EQUINE LAMINITIS: VASOACTIVE EFFECTS OF EICOSANOIDS AND INFLAMMATORY GENE EXPRESSION

by

ERIK NOSCHKA

(Under the Direction of James N. Moore)

ABSTRACT

Equine laminitis is debilitating and life-threatening disease that affects horses worldwide. This condition is exteremely painful and is associated with a variety of systemic diseases. Although the mechanisