signal transducing activators of transcription factors (STATs) are latent intracytoplasmic proteins that once activated via phosphorylation are translocated to the nucleus and serve as transcription factors that result in the production of various
inflammatory genes as well as those regulating cell growth and development.\textsuperscript{10} Seven different STATs are known including STAT 1, 2, 3, 4, 5a, 5b, and 6. Although they vary in their functions, the STATs share a fairly well-conserved structure consisting of a series of six domains including, a N-terminal region that stabilizes STAT dimers, a coiled coil region, a DNA binding domain, a linker domain, Src-homology 2 domain (SH2) domain which is responsible for receptor binding, and a C-terminal transcriptional activation (TAD) domain, which also serves as the location of tyrosine and serine phosphorylation.\textsuperscript{11, 12}

Phosphorylation or activation of STATs can occur by several different pathways. The most studied and first described pathway is JAK-STAT pathway activated by cytokine binding to a cell surface receptor.\textsuperscript{13} Transmembrane cytokine (such as IFN or IL-6) receptor dimerization and ligand binding allows for two non-covalently bound Janus kinase (JAK) proteins to be apposed, thereby allowing subsequent phosphorylation of the JAK protein. JAK phosphorylation on tyrosine residues leads to release of the JAK catalytic site which allows for binding of the SH2 domain of the STAT protein. STATs are then phosphorylated at a tyrosine residue where they then form dimers and are translocated to the nucleus through associations with importins to serve as transcription factors.\textsuperscript{14, 15} In addition to tyrosine phosphorylation, serine phosphorylation of STAT1, 3, 4 and 5a/b has also been reported to occur.\textsuperscript{16} Initially it was reported that serine phosphorylation was necessary for full transcriptional activation;\textsuperscript{17, 18} however, more recent reports suggest that is not necessarily the case and requirement of serine phosphorylation varies depending on type of cell and source of extracellular
stimulation. The particular protein kinase necessary for serine phosphorylation has also been reported to be variable depending on cell type and stimulatory factor. Protein kinase-C (PKC), Jun-N-terminal kinase, Extracellular signal-regulated kinase (ERK), p38MAPK, and mammalian target of rapamycin (mTOR) have all been reported to phosphorylate serine residues on STAT molecules.

STAT1 and STAT3, despite being very similar in structure, have reportedly many opposing actions of each other. STAT1 activation promotes apoptosis and cell 
cycle arrest while STAT3 activation inhibits apoptosis and stimulates cell growth and 
migration. In addition, it has been reported that STAT1 activation results in a 
proinflammatory response, whereas STAT3 activation is more anti-inflammatory. 
However, as with many signaling mediators these facts are not so black and white. 
STAT3 in particular, appears to have both pro- and anti-inflammatory effects secondary 
to either tyrosine or serine phosphorylation alone or in combination.

Since a significant inflammatory response resulting from cascade of cytokine and 
chemokine production occurs in patients with sepsis/SIRS, it is not unexpected that both 
STAT1 and STAT3 would have important cell signaling roles in these conditions. While 
STAT1 activation via IFNγ results in activation of macrophages and enhanced killing of 
bacteria, it also can result in the production of deleterious inflammatory mediators such 
as TNFα or HMGB1 which may increase morbidity and mortality in septic patients. STAT3 also appears to have conflicting roles in sepsis/SIRS with some reports stating a 
protective effect of STAT3 activation and other reports suggesting that STAT3 may 
support continued inflammation in these patients. For example, substantial increase

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in multiple cytokines (IL-1β, TNFα, IL-6, IL-12 and IFNγ) lead to uncontrolled local and systemic inflammation and increased lethality in a group of conditional STAT3 knockout mice (no STAT3 in neutrophils or macrophages) with septic peritonitis secondary to CLP when compared with the wild type. However, inhibitors of STAT3 also protected mice against CLP induced multiple organ damage and death when compared with an untreated group.

Equine laminitis secondary to sepsis/SIRS is known to result in a significant proinflammatory laminar response both in the developmental stages and at the onset of lameness. IL-1β, IL-6, IL-12, CXCL1, and CXCL8 are cytokines and chemokines that have been reported to be significantly increased in laminar tissue in the developmental and onset of lameness after administration of black walnut extract or carbohydrate overload. Due to the roles that STAT1 and STAT3 play in inflammatory diseases, such as sepsis, it was the objective of this study to determine if STAT1 and/or STAT3 played role in cellular signaling within the lamina during the development of laminitis.

**MATERIALS AND METHODS**

**Laminar tissue samples**

Frozen (-80°C) archived laminae tissue samples from previous studies were used to assess STAT1 and STAT3 tyrosine and serine phosphorylation as well as the production of genes associated with the activation of STAT1 and/or STAT3. Laminar samples were collected from horses that received either black walnut extract (BWE) or an
overload of carbohydrate (CHO; in the form of cornstarch and wood flour). Laminar samples were also collected from horses that received only deionized water to serve as a control. Tissue samples from the BWE horses were collected from horses at 1.5h and 3h post BWE administration and at the onset of lameness (LAM). Samples from control horses for this treatment were collected at 3h (E. CON) and 12h (L.CON) post administration of water. Laminar samples from the CHO horses were collected at the onset of fever (developmental group, DEV) and at the onset of lameness (LAM). Laminar tissue was collected from horses at 24h post water administration (CON) to serve as controls for the CHO treatment. All animal protocols were approved by the Animal Care and Use Committees of The Ohio State University or the University of Missouri. Induction of laminitis with BWE\textsuperscript{39} and CHO\textsuperscript{7} and collection of laminar tissue was performed as previously described.

**Protein extraction**

Protein was extracted from previously snap frozen laminar tissue. Briefly laminar tissue was pulverized and homogenized on ice in M-PER lysis buffer (Pierce; Rockford, IL) with the addition of protease cocktail inhibitor (Roche; Indianapolis, IN), PMSF (Pierce; Rockford, IL), and phosphatase inhibitor cocktail (Pierce; Rockford, IL). After incubation, the supernatant was collected by centrifugation and protein concentrations were determined using Bradford reagent (BioRad). After quantification samples were aliquotted and stored at -80°C until use.

**Western blot hybridization**
Laminar protein from horses receiving BWE and CHO were used to assess total and phosphorylated tyrosine and serine STAT1 and STAT3. Laminar protein samples from each group of horses (CON, DEV, LAM for the CHO horses and E.CON, L.CON, 1.5H, 3H, and LAM for the BWE horse) were equally pooled and 30 μg of protein was loaded per group for the CHO treated horses and for the BWE treated horses. Proteins were run on an 8% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membranes were blocked 3% BSA in PBS for 2 hours at room temperature. After washing blots were probed with rabbit anti-phospho-STAT3 Tyr705 (Cell Signaling Technology Inc, Danvers, MA), anti-phospho-STAT Ser727 (Invitrogen, Carlsbad, CA), anti-phospho-STAT1 Tyr701 (Cell Signaling Technology Inc, Danvers, MA) or anti phospho-STAT1 Ser727 (Invitrogen, Carlsbad, CA) at a 1:1000 dilution and incubated overnight at 4°C. After washing, the membranes were probed with an anti-rabbit IgG secondary antibody coupled to a horseradish peroxidase at a 1:20,000 dilution for 1 hour at room temperature. The labeled proteins were detected using west femto developing reagent. Blots were stripped after determination of the presence of STAT1 or STAT3 tyrosine and serine phosphorylation and reprobed with rabbit anti-STAT3 or rabbit anti-STAT1 antibody (Cell signaling, Danvers, MA) for determination of the total amount of the respective STAT protein. The blots were stripped one more time and reprobed with anti-β actin antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA) for the conformation of equal loading among groups. All blots were exposed to film and imaged on the Kodak imager.

Immunohistochemistry
Laminar sections from control horses and from horses at the onset of lameness (LAM) of both the BWE and CHO models were used to immunolocalize the production of tyrosine 705 phospho-STAT3. The paraffin-embedded samples were sectioned at 5-µm thickness, deparaffinized, and treated by boiling with EDTA for antigen retrieval. After blocking, sections were incubated at 4°C overnight with a rabbit mAb phospho-tyrosine 705 STAT3 antibody at a 1:50 dilution (Cell Signaling Technology, Inc; Danvers, MA). A biotinylated anti-rabbit antibody (VECTASTAIN ABC kit; Vector Laboratories, Burlingame, CA) was then applied at a 1:100 dilution at room temperature for 30 minutes. The immunoreactivity was observed using a peroxidase (DAB kit; Vector Laboratories, Burlingame, CA) following the instructions of the manufacturer and Harris hematoxylin (Fisher Scientific, Kalamazoo, MI) was used for the counterstain. Whole slide digital images (WSIs) were obtained from stained sections using an automated scanning robot (T2 Scan Scope) with a 40× magnification at a spatial sampling period of 0.2 µm per pixel. WSIs were assessed using a software program (Aperio Image Scope, Vista CA) to capture images from each section, which were then transformed into jpg files.

**RNA isolation and cDNA synthesis**

Total RNA was extracted previous snap frozen laminar tissues using a kit (Absolutely RNA Miniprep; Stratagene, LaJolla, CA) which includes a DNase treatment to remove genomic DNA contamination. PolyA mRNA was then isolated (mRNA extraction kit; Roche, Indianapolis, IN) and used to make complementary DNA (cDNA) for each sample via reverse transcription (Retroscript; Ambion Inc; Austin, TX) using at
total of 400 ng of mRNA. The cDNA was frozen at -20°C and stored until used for real-time quantitative PCR (RT-qPCR) analysis.

**Real-time qPCR procedure**

Real-time quantitative PCR was performed using a thermocycler (LightCyler, Roche Molecular Biochemical, Indianapolis, IN) and quantified with external standards with the fluorescent format for SYBR Green I dye as previously described. Primers were designed from equine-specific sequences. The housekeeping genes, β-actin, β-2 microglobulin, glyceraldehyde-3 phosphate dehydrogenase, and TATA-box binding protein, have been previously reported (Waguespack et al. 2004a and Waguespack et al. 2004b). The primers for ICE-1, IRF-1, CIS, and SOCS-3 were as follows: ICE-1 f- 5'-TGGTCGAAAGGATATGGAAAGAAAACTCAG-3' and r- 5'-TGGGCGGGCGACAAATCGAAGAAAACCTCAG-3'; IRF-1f- 5'-GTGAAGGACCAGAGCAGGAACA-3' and r- 5'-TCTTGGTGAGGGGTGGGAGCAT-3'; CIS f- 5'-CCAGCGAGGCCCGGCAACAC-3' and r- 5'-TCTTGTTGAGGGGTGGGAGCAT-3'; and SOCS3 f- 5'-CCCGCCGGCACCTTTCT-3' and r- 5'-GCACCAGCTTGAGCACCGACGCAGTC-3'. All primers were screened using gel electrophoresis and melt curve analysis (LightCyler, Roche Molecular Biochemical, Indianapolis, IN) to confirm amplification of a single cDNA fragment of the correct melting temperature and size. Amplified cDNA fragments of each gene were ligated into a vector (TOPO 010 E.Coli; Invitrogen, Carlsbad, CA) and the vectors linearized with Hind III restriction enzyme (Invitrogen, Carlsbad, CA) for the use of templates to generate a standard curve for the RT-qPCR reaction. Amplified cDNA fragments
were sequenced after cloning to confirm correct DNA sequence for the products of each primer.41

Each cDNA sample was diluted 1:5 and 1:500 with 1X TE buffer to be used in the cytokine and housekeeping PCR reactions, respectively. PCR reactions were performed in glass capillaries containing 5 μl of diluted sample and 15 μl of PCR master mixture. Master mix included the following: 1 unit of Taq polymerase (Invitrogen, Carlsbad, CA), 0.2 units of uracil-N-glycosylase (Roche Molecular Biochemical, Indianapolis, IN), 1:10,000 dilution of SYBR Green stock solution, forward and reverse primers, PCR nucleotide plus (Roche Molecular Biochemical, Indianapolis, IN) and PCR buffer. The PCR buffer (20mmol/l Tris-HCL) contained 0.05% each of Tween 20 and nonionic detergent. Primers were used at a concentration of 5 μmol/L.

Uracil-N-glycosylase activation, to prevent PCR product carryover, was conducted at 50°C for 2 minutes and was followed by denaturation at 95°C for 2 minutes. Amplification occurred for 40 to 45 cycles, with the annealing temperature set at 1-5°C below the melting temperature for each specific set of primers, extension was set at 72°C for 5 seconds, and fluorescence acquisition for 10 seconds in the SYBR Green format. Single fluorescence acquisition in each cycle was set at either 80 or 82°C, depending on the melting temperature of the cDNA product of interest as previously described.40,41 After amplification cycling, melting curves of the PCR product were acquired through a stepwise increase in temperature from 65-95°C.

Standard and target samples were prepared in separate capillaries. Standard curves and water for negative control were performed for each gene of interest and
amplified with each series of reactions. Standards were made up of 10-fold serial
dilutions of linearized plasmids containing the different gene-specific cDNA inserts. All
samples were run in duplicate.

qRT-PCR data analysis

Average copy number from each sample was determined for each gene
(housekeeping and inflammatory marker). As, previously reported, RT-qPCR data from
the four housekeeping genes for each sample was evaluated by the computer software
program geNorm (Ghent University; Ghent, Belgium) to determine which two genes
received the best acceptable score to be used for normalization.35 β-actin and
glyceraldehyde-3 phosphate dehydrogenase were selected and then used by the geNorm
software to create a normalization factor for each sample. To determine the corrected
copy number value for each sample the amplification data obtained by RT-qPCR for each
gene was divided by the normalization factor of the selected housekeeping gene for the
same sample. After normalization, the fold change from the average control value was
calculated for each sample. Data were analyzed non-parametrically using the Kruskal-
Wallis with Dunn’s multiple comparisons test to compare groups between the CHO
horses and were analyzed using a Mann-Whitney unpaired t-test to compare the control
groups to 1.5H, 3H and the LAM BWE groups. Statistical significance was set at P<0.05
to maintain type I error for all tests.

RESULTS

Western blot hybridization – BWE model
There was no difference in tyrosine 701 or serine 727 phosphorylation of STAT1 between groups in the lamina of the 1.5H, 3H, and LAM groups compared with their respective controls (Figures 4.1 and 4.2). Increased tyrosine 705 and serine 727 phosphorylation of STAT3 in lamina was present in the 1.5H, 3H, and the LAM group of horses when compared with their respective controls (Figures 4.5 and 4.6); however, there was no difference in signal intensity between the early (1.5H and 3H) horses and the LAM horses.

*Western blot hybridization – CHO model*

No difference in tyrosine 701 and serine 727 STAT1 phosphorylation was noted between groups of horses administered a carbohydrate overload (Figures 4.3 and 4.4); however, increased tyrosine 705 and serine 727 phosphorylation of STAT3 in the lamina was present in the DEV and LAM group of horses when compared with the CON group (Figures 4.7 and 4.8). Greater signal was present in the LAM horses compared with the DEV for both tyrosine 705 and serine 727 STAT3 phosphorylation.

*Immunohistochemistry*

Immunohistochemistry of paraffin embedded laminar sections from CON and LAM horses were used to demonstrate nuclear translocation of phosphorylated STAT3 tyrosine 705. Immunostaining of nuclei was present in the laminar epithelial cells as well as cells within the laminar dermis (many of which are suspected to be neutrophils or macrophages) of the LAM horses from both the BWE and CHO models when compared to their respective controls (Figure 4.9).

*qRT-PCR*
STAT1 (CIS, ICE-1, IRF-1) and STAT3 (CIS, SOCS3) responsive genes were evaluated in both the BWE and CHO models of laminitis (Table 1 and 2). Increases in expression of both STAT1 and STAT3 responsive genes were present in horses administered BWE (Table 4.1). Increased mRNA concentrations of IRF-1, ICE-1, and CIS were present in the 1.5h horses when compared with the E. CON horses. Increases in mRNA concentrations of ICE-1, CIS, and SOCS3 were also present in the 3h horses when compared with the E. CON horses. Only SOCS3 mRNA concentrations were increased in the LAM group when compared with L. CON horses. In the CHO model, only increases in SOCS3 mRNA concentrations were found in the LAM horses when compared with controls (Table 4.2). There were no differences in expression of STAT1 responsive genes between groups in the CHO model.

**DISCUSSION**

As the presence of laminar inflammation has been well documented early in the developmental phases and onset of lameness in both the BWE and CHO models of laminitis, it is now essential that signaling pathways involved in laminar inflammation be evaluated in these models in order to discover targets for pharmaceutical intervention. This study evaluated two STAT signal transduction molecules, STAT1 and 3, which have been documented to play a role in sepsis/SIRS in humans and rodent models and were suspected to play a role in laminar inflammation and subsequent failure. As STAT1 is primarily activated by type I and type II interferons and as there has been no significant increases in mRNA concentrations of IFNα, IFNβ
(unpublished data, Belknap), or IFNγ documented at any stage in lamina from horses in horses administered BWE or CHO the absence of a significant STAT1 response is not completely unexpected. The lack of STAT1 activation in the CHO model, represented by no difference in phosphorylation of tyrosine 701 or serine 727 between groups, was also supported by the RT-PCR, results demonstrating no increase in the STAT1 responsive genes IRF-1 and ICE-1. In the BWE model, significant increases in the STAT1 responsive genes IRF-1 and ICE-1 were present in the lamina of the 1.5h and 1.5h and 3h horses, respectively; however they were not found to be increased in the lamina of the LAM horses. It is suspected that other signal transducers are involved in the activating the increased gene expression of IRF-1 and ICE-1 in the developmental phases of laminitis secondary to BWE administration, since neither tyrosine 701 or serine 727 STAT1 phosphorylation were found to be increased in the 1.5h and 3h laminar samples. For example, IRF-1 has been found to be activated via toll-like receptor 9 signaling through the adaptor MyD88 and ICE-1 (also known as caspase-1) has been found to be activated secondary to flagellin binding to the intracellular NOD-like receptor Ipaf in macrophages.

Increased phosphorylation of STAT3 tyrosine 705 and serine 727 in lamina from both BWE and CHO in the DEV and LAM horses was found in this study. Phosphorylation of STAT3 was greater in the LAM horses then in the DEV horses from both models. Since one of the major activators of STAT3 working through the gp130 receptor is IL-6 and IL-6 type cytokines, and IL-6 mRNA concentrations have been reported to be greatly increased in the laminar tissue after BWE and CHO
increased tyrosine and serine phosphorylation of STAT3 in laminar tissue was predictable. Increased phosphorylation of serine and tyrosine moieties of STAT3 were found as early as the DEV period after CHO administration; however, increased IL-6 mRNA concentrations were only found in the LAM horses after CHO indicating that STAT3 activation may be occurring through a pathway not involving IL-6. As IL-10 and growth factors, including epidermal growth factor (EGF) and platelet derived growth factor (PDGF), can also activate STAT3, it is possible that one of these factors are involved in its activation. It is unlikely, however, that STAT3 is stimulated by IL-10 as it has only been found to be down regulated in lamina 3h after BWE administration and not changed after CHO administration. EGF receptors have been reported to be present in laminar tissue, particularly in the laminar basal and parabasal cells with increased number of receptors present in the hyperproliferative epidermis from horses with chronic laminitis; therefore, it is possible that EGF could be playing a role in STAT3 activation in the laminar tissue. It is also possible that even though laminar mRNA concentrations of IL-6 were not increased in the DEV horses after CHO administration, the laminar cells may still be exposed to circulating IL-6 protein.

Phosphorylation of STAT3 tyrosine and serine inhibits apoptosis, induces cellular proliferation and migration and serves to promote the production of both pro- and anti-inflammatory mediators. Examples of inflammatory mediators that are up-regulated by STAT3 include MMP-9, MMP-2, ICAM1, and COX-2, whereas IL-1β, IL-6, IL-8 and MCP-1 have been reported to be both up-regulated and down-regulated depending on various conditions. Interestingly, all of these mediators have been reported to be
increased in laminar tissue of horses either at the developmental period and/or the onset of lameness in the BWE and CHO models of laminitis. Although NF-κB plays an important role in IL-1β and IL-6 expression, STAT3 tyrosine phosphorylation has been reported to be critical for both IL-1β and IL-6 gene expression and protein production of murine macrophages in response to LPS and live bacteria. Circulating endotoxin has been documented in both the oligofructose model and in the carbohydrate overload model and could serve as a stimulator of tyrosine STAT3 phosphorylation in this model. In addition, administration of stattic, a STAT3 inhibitor, blocks the LPS stimulation of IL-1β and IL-6 in macrophages but has no effect on TNFα production; therefore, consistent absence of a TNFα response in lamina from models of experimentally induced laminitis may be explained by the inability of STAT3 to stimulate TNFα gene expression, while still stimulating IL-1β and IL-6 expression. It is also possible that STAT3 activation works directly with NF-κB resulting in a continuation of an inflammatory response, as in tumors where STAT3 interacts with the NF-κB family member RELA (p65), trapping it in the nucleus resulting in constitutive NF-κB activation prolonging inflammation. RELA (p65)-p50 heterodimer is known to be crucial for the genes encoding proinflammatory mediators such as IL-1β, IL-6, and COX-2. Inactivation of STATs occur through the production of inhibitors targeting various steps in the pathway, through the production of phosphatases, or by protein degradation via the ubiquitin-proteosome pathway. STAT inhibitors include suppressor of cytokine signaling (SOCS), cytokine-inducible SH2-containing protein
(CIS), and protein inhibitor of activated STAT (PIAS). In this study, we assessed two inhibitors of STATs, SOCS-3 and CIS, which have inducible expression by ligand binding. Significant increases of SOCS-3 mRNA concentrations were present in the LAM horses from both the BWE and CHO models and were also present in the 3h developmental horses after BWE administration. The increase in SOCS3 most likely occurs secondary to STAT3 activation in these horses as a method to limit the STAT3 transcriptional response in the lamina. SOCS3 has been reported to negatively regulate the gp130-STAT3 response in keratinocytes and immune cells preventing excessive neutrophil accumulation and production of MIP-1α, and is believed to be important in controlling cytokine expression secondary to mild to moderate ischemia-reperfusion injury in the liver. Increases in CIS mRNA concentrations were only present in the developmental horses (1.5h and 3h) after BWE administrations with no change found after CHO administration. The exact reason for this is unknown, but the duration of SOCS and CIS responses can be variable, and although cytokines like IL-6 can stimulate CIS mRNA production, their biological effects are mostly related to growth factors such as EPO, GH and prolactin.

Although activation of both STAT1 and STAT3 has been reported in models of human sepsis/SIRS, only STAT3 activation was seen in the lamina from the CHO and BWE models of laminitis. With the development of STAT inhibitors to be used in the treatment of many cancers, determination of the affect STAT3 activation has on sepsis/SIRS is important in order to decide whether these therapies can be used in septic patients to improve outcome. The use of STAT3 inhibitors has been found to improve
survivability in a CLP model of sepsis.\textsuperscript{33} In contrast, liver hyporesponsiveness to IL-6 in a CLP model of sepsis has been reported to occur in fulminant sepsis, suggesting that STAT3 activation could be important in the hepatic response to sepsis\textsuperscript{44} and liver specific STAT3 knockout mice had increase mortality and decreased amounts of acute phase proteins than compared to controls.\textsuperscript{45} Therefore, due to the mixed results in studies involving rodent models of sepsis, more research is needed to determine if STAT3 results in a beneficial or detrimental effect in the development of laminitis.

REFERENCES


Figure 4.1 STAT1 tyrosine responses in the lamina from horses receiving BWE.

Western blot analysis of STAT1 tyrosine 701 phosphorylation is shown in the top image from control horses (E. CON = early control; L. CON = late control), horses during the developmental phases (1.5H post BWE and 3H post BWE), and from horses at the onset of lameness (LAM) that received BWE. Note there is no difference between groups. Total STAT1 tyrosine 701 and β-actin images were acquired after stripping membrane probed for phosphor-tyrosine and demonstrate even loading of the gel.
Figure 4.2. STAT1 serine responses in the lamina from horses receiving BWE.

Western blot analysis of STAT1 serine 727 phosphorylation is shown in the top image from control horses (E. CON = early control; L. CON = late control), horses during the developmental phases (1.5H post BWE and 3H post BWE), and from horses at the onset of lameness (LAM) that received black walnut extract. Note there is no difference between groups. Total STAT1 serine 727 and β-actin images were acquired after stripping membrane probed for phosphor-tyrosine and demonstrate even loading of the gel.
Figure 4.3. STAT1 tyrosine responses in the lamina from horses receiving CHO.

Western blot analysis of STAT1 tyrosine 701 phosphorylation is shown in the top image from Control (CON), onset of fever (DEV), and onset of lameness (LAM) horses that received an overload of carbohydrate. Note there appears to be no real difference between the groups. Total STAT1 tyrosine701 and β-actin demonstrated equal loading of the gel.
Figure 4.4. STAT1 serine responses in the lamina from horses receiving CHO.

Western blot analysis of STAT1 serine 727 phosphorylation is shown in the top image from control (CON), onset of fever (DEV), and onset of lameness (LAM) horses that have received an overload of carbohydrate. Note there is no difference between groups. Total STAT1 serine 727 and β-actin images were acquired after stripping membrane probed for phosphor-serine and demonstrate even loading of the gel.
Figure 4.5. STAT3 tyrosine responses in the lamina from horses receiving BWE.

Western blot analysis of STAT3 tyrosine 705 phosphorylation is shown in the top image from control horses (E. CON = early control; L. CON = late control), horses during the developmental phases (1.5H post BWE and 3H post BWE), and from horses at the onset of lameness (LAM) after receiving black walnut extract. Note increased signal in the 1.5H, 3H, and LAM horses compared to the two CON groups. Total STAT3 tyrosine 705 and β-actin images were acquired after stripping membrane probed for phosphotyrosine and demonstrate even loading of the gel.
**Figure 4.6. STAT3 serine responses in the lamina from horses receiving BWE.**

Western blot analysis of STAT3 serine 727 phosphorylation is shown in the top image from control horses (E. CON = early control; L. CON = late control), horses during the developmental phases (1.5H post BWE and 3H post BWE), and from horses at the onset of lameness (LAM) after receiving black walnut extract. Note increased signal in the 1.5H, 3H, and LAM horses compared to the two CON groups. Total STAT3 serine 727 and β-actin images were acquired after stripping membrane probed for phosphor-tyrosine and demonstrate even loading of the gel.
Figure 4.7. STAT3 tyrosine responses in the lamina from horses receiving CHO.

Western blot analysis of STAT3 tyrosine 705 phosphorylation is shown in the top image from control horses (CON), horses at the onset of fever (DEV), and from horses at the onset of lameness (LAM) that received an overload of carbohydrate. Note there an increase in tyrosine phosphorylation in the DEV and LAM horses compared to controls, with horses developing lameness (LAM) having the greatest response. Total STAT3 tyrosine 705 and β-actin images were acquired after stripping membrane probed for phosphor-tyrosine and demonstrate even loading of the gel.
Figure 4.8. STAT3 serine responses in the lamina from horses receiving CHO.

Western blot analysis of STAT3 serine phosphorylation is shown in the top image from control horses (CON), horses at the onset of fever (DEV), and from horses at the onset of lameness (LAM) that received an overload of carbohydrate. Note there an increase in serine phosphorylation in the DEV and LAM horses compared to controls, with horses developing lameness (LAM) having the greatest response. Total STAT3 tyrosine 705 and β-actin images were acquired after stripping membrane probed for phosphor-tyrosine and demonstrate even loading of the gel.
Figure 4.9. Immunohistochemical staining of STAT3 tyrosine705 phosphorylation. Images demonstrate increased uptake in the nucleus of the laminar epithelial and dermal cells at the onset of lameness (LAM) from both the BWE and CHO models of laminitis when compared to no uptake in the respective control (CON) horses.
### Table 4.1. Laminar STAT responsive genes after black walnut extract administration

<table>
<thead>
<tr>
<th>Gene</th>
<th>1.5H CON</th>
<th>1.5H</th>
<th>3H CON</th>
<th>3H</th>
<th>L. CON</th>
<th>LAM</th>
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<tbody>
<tr>
<td>ICE-1</td>
<td>mRNA concentration*</td>
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<td>141</td>
<td>111</td>
<td>153</td>
<td>449</td>
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<td></td>
<td>(25%-75% percentile)</td>
<td>(51 - 101)</td>
<td>(98 - 249)</td>
<td>(82 - 123)</td>
<td>(71 - 341)</td>
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<td>Fold increase^</td>
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<td>0.7</td>
<td>1.5</td>
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</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>IRF-1</td>
<td>mRNA concentration*</td>
<td>476</td>
<td>5070</td>
<td>220</td>
<td>1870</td>
<td>1350</td>
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<tr>
<td></td>
<td>(25%-75% percentile)</td>
<td>(298 - 592)</td>
<td>(1,412 - 9,200)</td>
<td>(118 - 259)</td>
<td>(1,221 - 5,940)</td>
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<td>NS</td>
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<td>NS</td>
<td></td>
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<tr>
<td>CIS</td>
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<td>3,440</td>
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<td>4,720</td>
<td>2,810</td>
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<td></td>
<td>(25%-75% percentile)</td>
<td>(1,186 - 2,245)</td>
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<td>P&lt;0.01</td>
<td>NS</td>
<td></td>
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<tr>
<td>SOCS3</td>
<td>mRNA concentration*</td>
<td>0.15</td>
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<td>0.09</td>
<td>3.12</td>
<td>0.13</td>
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<td>(25%-75% percentile)</td>
<td>(0.1 - 0.45)</td>
<td>(0.24 - 0.75)</td>
<td>(0 - 0.49)</td>
<td>(1.2 - 5.9)</td>
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<td>P&gt;0.01</td>
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* mRNA values expressed as median cDNA copies per normalization factor

^ Fold increase expressed in medians from control.  NS= non significant fold change from control.
Table 4.2. Laminar STAT responsive genes after carbohydrate overload administration

<table>
<thead>
<tr>
<th>Gene</th>
<th>CON mRNA concentration*</th>
<th>DEV mRNA concentration*</th>
<th>LAM mRNA concentration*</th>
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<td>(25%-75% percentile)</td>
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<td>(25%-75% percentile)</td>
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<tr>
<td>ICE-1</td>
<td>536</td>
<td>681</td>
<td>1,345</td>
</tr>
<tr>
<td></td>
<td>(373 - 810)</td>
<td>(299 - 1,720)</td>
<td>(902 - 2,560)</td>
</tr>
<tr>
<td>Fold increase^</td>
<td>1.17</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>NS</td>
<td>NS</td>
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</tr>
<tr>
<td>IRF-1</td>
<td>53,600</td>
<td>110,450</td>
<td>76,850</td>
</tr>
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<td></td>
<td>(29,825 - 61,025)</td>
<td>(50,625 - 313,250)</td>
<td>(53,375 - 194,500)</td>
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<td>Fold increase^</td>
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<tr>
<td>CIS</td>
<td>2,945</td>
<td>3,335</td>
<td>3,395</td>
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<td>(2,295 - 4,130)</td>
<td>(2,715 - 4,785)</td>
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<td>SOCS3</td>
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<td></td>
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* mRNA values expressed as median cDNA copies per normalization factor
^ Fold increase expressed in medians from control. NS= non significant fold change from control.
ABSTRACT

Objective: To determine the inflammatory events occurring in hepatic, pulmonary, and renal tissue after administration of carbohydrate overload (CHO) to induce laminitis in the horse.

Methods: Archived pulmonary, hepatic and renal tissue samples were used from eighteen horses administered either water (CON group, n=6, euthanized 24 h after water administration) or 17.6 g of starch/kg body weight via nasogastric tube. Horses administered CHO were euthanized after a two degree increase in rectal temperature (DEV group, n=6) or at the onset of Obel grade 1 lameness (OG1 group, n=6). Real time-quantitative PCR for IL-1β, IL-6, CXCL8, IL-10, TNFα, COX-1 and COX-2 was performed using snap frozen tissue samples. MAC387 immunohistochemistry from formalin fixed tissues was performed to determine the presence and magnitude of leukocytes migration in the liver and lung. Serum chemistries were performed from 3 time points (pre, onset of fever, and onset of lameness) from the OG1 horses.

Results: Significant increases in pro-inflammatory mediators were present in the liver, lung, and kidney. The hepatic tissue had the earliest response with increase in mRNA
concentrations of IL-1β, CXCL8, IL-10 and TNFα in the DEV horses when compared with controls. Increases in these inflammatory mediators, along with COX-2 were also present in the liver of the OG1 horses. Only increases in mRNA concentrations of IL-1β were present in all three tissues in the OG1 horses when compared with controls. Significant increases in leukocyte counts were also present in the lung in both the DEV and OG1 horses, but no differences were noted in the hepatic tissue between groups.

**Conclusions:** Although no significant difference in leukocyte counts were seen, the liver underwent the greatest inflammatory signaling of the three tissues evaluated, most likely reflecting function of this organ in processing toxins from the gastrointestinal tract. Surprisingly, the kidney appears to have the least amount of inflammatory signaling. Furthermore, increases in inflammatory gene expression of the organs were less than previously reported for the primary “target tissue” in the septic horse, the lamina.

**INTRODUCTION**

In humans with endotoxemia/sepsis, a systemic inflammatory response syndrome (SIRS) occurs, leading to injury and dysfunction/failure of several organs including most commonly the liver, lungs, kidney, and intestinal tract. Compartmentalization or the occurrence of varying inflammatory responses in different organs or blood compared with each other has been shown to be present during sepsis/SIRS and is believed to be a result of cellular composition of these organs. Although each organ may have their own distinct inflammatory gene expression pattern, the ability exists for these organs to release inflammatory mediators into circulation and
thereby have a resulting affect on other organs and their production of inflammatory mediators. It is this overwhelming systemic inflammatory response and recruitment of inflammatory cells into organs that is believed to result in organ failure in humans with sepsis.\textsuperscript{4, 5}

Sepsis in the adult horse, the most common cause resulting from gastrointestinal tract diseases such as enterocolitis or colon torsions, most commonly leads to injury of the digital laminae in the form of laminitis. A similar SIRS has been reported in the black walnut extract (BWE) model of laminitis evidence by leukopenia, leukocyte migration into the tissues and increased inflammatory gene expression in the lung and liver.\textsuperscript{6, 7} Interestingly, the carbohydrate overload (CHO) laminitis model, a model that more closely reflects the severity of laminar injury observed in clinical sepsis-related laminitis cases, does not have the same SIRS-like events (i.e. no leukopenia) as the BWE model. Furthermore, it has been recently reported that the laminar inflammatory events occur at a later onset in the CHO model compared with the BWE. The difference in response is believed to be similar to the differences seen between the cecal ligation puncture (CLP) and endotoxin bolus models of sepsis. Therefore, the purpose of this study was to determine inflammatory signaling events in hepatic, pulmonary, and renal tissue in the CHO laminitis model and compare it to previous reports involving the BWE laminitis model.

**MATERIALS AND METHODS**

*Animals*
The experimental protocol was approved by the Institutional Animal Care and Use Committee. Twenty-four adult horses with a median body weight of 421 kg (ranging from 341 kg to 524 kg) and a median age of 5 years (ranging from 3 to 12 years old) were used in this study. Each horse was determined to be healthy and free of inflammatory disease and foot lameness by physical and lameness examinations and radiographic evaluation of the distal phalanx. Horses were divided into three groups: a control group (CON, n=8), a developmental period group (DEV, n=8), and an Obel Grade I lameness group (OG1, n=8). Prior to the experiment, the horses were quarantined for two weeks at the University of Missouri, housed in stalls and fed a regular ration of free choice hay.

**Carbohydrate overload model**

A carbohydrate gruel consisting of 85% cornstarch and 15% wood flour (17.6 g/kg body weight) was administered to each horse in the DEV and OG1 groups by nasogastric tube as previously described. Six liters of deionized water were administered via nasogastric tube to each horse in the control group. All horses received complete physical examinations, consisting of rectal temperature, heart rate, respiratory rate, abdominal sounds, digital pulses, evaluation with hoof testers, and gait evaluation immediately prior to nasogastric tube passage, and at 2 hour intervals following administration of either carbohydrate or water. Blood was taken from an intravenous jugular catheter at 0, 2, 4, 8, 12, 16 hours in all horses, and every 4 hours after 20 hours in the OG1 group until onset of Obel grade 1 lameness (horse lifts feet incessantly, short, stilted gait at trot). Anesthesia was induced in the DEV group within 2 hours of developing a temperature greater than 102°F (occurring between 12 and 22 hours) and in
the OG1 group at the onset of lameness (occurring between 20 and 48 hours). Although classically a 20% drop in central venous pressure is often used to determine the DEV time point in the CHO model, fever was used instead as it occurs at approximately the same time as the drop in CVP and is a much more repeatable and accurate variable to measure.\textsuperscript{10} Control horses were anesthetized at 24 hours after administration of water. After euthanasia with pentobarbital sodium and phenytoin sodium (20 mg/kg of body weight IV) representative samples of liver, lung and kidney tissue were collected and snap frozen in liquid nitrogen or placed in formalin. Samples were either stored at -80°C until used for RNA isolation or transferred from formalin to 70% ethanol until embedded in paraffin.

**RNA isolation and cDNA synthesis**

Total RNA was extracted from three separate pieces each of lung, liver, and kidney for horse in the CON, DEV, and OG1 groups using a kit (Absolutely RNA Miniprep; Stratagene, LaJolla, CA) which includes a DNase treatment to remove genomic DNA contamination. Complementary DNA (cDNA) was made for each sample via reverse transcription (Retroscript; Ambion Inc; Austin, TX) using at total of 1000 ng of RNA. The cDNA was frozen at -20°C and stored until used for real-time quantitative PCR (RT-qPCR) analysis.

**Real-time qPCR procedure**

Real-time quantitative PCR was performed using a thermocycler (LightCyler, Roche Molecular Biochemical, Indianapolis, IN) and quantified with external standards with the fluorescent format for SYBR Green I dye as previously described.\textsuperscript{11,12} Primers
were designed from equine-specific sequences and for IL-1β, IL-6, CXCL1, CXCL8, TNF-α, COX-1, COX-2, and the housekeeping genes (β-actin, β-2 microglobulin, and glyceraldehyde-3 phosphate dehydrogenase (GAPDH)) have been previously reported.11-13 Amplified cDNA fragments of each gene were ligated into a vector (TOPO 010 E.Coli; Invitrogen, Carlsbad, CA) and the vectors linearized with Hind III restriction enzyme (Invitrogen, Carlsbad, CA) for the use of templates to generate a standard curve for the RT-qPCR reaction.11, 12

Each cDNA sample was diluted 1:5 and 1:500 with 1X TE buffer to be used in the cytokine and housekeeping PCR reactions, respectively. PCR reactions were performed in glass capillaries containing 5 μl of diluted sample and 15 μl of PCR master mixture. Master mix included the following: 1 unit of Taq polymerase (Invitrogen, Carlsbad, CA), 0.2 units of uracil-N-glycosylase (Roche Molecular Biochemical, Indianapolis, IN), 1:10,000 dilution of SYBR Green stock solution, forward and reverse primers, PCR nucleotide plus (Roche Molecular Biochemical, Indianapolis, IN) and PCR buffer. The PCR buffer (20mmol/l Tris-HCL) contained 0.05% each of Tween 20 and nonionic detergent. Primers for CXCL8 were used at a concentration of 2.5 μmol/L. All remaining primers were used at a concentration of 5 μmol/L.

Uracil-N-glycosylase activation, to prevent PCR product carryover, was conducted at 50°C for 2 minutes and was followed by denaturation at 95°C for 2 minutes. Amplification occurred for 40 to 45 cycles, with the annealing temperature set at 1-5°C below the melting temperature for each specific set of primers, extension was set at 72°C for 5 seconds, and fluorescence acquisition for 10 seconds in the SYBR Green format.
Single fluorescence acquisition in each cycle was set at either 80 or 82°C, depending on the melting temperature of the cDNA product of interest as previously described.\textsuperscript{11, 12} After amplification cycling, melting curves of the PCR product were acquired through a stepwise increase in temperature from 65-95°C.

Standard and target samples were prepared in separate capillaries. Standard curves and water for negative control were performed for each gene of interest and amplified with each series of reactions. Standards were made up of 10-fold serial dilutions of linearized plasmids containing the different gene-specific cDNA inserts. All samples were run in duplicate.

\textit{Immunohistochemistry}

Paraffin embedded sections of hepatic and pulmonary tissue from all three groups of horses (CON, DEV, OG1) were used to determine leukocyte migration into the tissue through the immunostaining of calprotectin, which stains both activated macrophages and neutrophils, as well as stressed laminar epithelium.\textsuperscript{14} The paraffin-embedded samples were sectioned at 5-µm thickness, deparaffinized, and treated with a protease solution at 0.5 protease units/ml for 4 minutes (Proteinase K; Invitrogen, Carlsbad, CA). After blocking, sections were incubated with a mouse monoclonal anti-human calprotectin antibody using a 1:250 dilution at 37°C for 90 minutes (MAC387; Abcam, Cambridge, MA). A biotinylated anti-mouse antibody (VECTASTAIN ABC kit; Vector Laboratories, Burlingame, CA) was then applied using a 1:100 dilution at room temperature for 60 minutes. The immunoreactivity was observed using a peroxidase (DAB kit; Vector Laboratories, Burlingame, CA) following the instructions of the
manufacturer and Harris hematoxylin (Fisher Scientific, Kalamazoo, MI) was used for the counterstain. The front lamina from an OG1 horse served as a positive control.15

Image analysis

All slides were examined by light microscopy in order to identify the presence and location of stained cells and counting of cells for each image was blinded to remove bias from data collection. Whole slide digital images (WSIs) were obtained from stained sections using an automated scanning robot (T2 Scan Scope) with a 40× (lung) and 20× (liver) magnification at a spatial sampling period of 0.2 μm per pixel. WSIs were assessed using a software program (Aperio Image Scope, Vista CA) to randomly capture images from each section, which were then transformed into jpg files. Ten random images were captured for the lung and the liver from each horse in each group. The number of immunoperoxidase-positive cells per high power field (40X, lung and 20X, liver) of each image was determined manually. The average number of immunoperoxidase-positive cells for each horse in each group (CON, DEV, and OG1) were calculated and used for statistical analysis.

Serum chemistries

After collection, blood was stored on ice until processing by centrifugation at 1500 rpm for 20 minutes. Serum was obtained from each sample and stored at -20°C until analyzed by the OSU clinical chemistry laboratory (Columbus, OH). Only the time 0 (pre-CHO administration), time of fever onset (between 12-22 hours), and time of lameness onset (20-48 hours) from the horses in the OG1 group were analyzed. The following clinical chemistry parameters were measured for each sample: blood urea
nitrogen (BUN), creatinine, sodium, chloride, potassium, phosphorus, calcium, magnesium, bicarbonate, anion gap, serum osmolality, total protein, albumin, globulin, alkaline phosphatase, GGT, SDH, total bilirubin, direct bilirubin, CK, AST, glucose, and triglycerides. Average values for each time point was determined for each parameter evaluated.

Data analysis

Average copy number from each sample was determined for each gene (housekeeping and inflammatory marker). As, previously reported, RT-qPCR data from the four housekeeping genes for each sample was evaluated by the computer software program geNorm (Ghent University; Ghent, Belgium) to determine which two genes received the best acceptable score to be used for normalization.\textsuperscript{16} β-actin and β2 microglobulin or GAPDH were selected and then used by the geNorm software to create a normalization factor for each sample. To determine the corrected copy number value for each sample the amplification data obtained by RT-qPCR for each gene was divided by the normalization factor of the selected housekeeping gene for the same sample. After normalization, the fold change from the average control value was calculated for each sample. All data were tested for normality. qRT-PCR and serum chemistry data were analyzed non-parametrically using the Kruskal-Wallis and Dunn’s multiple comparisons test to compare groups. Calprotectin positive leukocyte data from pulmonary and hepatic tissue were analyzed using a one way ANOVA with Tukey’s multiple comparisons test to compare groups. Statistical significance was set at P<0.05 to maintain type I error for all tests.
RESULTS

Clinical findings

Horses in the DEV group developed fevers with an average high temperature of 103.6°F (range 103.2°F to 105.1°F) which occurred between 12 and 22 hours post-CHO administration (median 17 hours). Horses in the OG1 group developed signs of Obel grade 1 lameness (lifting feet incessantly and demonstrating a short, stilted gait at trot) between 20 and 48 hours post-CHO administration (median 25 hours).

qRT-PCR

All three tissues, liver, lung and kidney, demonstrated an inflammatory response after administration of CHO. However, the onset of response and magnitude, determined by number of inflammatory mediators increased, was variable from tissue to tissue. Liver had the earliest and greatest response with mRNA concentrations of IL-1β, TNFα, CXCL8, and IL-10 significantly (P<0.05) increased in the DEV horses compared with controls (Figure 5.1). These mediators, in addition to COX-2, remained significantly increased in the OG1 horses compared with controls (Figure 5.1). The lung had a different response with significant increases in IL-1β, IL-10, and COX2 mRNA concentrations only occurring in the OG1 horses (Figure 5.2). Administration of CHO had the least effect on the kidney, with only a small, but significant increase in IL-1β mRNA concentration in the OG1 horses compared with controls (Figure 5.3).

Immunohistochemistry
Leukocytes, evidence by calprotectin staining, were present in the liver and lung in all groups of horses (Figures 5.4 and 5.5). Administration of CHO significantly (P<0.05) increased the number of leukocytes in the lung in both DEV and OG1 horses compared with controls (Figure 5.5); however, no significant change in leukocyte numbers was found in the liver in either the DEV or OG1 horses (Figure 5.4).

**Serum Chemistries**

Administration of CHO to horses resulted in minor serum biochemical changes in total protein concentrations, anion gap, and bicarbonate. Serum total protein concentrations were significantly (P<0.05) increased at both the onset of fever and onset of lameness time points compared with controls; however, no significant differences in albumin or globulins were noted (Figure 5.6). Significantly increased serum anion gap and decreased serum bicarbonate concentrations were present at both the onset of fever and onset of lameness time points (Figure 5.6). A small but significant decrease in serum blood urea nitrogen was noted at the onset of lameness compared with controls. Despite inflammatory changes in the liver and kidney no significant differences were found in the liver parameters GGT, SDH, and bilirubin or in the kidney enzyme creatinine. In addition, there were no significant changes in electrolyte concentrations or in serum glucose concentrations.

**DISCUSSION**

As laminitis has been reported to be a sequela to many systemic diseases, particularly conditions of sepsis or SIRS,\(^17,18\) and as sepsis/SIRS can frequently result in
multiple organ failure,\textsuperscript{19, 20} it is reasonable to explore differences between the lamina and other organs in order to better understand why the foot of the horse is the predominate organ that fails\textsuperscript{18} and not organs such as the liver, lung or kidney. Results from this study support that an inflammatory response does occur in the liver, lung and kidney, but the response varies in onset, magnitude, and type of mediators produced compared with each other and to previous reports from the lamina after CHO administration.\textsuperscript{13, 15, 21, 22}

Of the three tissues evaluated for inflammatory mediator mRNA concentrations in this study, the hepatic response not only occurred earlier but also had the greatest number of mediators increased. The liver is a major site for removal of bacteria and endotoxin from the circulation\textsuperscript{23} and as increases in endotoxin have been reported after CHO administration\textsuperscript{24-26} it is not unexpected that the liver would be able to respond rapidly in its production of inflammatory mediators to CHO administration. The pulmonary tissue had the second greatest response with mRNA concentration of IL-1\textbeta, IL-10 and COX-2 being significantly increased in the OG1 horses compared with controls. Unexpectedly, the kidney did not have a strong inflammatory response after CHO administration as only small increases in IL-1\textbeta were present in the OG1 horses compared with controls. Since the kidney is known to have TLR4 receptors that can be up-regulate to produce inflammatory mediators in cases of sepsis/SIRS\textsuperscript{27, 28} and clinically renal failure occasionally occurs in horses\textsuperscript{17, 29} with sepsis or diseases with severe systemic inflammatory responses we expected a more prominent inflammatory response from this tissue. However, it is uncommon for horses with grain overload to develop renal dysfunction, unless they become severely hypovolemic secondary to fluid losses in the
gastrointestinal tract; therefore, it is not the inflammatory response that damages the kidney in these diseases but simply decreases in renal blood flow.

Interestingly, the response of the liver and lung to CHO is slightly different than their response to BWE administration. There was no increase in IL-6 production by the liver or lung after CHO as was reported after BWE administration. There also was an increase in IL-10 and COX-2 mRNA concentrations in the liver after CHO compared with a decrease in IL-10 and no change in COX-2 reported after BWE administration. Inflammatory mediator expression in the lung was reported to occur within 3 hours of BWE administration; however, there were no significant increases in inflammatory mediator mRNA concentrations until the onset of lameness after CHO administration. TNFα, which was reported to be increased in the lung after BWE administration, was not found to be increased in horses receiving CHO. The differences in the types of inflammatory mediators produced and onset of production between the two models has also been reported to occur in the lamina and may be explained by comparing CHO and BWE administration in horses to the CLP and endotoxin models of sepsis in rodents. The CHO model is believed to be a more clinically relevant model of laminitis in regard to its time course and clinical outcome. This is similar to the CLP model in that it is slower in onset and decreased in magnitude, but increased in duration for inflammatory mediator production compared with the administration of an endotoxin bolus.

As leukocyte emigration into the tissues is believed to play a role in organ failure associated sepsis, we evaluated the presence of leukocytes in the pulmonary and hepatic parenchyma after administration of CHO and compared it to control horses. Increases in
leukocyte numbers, demonstrated by calprotectin staining, were found after CHO administration in the lung in both the DEV and OG1 horses. This is in agreement with reports of increased CD13 positive leukocyte staining in the lungs of horses 3 hours after BWE administration. A hallmark of acute respiratory distress syndrome (ARDS) in humans is excessive inflammation into the alveolar interstitium and air spaces involving massive neutrophil influx secondary to chemotaxic signals. Although horses in this study had significant increase in calprotectin staining in leukocytes (macrophages and neutrophils), we did not see any clinical evidence of acute lung injury or ARDS in our horses. One possible explanation may be that neutrophils and macrophages are recruited to the lung, but are not activated in this model. It is also possible that humans are much more sensitive to leukocytes and their production of inflammatory mediators, proteases and vasoactive substances than the horse.

In contrast to previous reports after BWE administration, there were no increases in leukocyte numbers in the liver after CHO administration. With the significantly increased gene expression of numerous inflammatory mediators in both DEV and OG1 horses in this study, it is initially surprising that there was no increase in leukocyte numbers within the hepatic parenchyma. However, since the liver is known to have the largest resident population of macrophages (Kupffer cells) it would be possible for the liver to produce inflammatory mediators from these cells without having to recruit a significant number of cells into the tissue. Furthermore, perhaps it is the lack of recruitment that keeps significant liver damage from occurring in this model as
neutrophils are known for having a major role in local tissue damage in cases of sepsis and SIRS in humans.\textsuperscript{23}

The serum biochemical changes found in this study after CHO administration are similar to previous reports. Increases in serum total protein concentrations have been previously reported and are suspected to occur from compartmental fluid shifts from the vasculature into the lumen of the cecum and colon after CHO administration.\textsuperscript{33} Increases in acute phase proteins (including ceruloplasmin, c-reactive protein, α\textsubscript{1}-antitrypsin I, and haptoglobin) have also been demonstrated to increase in the plasma of ponies after CHO administration and may be of minor significance in total serum protein concentrations.\textsuperscript{34} Interestingly, the major source for these inflammatory proteins is the liver and they are known to be produced secondary to inflammatory mediators, particularly IL-6 which was not found to be significantly increased in our study. Furthermore, no changes in the liver parameters GGT, SDH, and total bilirubin were found in this study. This suggests that although inflammatory mediators are produced by the liver it was not a significant enough response to result in hepatocellular damage (SDH), cholestasis (GGT), or liver function (total bilirubin).

The increased serum anion gap is likely due to either the presence of the unmeasured anion lactate or due to the decrease in bicarbonate. Increases in plasma lactate\textsuperscript{25} have been previously reported as well as the presence of a metabolic acidosis\textsuperscript{35} in horses and ponies administered CHO. Although not measured in this study, increases in lactate/lactic acid most likely is a result of increased intestinal production, which has been documented to occur in the cecum after CHO administration\textsuperscript{26} or could be a result
from decreased tissue perfusion, as various hemodynamic alterations have been reported
to occur in horse in various stages of laminitis development after CHO.\textsuperscript{10, 36} As anion gap
is a calculated value using the difference between the cations sodium and potassium and
the anions chloride and bicarbonate, it is possible that the decrease in serum bicarbonate
resulted in the increased anion gap. The loss of serum bicarbonate most likely resulted
from gastrointestinal loss secondary to the diarrhea many horses will develop after CHO
administration or may be a compensatory loss in attempts to buffer the suspected increase
in lactic acid production. In contrast to previous reports there was no change in serum
electrolytes or glucose in this study.\textsuperscript{33, 35, 37} Perhaps significant changes in these
parameters would have been seen if more frequent samples have been analyzed. There
was also no change in serum creatinine, a marker for renal function. This is not
surprising as the inflammatory response in the kidney was minimal and most likely not
enough to result in significant renal damage in this model.

Overall the liver, lung and kidney of the horse respond to administration of CHO
through the production of inflammatory mediators. However, this production is variable
among the organs and does not appear to result in any significant clinical abnormalities in
these horses. The lamina still remains the tissue with the greatest inflammatory response
and the only one with subsequent structural damage leading to ultimate failure after CHO
administration. The exact reasons for this remains to be elucidated; however, the
continued search to determine why this occurs may provide clues in developing
successful treatment and prevention regimes for laminitis in the future.
REFERENCES


Figure 5.1. Inflammatory mediator mRNA concentrations in the liver after CHO administration. *P<0.05 when compared with control horses. No significant differences between groups for IL-6 and COX-1 (graphs not shown).
Figure 5.2. Inflammatory mediator mRNA concentrations in the lung after CHO administration. *P<0.05 when compared with control horses. No significant differences between groups for IL-6, TNFα, CXCL8, and COX-1 (graphs not shown).
Figure 5.3. IL-1β mRNA concentrations in the kidney after CHO administration.

*P<0.05 when compared with control horses. No significant differences between groups in any of the other inflammatory mediators evaluated (graphs not shown).
Figure 5.4 Calprotectin (MAC387) positive cells in the liver after CHO administration. Arrows demonstrate positively stained leukocytes. There was no significant difference in calprotectin positive cells between groups of horses.
Figure 5.5 Calprotectin (MAC387) positive cells in the lung after CHO administration. Arrows demonstrate positively stained leukocytes. *P<0.05 when compared with number of calprotectin positive cells of CON horses.
Figure 5.6 Serum bicarbonate, anion gap, and total protein concentrations in the OG1 horses at time 0 (PRE), onset of fever (FEVER), and onset of lameness (LAME). *P<0.05 when compared with the PRE sample. There were no significant differences between groups in any of the other serum chemistry parameters measured.
Chapter 6: Proinflammatory Cytokine Responses of Cultured Equine Keratinocytes to Bacterial Pathogen-Associated Molecular Pattern (PAMP) Motifs

ABSTRACT

Objectives: To determine if equine keratinocytes have a proinflammatory response to various bacterial pathogen-associated molecular pattern molecules (PAMPs) likely to be present in equine sepsis.

Methods: Keratinocytes were isolated from skin of two horses and primary cultures performed. Immunohistochemical analysis for cytokeratin and cobblestone phenotype confirmed purity of epithelial cell cultures. Keratinocytes were harvested for RNA extraction after exposure to lipopolysaccharide (LPS), lipoteichoic acid (LTA), peptidoglycan (PGN), bacterial DNA (CpG), flagellin or maintained in media (controls) for 4 or 24 hours. Real time-quantitative PCR was used to quantify interleukin-1β (IL-1β), interleukin-6 (IL-6), and CXCL8 mRNA concentrations.

Results: Increases (P<0.05) in IL-1β, IL-6, and CXCL8 mRNA concentrations were induced by LPS exposure compared with controls. Increased mRNA concentrations of both IL-6 and CXCL8 were also noted (vs. controls) upon exposure to flagellin. Overall, responses were greater at 4 hours. No increases (P>0.05) in cytokine expression by keratinocytes were present after LTA, PGN, or CpG exposure.
**Conclusions:** Increased proinflammatory cytokine expression in response to LPS and flagellin indicate that equine keratinocytes have functional TLR4 and TLR5 receptor signaling. However, the lack of keratinocyte stimulation by PGN, LTA, or CPG provides no evidence for functional TLR2, TLR9, or NOD receptor signaling. These results suggest that equine keratinocytes are more responsive to PAMPs usually associated with gram negative sepsis and unresponsive to PAMPs most commonly associated with gram positive sepsis.

**INTRODUCTION**

Due to the dynamic role that the keratinocyte is now known to play in inflammatory and septic conditions of the skin, the human and rodent skin keratinocyte has been well characterized in vitro in order to assess the cell’s responsiveness to different bacterial products. The equine keratinocyte has recently been successfully isolated, cultured and characterized. Although the cells’ phenotype was well described in culture, no further studies involving the equine keratinocyte’s response to inflammatory stimuli have been reported. Further knowledge of equine keratinocyte physiology and response to various stimuli is important in developing a better understanding of disease states involving the epidermis including wound healing, squamous cell carcinoma, and skin diseases such as pemphigus and bacterial folliculitis. In addition to the various diseases and conditions involving the skin, the horse has the unique involvement of the epidermal epithelial cell in the laminae, where systemic diseases most commonly involving gram negative sepsis can lead to intense inflammation.
of the dermal/epidermal tissue and dysadhesion of the laminar epithelial cells from the underlying dermis. Therefore, assessing the response of the equine keratinocyte to various microbial components from both gram positive and gram negative organisms will not only provide information important for inflammatory and septic processes of the skin, but may also provide insight to the unique response of the laminar epidermal/dermal structure to gram negative sepsis.

The epidermis in all species serves as part of the innate immune system as a physical barrier to various microbes. Wounding and disease can alter this barrier, inducing the involvement of other components of the innate immune system to assist in the response to skin trauma and microbial contamination. Recognition of conserved cellular components of microbial pathogens, termed pathogen-associated molecular patterns (PAMPs), by the host is one of the primary mechanisms of defense of the innate immune system. PAMPs are recognized by pattern recognition receptors (PRRs) in the host cells. The best characterized PRRs are the toll-like receptors (TLRs), which recognize microbial components ranging from bacterial cell wall components to bacterial DNA. These PRRs have been extensively studied in leukocytes due to the important role they play in the innate immune response. However, the keratinocyte has also been studied in regard to PRRs, as this cell is now known to not only contribute to the innate immune system as a physical barrier to pathogens, but also as a source of inflammatory mediators. The epithelial cell reportedly responds to bacterial toxins and ligands leading to amplification of the immune response via epithelial production of proinflammatory cytokines and by activation of leukocyte emigration from the vasculature to the epidermal
tissue through epithelial expression of chemokine molecules such as CXCL8.\textsuperscript{10-12} This immune response is critical as a protective mechanism against pathogen entry into the body through the epidermis.

Researchers have characterized the response of the human keratinocyte to multiple PAMPs including lipopolysaccharide, lipoteichoic acid, peptidoglycan, flagellin, and bacterial DNA. These PAMPs are now known to be recognized by different PRRs including TLRs and nucleotide-binding oligomerization domain (NOD) receptors. TLR2 has been widely described as a PRR for proteins from gram positive bacteria including lipoproteins and lipoteichoic acid.\textsuperscript{13} Although PGN was originally reported to also induce signaling via binding to TLR2, recent research has indicated that this toxin mainly binds to (and induces inflammatory signaling through) the NOD proteins in the cytoplasm.\textsuperscript{14} TLR4, the first-discovered and best-described TLR, is the primary PRR for LPS from gram-negative bacteria. Two additional TLRs reported to be present in the human epithelium but have received less attention are TLR5, which recognizes flagellin from gram positive and negative organisms, and TLR9, which recognizes bacterial DNA (unmethylated CpG DNA) from diverse bacterial organisms. Due to the importance of the inflammatory response of the keratinocyte to bacterial toxins both in physiologic and pathologic skin conditions, numerous studies have been performed on the response of human keratinocytes to different bacterial components. In these reports, the human keratinocyte in culture has been reported to respond to all ligands tested in the current study including LTA, LPS, PGN, CpG DNA, and flagellin.\textsuperscript{1-5}
Due to the increased incidence of injury to the epidermal structures of the equine laminae in clinical cases involving gram negative sepsis (i.e. Salmonella enterocolitis or gram negative pleuropneumonia) compared with those with gram positive sepsis (i.e. streptococcal respiratory infections), we were interested in determining the equine keratinocyte response to PAMPs reported to be important in both types of sepsis. In this study, we first investigated the mRNA concentrations of several cytokines/chemokines (Il-1β, IL-6 and CXCL8) in cultured equine keratinocytes exposed to the TLR2 ligand, LTA, and compared the response of LTA with the response elicited by TLR4 ligand, LPS. We then further assessed the response of the keratinocytes to different bacterial components (recognized by multiple TLRs and NOD proteins) likely to be present in the septic equid.

MATERIALS AND METHODS

Overview

Due to the possible genetic variability between animals regarding TLR signaling,15,16 we chose to perform the experiments in keratinocytes from two unrelated horses to ensure that the gene expression patterns were consistent between animals. The cells from each horse were analyzed separately and the results presented in separate graphs to demonstrate that, although the magnitude of response of the cells from the two different horses was different, the pattern of the response compared to other treatments were similar. In order to compare results from equine cells to those reported in human-related studies, we also attempted to address the variations in time points, concentrations
of TLR ligands, and inflammatory mediators assessed in the human keratinocyte studies\textsuperscript{1}, \textsuperscript{3-5} by the following methods: 1) we used several concentrations (reflecting concentrations used in human-related studies) of most PAMPs tested (only one concentration of CpG-ODN was used due to financial constraints); 2) we assessed two time points (4 and 24 hours) commonly assessed in other studies, and 3) we assessed three different proinflammatory mediators (Il-1\textbeta, IL-6, and CXCL8) relevant to equine sepsis and also assessed in human keratinocyte studies.\textsuperscript{7,17,18} Two experiments were performed using cells from each horse. We first assessed the response of the integumentary epithelial cells to two concentrations of the gram positive TLR2 ligand LTA and compared results to media only (negative control) and to LPS (5 µg/ml, positive control). LPS at this concentration had elicited a response in cultured equine keratinocytes in pilot studies. The second experiment was conducted to further assess the skin epithelial cells’ response to: multiple concentrations of LPS, the gram negative ligand flagellin, a NOD receptor ligand peptidoglycan (PGN, \textit{S. aureus} origin; also assessed a gram negative PGN in pilot study (data not shown)), and the response to bacterial DNA (CpG).

\textit{Isolation and culture of equine keratinocytes}

Thorough physical examination and history determined that horses were free of systemic disease or systemic inflammatory conditions prior to collection of tissue for culture; however, they were euthanized for chronic musculoskeletal or neurological conditions. Sections of skin from the shoulder region of two horses were obtained immediately after euthanasia to establish cultures of equine primary keratinocytes. The region was aseptically prepared after shaving the hair and 1 x 20 mm strips of skin were
removed and placed in Hanks buffered saline solution (HBSS). A dissection microscope was used to completely remove the subcutaneous tissues in order to avoid contamination of fibroblasts within the culture. The remaining epidermis was minced and trypsinized (0.25% in HBSS) for 4 hours at 37°C. After trypsinization, the cells were collected and placed in T-25 flasks coated with collagen type I (Bovine collagen type I; BD Biosciences, Billerica, MA). Cells were cultured in epithelial cell culture media (Epi-CM; ScienCell Research Laboratories, Carlsbad, CA) supplemented with 2% fetal bovine serum, epidermal growth factors, and penicillin and streptomycin at 37°C with 5% CO₂. Keratinocytes were passaged at approximately 80% confluency and frozen after the second passage. Epithelial cells, at third passage, were plated in 12-well cell culture plates (Corning, Corning, NY) at 2 x 10⁴ cells per well. Each bacterial ligand concentration tested was assayed in triplicate wells.

**Immunohistochemical analysis**

Immunofluorescence staining for cytokeratin (Dako, Carpinteria, CA) was performed in the same passaged epithelial cells cultured in chamber slides (Fisher Scientific, Pittsburgh, PA) on collagen type I matrix to verify pure culture of keratinocytes. Cultured cells were fixed with acetone once they reached confluency. Slides were blocked with 10% normal goat serum for 1 hour. After washing with PBS-Triton x100, the primary antibody for cytokeratin (mouse anti-human A1/A3 clone; 1:50 dilution) was applied and incubated for 1½ hours. After repeating the wash, the secondary antibody (Molecular Probes, Carlsbad, CA; goat anti-mouse IgG at 1:400) was
applied and incubated in the dark for one hour. Slides were examined after mounting to verify the presence of epithelial cells.

**Stimulation of keratinocytes**

The experiments were performed immediately after the epithelial cells reached confluency; the bacterial components described below were added to the same commercial epithelial medium with 2% calf serum in which the cells were cultured. In the first experiment, keratinocytes were exposed to two concentrations of LTA (from *Staphylococcus aureus*; Sigma-Aldrich, St. Louis, MO) (1 μg/ml and 10 μg/ml), media, or LPS (from *E. Coli*; Invivogen, San Diego, CA) (5 μg/ml). In the second experiment, keratinocytes were exposed to three concentrations (100 ng/ml, 500 ng/ml, and 5 μg/ml) of LPS, four concentrations (100 ng/ml, 500 ng/ml, 5 μg/ml and 10 μg/ml) of PGN (from *Staphylococcus aureus*; Invivogen, Carlsbad, CA), three concentrations (100 ng/ml, 500 ng/ml, 5 μg/ml) of flagellin (*Salmonella typhurimium*; Invivogen, San Diego, CA), and one concentration (1 μM) of unmethylated CpG DNA (Invivogen, San Diego, CA) in epithelial cell culture media. Each bacterial ligand concentration tested was assayed in triplicate wells. Media only was placed in four wells with keratinocytes to serve as a negative control. The keratinocytes were incubated with the TLR ligands at 37°C for four and twenty-four hours. After incubation the cells were trypsinized and collected for future RNA analysis.

**RNA isolation and cDNA synthesis**

Total RNA was extracted from the cultured keratinocytes for each horse in each treated and control wells using a kit (Stratagene, Inc., LaJolla, CA) which includes a
DNase treatment to remove genomic DNA contamination. Complementary DNA (cDNA) was then made for each sample using 1000 ng of total RNA via reverse transcription (Ambion, Inc., Austin, TX). The cDNA was frozen at -20°C and stored until used for real-time quantitative PCR (RT-qPCR) analysis.

**Real-time qPCR procedure**

Real-time quantitative PCR was performed using a thermocycler (Roche Molecular Biochemical, Indianapolis, IN) and quantified with external standards with the fluorescent format for SYBR Green I dye as previously described. Primers for IL-1β, IL-6, CXCL8, and the housekeeping genes (β-actin, β-2 microglobulin, glyceraldehyde-3 phosphate dehydrogenase, and TATA-box binding protein) were designed from equine specific sequences and have been previously reported by Waguespack and coworkers. Amplified cDNA fragments of each gene were ligated into a vector (TOPO 010 E.Coli; Invitrogen, Carlsbad, CA) and the vectors linearized with Hind III restriction enzyme (Invitrogen, Carlsbad, CA) for the use of templates to generate a standard curve for the RT-qPCR reaction.

Each cDNA sample was diluted 1:5 and 1:500 with 1X TE buffer to be used in the cytokine and housekeeping PCR reactions, respectively. PCR reactions were performed in glass capillaries containing 5 μl of diluted sample and 15 μl of PCR master mixture. Master mix included the following: 1 unit of *Taq* polymerase (Invitrogen, Carlsbad, CA), 0.2 units of uracil-N-glycosylase (Roche Molecular Biochemical, Indianapolis, IN), 1:10,000 dilution of SYBR Green stock solution, forward and reverse primers, PCR nucleotide plus (Roche Molecular Biochemical, Indianapolis, IN) and PCR buffer. The
PCR buffer (20mmol/l Tris-HCL) contained 0.05% each of Tween 20 and nonionic detergent. All primers were used at a concentration of 5 μmol/L.

Uracil-N-glycosylase activation, to prevent PCR product carryover, was conducted at 50°C for 2 minutes and was followed by denaturation at 95°C for 2 minutes. Amplification occurred for 40 cycles, with the annealing temperature set at 1-5°C below the melting temperature for each specific set of primers, extension set at 72°C for 5 seconds, and fluorescence acquisition for 10 seconds in the SYBR Green format. Single fluorescence acquisition in each cycle was set at either 80 or 82°C, depending on the melting temperature of the cDNA product of interest as previously described.19, 20 After amplification cycling, melting curves of the PCR product were acquired through a stepwise increase in temperature from 65-95°C.

Standard and target samples were prepared in separate capillaries. Standard curves and water for negative control were performed for each gene of interest and amplified with each series of reactions. Standards were made up of 10-fold serial dilutions of linearized plasmids containing the different gene-specific cDNA inserts.

Data analysis

Copy number was determined for each gene (housekeeping and cytokines) for each replicate (three samples per treatment). As previously reported, RT-qPCR data from the four housekeeping genes for each sample was evaluated by the computer software program geNorm (Ghent University; Ghent, Belgium) to determine which two genes received the best acceptable score to be used for normalization.21 β-actin and glyceraldehyde-3 phosphate dehydrogenase were selected and then used by the geNorm
software to create a normalization factor for each sample. To determine the corrected copy number value for each sample the amplification data obtained by RT-qPCR for each gene was divided by the normalization factor of the selected housekeeping gene for the same sample. After normalization the mean (± SEM) copy number for IL-1β, IL-6, and CXCL8 mRNA concentrations were determined for each treatment (cells stimulated in triplicate) and for each horse separately. Due to the variation of the magnitude of the response between the two horses, each horse was analyzed separately and reported separately throughout the study. A one-way analysis of variance was used to analyze (GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California) the data and Tukeys’ multiple comparison tests were used for post-hoc analysis. Statistical significance was set at P<0.05 to maintain type I error for all tests.

RESULTS

Isolation and culture of equine keratinocytes

Isolation and subsequent culture of equine keratinocytes from skin over the shoulder region was successful. Cells portrayed the classical cobblestone appearance of epithelial cells and stained positive for cytokeratin, a marker of epithelial origin (Figure 2.1); no fibroblast contamination was apparent in the cultured cells (assessed by cell morphology and cytokeratin staining).

Interleukin-1β mRNA concentrations

LPS stimulation (5 μg/ml) increased IL-1β mRNA concentrations at 4 and 24 hours (compared to controls) whereas LTA had no effect (Figures 1 and 2). In the second
experiment, increased IL-1β mRNA concentrations were present in keratinocytes from both horses at 4 and 24 hours post stimulation with 5 μg/ml LPS; however, no effect was observed at either time point using lower concentrations of LPS or with any of the other bacterial ligands tested (Figures 3 and 4).

**Interleukin-6 mRNA concentrations**

LPS stimulation (5 μg/ml) increased IL-6 mRNA concentrations at 4 and 24 hours (compared to controls; Figures 1-4) whereas LTA had no effect (Figures 1 and 2). Increased IL-6 mRNA concentrations were also present in both horses at 4 hours after stimulation with the lower concentrations of LPS (100 ng/ml and 500 ng/ml of LPS) when compared to the unstimulated cells (Figure 3). Increases in IL-6 mRNA concentrations were present in horse 1 at 4 hours post stimulation with 100 ng/ml and 500 ng/ml of flagellin when compared to the unstimulated cells (Figure 3). No change in IL-6 mRNA concentration was noted in cells from either horse at 4 or 24 hours post stimulation with PGN or CpG when compared to the unstimulated cells (Figures 3 and 4).

**CXCL8 mRNA concentrations**

LPS stimulation (5 μg/ml) increased CXCL8 mRNA concentrations at 4 and 24 hours (compared to controls; Figures 1-4) whereas LTA had no effect (Figures 1 and 2). Increased CXCL8 mRNA concentrations were also present at 4 hours in cells from both horses after stimulation with 500 ng/ml of LPS and additionally in horse 2 after stimulation with 100 ng/ml of LPS (Figure 3). In epithelial cells from both horses, flagellin induced a significant increase in CXCL8 mRNA concentration compared to the unstimulated cells at 4 hours after exposure to the lowest concentration of flagellin tested.
(100 ng/ml; Figure 3). Interestingly, higher concentrations of the flagellin did not consistently induce a significant increase in CXCL8. No significant change in CXCL8 mRNA concentration was present with stimulation of any concentration of PGN or CpG in cells from either horse over both time points (Figures 3 and 4).

**DISCUSSION**

The human keratinocyte has been reported to express multiple TLRs including TLRs 1-6, 9 and 10. These keratinocytes are also known to undergo multiple aspects of inflammatory signaling upon exposure to different PAMPs including NFκB activation/nuclear translocation and expression of numerous proinflammatory cytokines and chemokines. The reported results for the keratinocyte response to the different PAMPs vary, most likely due to the use of different epithelial cell types (i.e. cell lines vs. primary culture), different time points examined after exposure to the PAMPs, different culture conditions (i.e. TLR4 and CD14 protein expression dependant on growth media) and different inflammatory genes assessed in the different reports (CXCL8 is the most commonly assessed inflammatory molecule). In the current study, we based our choices of time points, concentrations of PAMPs, and choice of cytokines to assess in the equine keratinocyte on both physiologic relevance and on the protocols used in the human literature in order to allow comparison of responses. In this study, the equine epidermal epithelial cell exhibits an inflammatory response to bacterial toxins as has been reported for the human counterpart. However, as described below, the pattern of cytokine
expression in response to bacterial ligands in the present study varies markedly from findings in the human literature.

The PAMP which most consistently induced the expression of the different cytokines and chemokines by the equine keratinocyte was LPS. However, the sensitivity differed depending on the cytokine, with only the highest concentration of LPS examined (5 μg/ml) inducing an increase in IL-1β mRNA concentration, whereas lower LPS concentrations also induced increases in mRNA concentration for IL-6 and CXCL8. Although there are conflicting data regarding the response of primary human epithelial cells to LPS, similar patterns of mediator expression as that found in the current study have been reported in LPS-stimulated human keratinocytes with both IL-1β and CXCL8 gene expression and protein secretion being increased. In one study, the proinflammatory cytokine response to LPS was abrogated by preincubation with anti-CD14 and anti-TLR4 antibodies, demonstrating that the cytokine response was indeed secondary to LPS binding to the TLR4 receptor complex.

In the initial experiment, keratinocytes did not respond to LTA at either of the two concentrations (1μg/ml or 10μg/ml) used. This lack of proinflammatory cytokine response of the equine epidermal epithelial cells to LTA suggests that equine keratinocytes do not respond to TLR2 receptor ligands, which is in contrast to human keratinocyte. It is possible that, similar to some reports of human keratinocytes, full equine epidermal TLR2 response may require stimulation by TH1 cytokines (TNFα and IFNγ), which results in translocation of intracytoplasmic receptors to the membrane surface. However, the majority of reports involving human keratinocytes document
PGN alone as a stimulatory agent for the TLR2 receptor.\textsuperscript{2, 3, 5, 23} Thus, in the second experiment we evaluated the equine keratinocyte response to several concentrations of PGN. Once again, the response of the equine epithelial cell appears to be distinctly different when compared to the human keratinocyte. For example, whereas CXCL8 was found to be significantly increased (approx. 3-11 fold) in human keratinocytes in response to PGN,\textsuperscript{2, 3} we found no increase in the mRNA concentrations of IL-1\(\beta\), IL-6, or CXCL8 when exposed to identical concentrations of PGN. Although most studies use PGN from \textit{S. aureus}, we assessed PGN from both \textit{S. aureus} and \textit{E. coli} in pilot studies and found no response in equine keratinocytes regardless of the species of bacteria (data not shown for \textit{E. coli} PGN). Although PGN was originally reported to be a TLR2 ligand, recent data indicate that the intracellular NOD1 and NOD2 receptors are the important receptors for PGN,\textsuperscript{23} whereas the primary ligands of TLR2 are lipoproteins and LTA. It is likely that earlier reports of TLR2 activation with PGN were due to PGN being contaminated with other bacterial toxins (specifically with LTA or lipoproteins), as purification of PGN resulted in a loss of activation of the TLR2 receptor/receptor heterodimer (TLR2/1 or TLR2/6).\textsuperscript{23} TLR2 responds to a wide range of bacterial ligands, including LTA, lipoproteins, and PGN, and it is suggested that the variable responses to these different ligands from various bacterial sources may be related to dimerization with other TLRs (TLR1 or TLR6) and/or functional association with other adaptor proteins (such as CD14 or CD36).\textsuperscript{25-27} Thus, it is possible that under the culture conditions of this experiment, necessary adapter proteins or presence of other TLRs may have resulted in no response. However, human studies, which often evaluate primary keratinocytes from
single individuals, report a keratinocyte response in similar culture conditions using LTA.\textsuperscript{28} Additionally, although there is variance in the magnitude of the response to the different ligands, the overall gene expression patterns are very similar between these two horses. We did not have a positive control for TLR signaling in equine cells for PGN, LTA, and bacterial DNA, and can therefore not ensure that the lack of response is due to our specific culture conditions. However, the consistent absence of response of the equine epithelial cells to LTA and PGN in the same conditions that resulted in cytokine expression in human keratinocyte studies\textsuperscript{2,3,5,23} indicates that the equine keratinocytes are not as sensitive to TLR2 and NOD receptor ligands as human keratinocytes. Additionally, as there was a response to two other PAMPs of which the proteins involved in TLR binding and activation are very distinct from each other (flagellin and LPS, discussed below), we believe that it is unlikely that the culture conditions used in the present study are the reason for a negative response to PAMPs such as PGN, LTA, and bacterial DNA.

Flagellin, a bacterial protein present on both gram positive and gram negative organisms, has been found to be an important component of the inflammatory response, especially in gram negative sepsis. In sepsis from \textit{Salmonella} spp, flagellin is reported to amplify the inflammatory response to LPS due to flagellin’s ability to induce caspase 1/ICE (interleukin-1 beta converting enzyme).\textsuperscript{29} Subsequent to IL-1\beta protein expression induced by LPS, ICE is necessary to activate IL-1\beta, thus making its induction by flagellin important in LPS-associated sepsis.\textsuperscript{29} However, flagellin alone has been found to induce as severe a systemic inflammation as LPS in a rodent model of sepsis.\textsuperscript{30} Similar to the
induction of CXCL8 expression in human keratinocytes exposed to *S. typhimurium* flagellin,\textsuperscript{4, 5} we found that flagellin increased mRNA concentrations of both IL-6 and CXCL8 indicating the presence of functional TLR5 receptor on equine keratinocytes. Although the response was not significant for both horses for IL-6, graphically the trend of increased IL-6 mRNA concentration can be seen, when compared to CpG, PGN and media control. Interestingly, unlike that reported in the human keratinocyte, the peak effect of flagellin on IL-6 in horse 1 and CXCL8 mRNA concentrations appeared to occur with the lowest concentration of flagellin used, and occurred at an earlier time point than reported in the human cells.\textsuperscript{4, 5} Although several studies in human keratinocytes did not examine cell responses before the 24 hour time point, we found that, similar to the temporal pattern of inflammatory signaling reported in colonic epithelial cells,\textsuperscript{31} the greater cellular response to flagellin occurred at 4 hours and cytokine mRNA concentrations returned to baseline by 24 hours of exposure.

**TLR9** is of interest due not only to previous reports of documenting responsiveness of human keratinocytes to TLR9 ligands,\textsuperscript{4} but also due to the fact that the ligand, bacterial DNA, is present in all types of bacteria and therefore response to bacterial DNA would indicate a responsiveness of the equine keratinocyte to gram positive and negative bacteria. CpG-ODNs are synthetic oligonucleotides containing unmethylated CpG dinucleotides in particular sequences, known as CpG motifs. These motifs can elicit various responses but fall into two main categories, type A motifs which result in IFN production from dendritic cells and type B which results in activation of B cells and only type B has been shown to activate NF-κB.\textsuperscript{32} The CpG-ODN used in this
study was type C, which combines characteristics of both A and B and containing the phosphorothioate nuclease resistant backbone that provides the properties of high TLR9 affinity and activation.\textsuperscript{33} Equine dendritic cells, macrophages, and peripheral blood mononuclear cells have been exposed to CpG-ODN with variable response.\textsuperscript{32, 34} Adult dendritic cells responded with production of the TH\textsubscript{1} cytokines IFN\textgreek{a} and IL-12 but no response was noted in equine macrophages.\textsuperscript{32} The response also varied depending on type of CpG-ODN used and was variable depending on the presence of lipofectin.\textsuperscript{34} We did not detect a cytokine response in equine keratinocytes exposed to a CpG DNA concentration reported to elicit a response in human keratinocytes and similar to that used to stimulate equine dendritic cells.\textsuperscript{4, 5, 32} As sensitivity of keratinocytes to TLR9 ligands in other species is reportedly dependent on other factors including the presence of growth factors (i.e. TGF\textgreek{a})\textsuperscript{35} or type of CpG DNA (different CpG motifs),\textsuperscript{36} it is possible that equine keratinocytes may respond to TLR9 ligands under different experimental conditions. Therefore although these results do not support the presence of functional TLR9 signaling in the equine keratinocyte, future studies including the use of multiple culture conditions and multiple types of CpG DNA should be performed prior to unequivocally stating that no TLR9 signaling occurs in the equine keratinocyte.

In conclusion, whereas there are several studies in the medical literature reporting both mRNA and protein expression of IL-1\textbeta, IL-6, and CXCL-8 in human keratinocytes exposed to TLR ligands (PAMPs) from gram positive and gram negative bacteria, the results are difficult to compare to each other as the studies commonly used different concentrations of PAMPs, assessed only one cytokine or chemokine, used different time
points for assessment, and exposed different types of human keratinocytes (both primary culture and cell lines) in the studies. We addressed these concerns by using multiple concentrations of several PAMPs, assessing multiple cytokines, and examining two time points in cells from two unrelated horses. Using this protocol, some consistent differences appear to be present in the equine epidermal epithelium when compared to the human counterpart. Importantly, the unique lack of response of the equine epidermal epithelial cell to LTA and PGN indicates that, in contrast to human epidermal epithelial studies, gram positive sepsis does not induce a consistent inflammatory response in the equine epidermis. The lack of response to CpG DNA is consistent with the fact that the equine keratinocyte does not universally respond to all bacterial components. The lack of response or hyporesponsiveness to ligands characteristic of gram positive organisms may play a role in the fact that equine cases of pure gram positive sepsis are rarely associated with diseases involving epithelial injury such as laminitis. The repeatable cytokine response to LPS is consistent with our clinical experience in equine medicine of more severe laminar epithelial injury with gram negative sepsis. The early response of the equine keratinocyte to flagellin, the only other bacterial toxin which induced inflammatory gene expression in this study, is likely to amplify the inflammatory response to LPS in gram negative sepsis. Thus, the equine keratinocyte appears to have the innate ability to respond to various components of gram negative bacteria with the proinflammatory response reported in this study being consistent with the typical systemic response of the horse to gram negative bacterial infection. Future studies are
needed using laminar epithelial cells in a similar model to assess whether or not these cells exhibit the same response as their counterparts in the skin.

REFERENCES


Figure 6.1 Equine keratinocytes in culture with phase microscopy (top) and with cytokeratin immunofluorescent staining (bottom).
Figure 6.2. IL-1β response from equine keratinocytes after 4 and 24 hours of stimulation with LTA and LPS. †P<0.05 when compared to media controls. Horse 1 is represented by the checkered bars and horse 2 represented by solid bars.
Figure 6.3. IL-6 response from equine keratinocytes after 4 and 24 hours of stimulation with LTA and LPS. +P<0.05 and *P<0.001 when compared to media controls. Horse 1 is represented by the checkered bars and horse 2 represented by solid bars.
Figure 6.4. CXCL8 response from equine keratinocytes after 4 and 24 hours of stimulation with LTA and LPS. †P<0.05 and *P<0.001 when compared to media controls. Horse 1 is represented by the checkered bars and horse 2 represented by solid bars.
Figure 6.5. IL-1β response from equine keratinocytes after 4 hours of stimulation with LPS, CpG-ODN, PGN, and Flagellin. *P<0.001 when compared to media controls. Horse 1 is represented by the checkered bars and horse 2 represented by solid bars.
Figure 6.6. IL-6 response from equine keratinocytes after 4 hours of stimulation with LPS, CpG-ODN, PGN, and Flagellin. †P<0.05 and *P<0.001 when compared to media controls. Horse 1 is represented by the checkered bars and horse 2 represented by solid bars.
Figure 6.7. CXCL8 response from equine keratinocytes after 4 hours of stimulation with LPS, CpG-ODN, PGN, and Flagellin. $^{+}$P<0.05 and $^{*}$P<0.001 when compared to media controls. Horse 1 is represented by the checkered bars and horse 2 represented by solid bars.
Figure 6.8. IL-1β response from equine keratinocytes after 24 hours of stimulation with LPS, CpG-ODN, PGN, and Flagellin. *P<0.001 when compared to media controls. Horse 1 is represented by the checkered bars and horse 2 represented by solid bars.
Figure 6.9. IL-6 response from equine keratinocytes after 24 hours of stimulation with LPS, CpG-ODN, PGN, and Flagellin. \(+P<0.05\) and \(*P<0.001\) when compared to media controls. Horse 1 is represented by the checkered bars and horse 2 represented by solid bars.
Figure 6.10. CXCL8 response from equine keratinocytes after 24 hours of stimulation with LPS, CpG-ODN, PGN, and Flagellin. *P<0.001 when compared to media controls. Horse 1 is represented by the checkered bars and horse 2 represented by solid bars.
Chapter 7: Development of a Successful Technique to Isolate RNA from Laminar Basal Epithelial Cells of Horses using Laser Capture Microdissection

ABSTRACT

Objectives: To collect laminar basal epithelial cells (LBECs) using laser capture and microdissection (LCM) and to develop a technique for isolation of total RNA from the collected cells.

Methods: LBECs were collected from OCT embedded cyrosections of equine lamina using the PALM LCM microscope. Cryosections were stained with cresyl violet or hematoxylin. Isolated cells were collected into a cap either with an extraction/lysis buffer or without followed by isolation of RNA by several techniques to determine the combination that yielded the best quality RNA determined by Agilent analysis.

Results: Isolation of good to high quality RNA was consistently achieved by staining the sections with cresyl violet followed by the combination of collection of LBECs in adhesive caps, with no extraction/lysis buffer and isolation of total RNA using the Qiagen RNAeasy Micro kit.

Conclusions: The combination of staining and isolation of RNA techniques must be developed individually for each type of tissue used. The correct combination will most
likely involve steps that limit time spent a room temperature and steps that do not involve aqueous solutions.

**INTRODUCTION**

Laminitis is a devastating, life-threatening disease of the equine digit that is a common sequela to sepsis resulting from various conditions including strangulating intestinal lesions, enterocolitis, metritis, and pleuropneumonia. Laminar failure occurs in laminitis due to dysadhesion of laminar basal epithelial cells (LBECs) from the underlying dermis and subsequent displacement of the third phalanx due to extraordinary forces imposed on the laminar structure by its relationship to the musculoskeletal system. Similar events occur in laminar failure as occurs in organ failure in human sepsis including inflammatory mediator expression (i.e. cytokines and chemokines), leukocyte emigration, oxidant stress, and production of vasoactive molecules. Epithelial injury is a central component of sepsis-related injury to organs such as the kidney, lung, and intestinal tract. Although inflammatory injury to epithelial cells is commonly reported in sepsis/SIRS, the epithelial cell itself can amplify ongoing tissue inflammation in sepsis/SIRS by expressing numerous cytokines and chemokines. Epithelial cell exposure to reactive oxygen species can alter E-cadherin and actin cytoskeletal organization, resulting in loss of tight junctions and increased paracellular permeability. Finally, hypoxia can induce actin cytoskeletal rearrangements (reportedly through Rho and Rac1 GTPase proteins) in renal epithelial cells, disturbing
cytoskeletal interactions with integrins and adhesion molecules, which leads to disruption of tubular integrity, epithelial sloughing and breakdown of the basement membrane.31-33

Techniques such as immunohistochemistry9,34 and in situ PCR11,34 that evaluate the production of various mediators by laminar epithelial cells suspected to be involved in the development of laminitis are limited, most likely due to the lack of equine specific antibodies and skill/expense to develop these techniques. Furthermore, there are only two reports of successful isolation and culture of laminar epithelial cells35,36 and are historically difficult to isolate in pure culture and survive successfully through subsequent passages. Therefore, a technique that would allow for the study of the laminar epithelial cell would be ideal to develop.

Laser capture microdissection (LCM) is a technique that allows for the isolation of highly purified cell populations within a heterogeneous tissue section.37 Direct visualization with the microscope combined with the use of a motorized stage allows for selection of the cell population of interest and movement of the stage to the selected segments. A laser beam is then used to dissect and catapult the cells into an aqueous extraction buffer placed in a cap directly over the cut area. Collected samples can later be used for DNA, RNA or protein analysis. Laser capture microscopy was first described38 in 1996 and since has been used primarily for the study of cancer cells; however, the technique has increasingly been used to more accurately determine the role of specific cell types in various diseases including the endothelium in wound healing,39 myocytes in myocardial infarction,40,41 and endothelial42 and neuroprogenitor43 cells in stroke. In specific regard to basal epithelial cells, the LCM has also been used along with
cDNA microarray technology to determine a set of over 350 differentially expressed genes produced in basal cell carcinoma when compared to basal cells in normal epidermis of humans.\textsuperscript{44}

The purpose of this study was to use the LCM to collect LBECs from archived laminar samples from control horses and horses receiving an overload of carbohydrate to induce laminitis and to isolate high quality RNA from these collected cells that then can be used for qRT-PCR or microarray analysis.

**MATERIALS AND METHODS**

*Cryosectioning*

Archive laminar tissues, frozen in OCT and stored at -80°C, were cut into 10 μm sections using a cryostat (Leica Biosystems; Nussloch, Germany). Three sections were mounted onto a polyethylene naphthalate (PEN) covered slide (PALM Technologies; Bernreid, Germany) and either used immediately or stored at -80°C in a 50 ml conical until used (typically less than 48 hours from sectioning). Prior to sectioning the slides were treated with RNA-Zap (Ambion; Austin, TX), followed by two washes in nuclease free water (Ambion; Austin, TX) and allowed to dry for approximately 20 minutes before mounting.

*Staining*

Slides were stained using either hematoxylin QS (Vector Labs; Burlingame, CA) or cresyl violet (Ambion; Austin, TX). Slides that were stained with hematoxylin were initially treated with RNA later (Ambion; Austin, TX) for 4 minutes followed by 2
washes in diethyl pyrocarbonate (DEPC) treated water (Roy et al 2007). After staining, these slides were dehydrated with 95% followed by 100% molecular grade ethanol (Sigma-Aldrich, St. Louis, MO) solutions. Staining slides with cresyl violet was performed according to the LCM staining kit (Ambion; Austin, TX) manufacturer’s instructions by first rehydrating with 95%, 70% and 50% ethanol followed by staining for 30 seconds and sequential dehydration with molecular grade ethanol (Sigma-Aldrich; St. Louis, MO) solutions. The xylene step was excluded from the protocol. After staining slides were air dried and immediately used for LCM.

**Laser Capture Microdissection**

Slides were placed on the PALM LCM (Bernreid, Germany) for collection of tissue. Laminar basal epithelial cells were selected at 10X objective using the freelance drawing option on the PALM LCM software (Figure 7.1). Once selected the LBEC were cut and subsequently catapulted using an UV-energy of 50-60 and UV-focus of 80-82 (Figure 7.1). Laser dissections of each slide were limited to 30-45 minutes to minimize RNA degradation. An average of 500,000 to 1,000,000 μm² area of LBECs was collected for each sample. Collected tissue was captured into a cap that contained 25 μl of either PicoPure RNA isolation kit extraction buffer (Arcturus; Sunnyvale, CA), RNAqueous® Micro kit lysis buffer (Ambion; Austin, TX), Trizol (Invitrogen; Carlsbad, CA), or Micro-RNeasy RLT buffer with β-mercaptoethanol (β-ME) (Qiagen; Valencia, CA). LBECs were also collected in aqueous-free adhesive caps (PALM Technologies, Bernreid, Germany). After collection, the caps were place onto 200 μl RNase free tubes
and either incubated at 42°C if in extraction (PicoPure) or lysis (RNAqueous) buffer, or stored at -80°C until RNA could be isolated.

**RNA isolation**

RNA isolation techniques varied depending on collection technique but occurred within 48-72 hours of LCM. For samples collected in the Pico Pure extraction buffer, RNAqueous® Micro lysis buffer, and RLT buffer with β-ME the isolation protocol was performed according to manufacturer’s instructions. For samples collected in trizol, chloroform extraction was performed followed by the column based collection of RNA using the RNAqueous® Micro kit for LCM samples. For samples collected in adhesive caps, 75 μl of RLT buffer with β-ME was added to the tube and the sample was vortexed upside down to disperse the cells into the buffer for lysis. After centrifugation, RNA isolation was performed according to the Micro RNeasy kit manufacturer’s instructions. RNA from individual samples were quantified using a NanoDrop spectrophotometer and the quality assessed using the pico chip on the Agilent Bioanalyzer 2100 (Wilmington, DE).

**qRT-PCR and microarray**

To determine if RNA collected in this study would be successful in obtaining qRT-PCR data and microarray data, a few samples with acceptable RNA quality were used for both techniques. For the qRT-PCR samples, cDNA was made using the VILO kit (Invitrogen, Carlsbad, CA) and subsequently used for RT-PCR determination of CXCL1 and GADPH mRNA concentrations. Primers used were previously reported and had been designed from equine specific sequences. The LightCycler system (Roche;
Indianapolis, IN) in the fluorescent format, SYBR Green I dye was used for quantification of each gene by the addition external standard as previously described.\textsuperscript{45,46}

A 70-mer-based, ~21,000 element, equine whole genome expression microarray (WG-oligoarray) representing most equine genes was used. A sample of total RNA from the LCM captured LBECs and as a sample of equine kidney (to serve as a control) were used to make cDNA that was labeled with Cy3 or Cy5 dye via an indirect labeling method utilizing dendrimer technology.\textsuperscript{47} Labeling was carried out with the 3DNA Array 350 Expression Array Detection kit (Genisphere; Hatfield, PA).\textsuperscript{48,49} Hybridizations were performed in a SureHyb hybridization chamber (Agilent; Santa Clare, CA) at 55°C. Following post-hybridization washes, arrays were scanned with a GenePix 4000B scanner at 5 micron resolution (Molecular Devices; Sunnyvale, CA). GenePix Pro 6.1 software was then utilized for raw data acquisition, spot-finding, and quantification of the array.

RESULTS

Staining of the equine lamina cyrosections were successful with both the hematoxylin QS stain and the cresyl violet allowing for good determination of the LBECs (Figure 7.2); however, the cresyl violet staining protocol (ethanol rehydration-staining-ethanol dehydration) yielded resulted in greater quality RNA. Of the five different capture/RNA isolation protocols used in this study the combination that consistently yielded the best quality RNA, demonstrated by the Agilent Bioanalyzer’s RIN value, was the combination of capturing in the adhesive cap (PALM Technologies; Bernreid,
Germany) followed by isolation using the Micro RNAeasy kit (Qiagen; Valencia, CA). Electrophenograms and RNA integrity number (RIN) for the samples were generated and this combination resulted in RIN values ranging from 5 to 9 (Figure 7.3). RIN values can range from 1-10, with highly degraded values at 3 or below, partially degraded values between 4 and 6 and fully intact RNA values equaling 10 (Figure 7.3). Although capture of LBECs in trizol and subsequent isolation using the RNAqueous® Micro kit resulted in the greatest quantities of RNA (20-40 ng/ul vs. 2-10 ng/ul average from the other techniques) the ability to consistently obtain acceptable quality did not occur. Collection and isolation using either the extraction buffer (PicoPure) or lysis buffer (RNAqueous) resulted in the collection of poor quality RNA (Figure 7.3). Collection and isolation using RNAqueous® MicroRNA kit in combination with adhesive caps (using no lysis buffer during the LCM) did result in acceptable quality RNA; however, the technique was more time consuming than the Micro RNAeasy kit and resulted in similar quantities of RNA.

Successful qRT-PCR reactions were performed on one control horse and one lame horse from the CHO model for both CXCL1 and GAPDH. Single melting peaks at the correct temperature were present and amplification occurred in both samples (Figure 7.4). Also successful hybridization with the LBECs collected on the LCM and the equine kidney tissue was achieved with the microarray (Figure 7.4).

**DISCUSSION**
Studying the laminar epithelial cell is essential in determining how laminar failure occurs, as it is at the epidermal-dermal junction and in particular between the basement membrane and basal epithelial cells where dysadhesion occurs allowing for rotation and/or sinking of P3. Numerous attempts by our laboratory and others have been made to grow equine laminar epithelial cells in culture; however, they are difficult to obtain in pure culture and do not passage well. Cultured keratinocytes harvested from equine skin have been used as a model to study the equine epidermal epithelial cell response to bacterial ligands, as similar responses between skin and lamina have been reported in regards to leukocyte migration and cytokine production.

Immunohistochemical evaluation of the equine laminar epithelial cell is limited by the number of quality reagents that will cross react with the horse. Therefore, we have been left to evaluate the lamina by grinding up whole tissue, which contains not only epidermal cell, but dermal components including leukocytes and endothelial cells. However, with the development of laser capture microscopy we now have the ability to individually dissect cells of interest out and evaluate them individually. In addition, LCM provides the unique technique ability to allow scientist to study individual cells within the context of other surrounding cells affected by the same conditions that initiating the disease of interest.

Through trial and error we have determined a repeatable successful method for isolation of total RNA from LBECs using the LCM. As this technique has become increasingly available so have products been developed to help successfully perform experiments. Using these products and following strict protocols to maintain RNA
stability successful capture and isolation can result. Using frozen samples instead of formalin fixed tissue has been reported to result in improved quality of isolated RNA as formalin allows for crosslinking and it is more difficult to recover RNA from these samples. Keeping frozen sections as cold as possible until used is also important as thawing can activate RNases within the tissue and quickly degrade the sample.

Appropriate staining of the slides are necessary in order to appropriately identify the cells of interest and to limit any possibility of RNA degradation. The use of several common histological stains, including hemoxyllin, cresyl violet, MPA, and nissal, has been evaluated for LCM collection of RNA and results demonstrate that type of stain can affect RNA quality to some degree. In that same study the addition of RNase inhibitors improved RNA quality and were recommended for use; however, in our study the addition of RNA later was not successful and therefore eliminated from the protocol.

Another important rule in staining slides for LCM collection of RNA is that the staining procedure needs to be quick and ideally should be limited to a 5-10 minute protocol from start to finish. The staining protocol with cresyl violet used in this study was rapid and effective. One study evaluated time from staining to collection of sample on the LCM and found that RNA was stable for up to 90 minutes when ethanol was added as a solvent in all staining steps. This however appears to be variable depending on type of tissue samples are collected from as some tissues have more endogenous RNases than others. We were able to stain and collect LBECs at room temperature for at least 45 minutes and still obtain good quality RNA. In addition, using an aqueous solution (lysis buffer or trizol) to collect the captured sample, which should help to stabilize the collected RNA
can still allow RNases to degrade the sample. The development of adhesive caps by Zeiss to allow for capture without the use of aqueous solutions has been recommended to help to limit RNA degradation. The introduction of these caps into our protocol was essential in consistently recovering high quality RNA from the LBECs.

Several different RNA isolation procedures/kits can be used to isolate RNA from laser captured samples. A few of these kits are especially made for LCM samples and are useful when collecting small samples with only nanograms of RNA. DNase treatment is essential to remove genomic DNA contamination from samples particularly if being used for microarray analysis. We assessed several kits for RNA isolation, and modified protocols in attempts to develop an ideal protocol. While most kits appeared to work, the most consistent results were achieved with the Qiagen kit. The author believes that the key is elimination of any aqueous solution including that for collection in the caps, and other kits may still be successful if tried with this protocol.

Once the RNA was isolated the RNA quality was assessed using the Agilent bioanalyzer pico chip for small samples as this is reported to be the gold standard for RNA assessment. Having a 28S/18S ratio of >1.5, and a RIN value close to 10 is ideal. Better quality RNA is necessary for microarray studies (RIN values of 7-8 from LCM would be considered good) then if the sample is to be used for PCR. We obtained high quality RNA from our samples and they were successfully used for PCR and microarray analysis. Collection of control and affected tissue is still required to draw conclusions from these test, should be able to now be performed with the protocol designed in this study.
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Figure 7.1 Selection, cutting, and capture of LBECs from the LCM. Images from the PALM LCM microscope at 10X objective. (A) Demonstration of selection of the LBECs with the freehand drawing tool. (B) Image captured after cutting with the laser but before catapulting. (C) Image of captured piece in the cap.
Figure 7.2. Staining of equine lamina for LCM. Cryosections of equine lamina stained with hematoxylin QS (top) and cresyl violet (bottom). Images acquired with PALM LCM microscope at 10X.
Figure 7.3. Analysis of RNA quality isolated from LBECs. (A) Electropherogram from a RNA sample collected using a combination of adhesive caps and Micro RNeasy isolation kit. The RIN value for this sample was 9.0 and the 28S:18S ratio was 3.3. (B) Electropherogram from a sample of isolated LBEC RNA with an RIN value of 5.9 and a 28S:18S ratio of 2.4. (C) Electropherogram from a RNA sample collected in extraction buffer and isolated with the PicoPure isolation kit.
Figure 7.4. RT-PCR and Microarray from LCM samples. (A) Amplification plot and melting curves demonstrate amplification of CXCL1 from isolated LBECs (B) cDNA products generated from LBECs (Cy3/green) and equine kidney (Cy5/Red) RNA samples were hybridized to the equine whole genome oligoarray and a portion of the array is shown demonstrating successful hybridization.
Chapter 8: Conclusions

The work summarized in this dissertation was performed to determine the role of inflammation and the epidermal epithelial cell in the development of equine laminitis. Inflammation has been documented to occur early in the developmental phases (1.5h and 3h) of BWE induced laminitis. As reported in chapter 2, horses administered CHO also had increased mRNA concentrations of many inflammatory mediators including IL-1β, IL-6, IL-12p35, COX-2, ICAM-1, and E-Selectin; however, the increases in these mediators occurred at a later onset than with the BWE model. The differences in the time course of the inflammatory response between these two models appear to be similar to what is reported for models of human sepsis, where the BWE model more closely resembles administration of an endotoxin bolus and the CHO more closely resembling the cecal ligation and puncture (CLP) model in rodents. It is believed that the CLP model is more clinically relevant, having a delayed onset and slightly lower magnitude of inflammation, but longer in duration, whereas intravenous endotoxin administration results in a rapid and robust inflammatory response that is short lived. As with the CLP model, the CHO model is more clinically relevant and had a delayed onset of inflammation when compared to the BWE model.
The following study assessed the presence of inflammation in the hindlimb lamina after CHO administration through determination of inflammatory mediator gene expression and leukocyte emigration into the lamina. Inflammation was documented to occur in the hindlimb after administration of CHO, with the pattern of inflammation, particularly time of onset and overall magnitude, being similar to what was previously discovered in the forelimbs. The results from this study support that inflammation does occur in the hindlimb with equal magnitude to the front limbs and therefore is not the cause for the clinical observation that laminitis most commonly affects the forelimb. Other factors, such as weight bearing and hoof shape/size, may play an important role in the development of laminitis and should be assessed further to determine why the hindlimb appears to be less frequently affected than the fore.

STAT signaling was evaluated in the BWE and CHO models of laminitis (Chapter 4), as STAT1 and STAT3 activation in sepsis/SIRS has been reported and is known to be involved in inflammatory signaling and with the production of inflammatory mediators. STAT3 tyrosine and serine phosphorylation occurred in the lamina during both the developmental and onset of lameness stages of laminitis in horses receiving BWE or CHO, whereas no change in STAT1 activation occurred. Furthermore, STAT3 tyrosine phosphorylation was determined to be occurring in the laminar epithelial cells as well as in some cells within the dermis (which were suspected to be leukocytes). IL-6 is known to be greatly increased in the lamina of horses after BWE or CHO administration and is known to activate STAT3 phosphorylation; therefore, it was not unexpected that STAT3 phosphorylation was increased in these samples. The lack of a STAT1 response,
when it is known to be present in cases of sepsis, is suspected to be due to an absence of interferon production, as no change in IFNα, IFNβ, or IFNγ mRNA concentrations, have been reported in the lamina after CHO or BWE administration. Since STAT3 phosphorylation in suspected to occur secondary to IL-6 production, and IL-6 is known to be a pleiotropic cytokine having both pro- and anti-inflammatory functions, it is not known at this time if the use of STAT3 inhibitors would helpful or harmful. Further work is needed to determine how STAT3 phosphorylation affects laminitis before inhibitor treatment can be applied to clinical patients.

As sepsis/SIRS is known to result in multiple organ failure in people but often just results in single organ failure (laminar failure) within the horse, determination of inflammatory changes in other organs not frequently affected in horses was evaluated after CHO administration (Chapter 5). Of the three organs evaluated (liver, lung, and kidney), the liver had the earliest and greatest response in inflammatory mediator gene expression. As previously reported with the BWE model, horses administered CHO had increases in TNFα, IL-1β, IL-10, CXCL8 mRNA concentrations in the liver during the developmental and onset of lameness times as well as increases in COX-2 at the onset of lameness. Although the liver appeared to have the greatest inflammatory response in regards to gene expression, no significant changes in leukocyte counts were noted between groups. In the lung however, increases in leukocytes were noted in both the developmental period examined and at the onset of lameness after CHO administration. This was accompanied by increases in IL-1β, IL-10, and COX-2 mRNA concentrations from cellular components of affected the pulmonary tissue of horses at the onset of
lameness. As renal failure is probably the second most common condition associated with sepsis/SIRS of the adult horse, it was surprising that only minute increases in IL-1β concentrations were present in the onset of lameness horses after CHO administration relative to that observed in the lung or lamina.

Although other organs mount an inflammatory response to CHO, the cytokine profile and magnitude is not the same as what was previously found in the lamina. No increases in IL-6 were noted after CHO in the liver, lung or kidney, whereas this cytokine is significantly increased in the lamina. Additionally TNFα, which is known to be a prominent cytokine of sepsis/SIRS in humans, is not increased in the lamina, but is present in the liver after CHO administration. It appears that, similar to that reported in sepsis/SIRS of humans, each organ in the septic horse acts like a separate compartment, responding differently to the initiating cause. Perhaps it is the lack of a TNFα response in the visceral organs or the presence of a prominent laminar IL-6 response that results in laminar failure, but not multiple organ failure.

An in vitro study was conducted in order to further study inflammatory signaling mechanisms that may be involved in laminitis and to begin to critically evaluate the role of the epidermal epithelial cell (Chapter 6). Cell culture assays of equine keratinocytes, isolated from equine skin, were used in which the cells were stimulated with various bacterial ligands to determine if, similar to that reported in the human cultured keratinocyte, the equine epidermal epithelial cells would respond to PAMPs characteristic of gram positive and gram negative sepsis by producing the pro-inflammatory cytokines IL-1β and IL-6 and the chemokine CXCL8. Interestingly, the
keratinocytes only responded to bacterial ligands from of a gram negative source, LPS initiating its response through the TLR4 receptor and flagellin through TLR5 receptor. This is similar to what is reported clinically where laminitis, a disorder that affects the attachment of the epidermal lamina to dermal lamina, overwhelmingly results from sepsis of gram negative origin. Although it remains to be determined if laminar epithelial cells will respond similarly to the bacterial ligands used in this study, these results suggest that the use of TLR4 or TLR5 antagonists may be able to decrease the inflammatory response, thereby decreasing the risk for development of laminitis in horses.

Since the successful culture of equine laminar epithelial cells remains elusive, the development of another technique to study these important cells was performed and reported in Chapter 7. The use of laser capture microscopy allows for the microscopic dissection of basal laminar cells followed by collection and isolation of total RNA that can then be used in real-time PCR reactions or in microarray analysis. Successful capture of the laminar basal epithelial cells and isolation of good to high quality RNA was achieved. This technique will prove to be invaluable to the ongoing study of the laminar epithelial cells’ role in laminitis.

Overall, laminitis is a complex disease with many mechanisms involved in the process of laminar failure (Figure 8.1). The research conducted for this dissertation has proven that inflammation does occur in the BWE and CHO experimental models of laminitis and is mediated through STAT3 and/or TLR signaling. Since the overall patterns of inflammation differ between two models and between the various organs within the horse, further studies evaluating the differences in these patterns may be
essential in determining why the digit is the target organ of sepsis/SIRS in the horse. Furthermore, studies using STAT3 or TLR4 inhibitors must be performed to determine if they can be successful in preventing or limiting disease in our septic equine patients.

However, it is possible that factors other than inflammation may play a more important role in laminar failure, as no difference in type or magnitude of the inflammatory response was present between the front and hind lamina. Evaluation of the biomechanical structures within in the digit and lamina, both macroscopically and microscopically are necessary to determine why the front feet are at a greater risk of developing laminitis than the hind.

Additional characterization of the inflammatory response, evaluated by in vitro bacterial ligands stimulation of equine epidermal epithelial cells, supported the clinical observations that conditions involving gram negative bacteria are more likely to result in the development of laminitis. Since the epidermal epithelial cell was determined to be an active participant in the inflammatory response, the development of cutting edge techniques such as LCM are critical in order for us to study the laminar epithelial in association with surrounding cells during the development of laminitis. Collection of laminar basal epithelial cells via LCM, isolation of RNA, and subsequent gene analysis through microarray techniques will likely result in a better understanding of not only how these cells function in the inflammatory response, but how other mechanisms such as cytoskeletal signaling may be altered during the development of laminitis.
Figure 8.1. **Inflammatory processes within the lamina during laminitis.** Diagram demonstrates laminar basal epithelial cells (LBEC - insert A) and their production of inflammatory mediators in response to TLR ligand binding and/or IL-6 cytokine binding activating the JAK-STAT3 pathway. Also leukocyte (insert B) emigration from the blood vessels into the dermal lamina can produce inflammatory mediators. Other possible mechanisms involved in inflammatory injury in laminitis include alterations to local laminar blood flow (perhaps through vasoactive inflammatory mediators, and may exacerbate cell dysfunction), cytoskeletal alteration within epithelial cells (perhaps responding to STAT3 activation, TLR activation, or ischemia), and biomechanical forces of the digit exacerbating any cytoskeletal failure and /or failure of attachment of the LBEC to the underlying dermis.
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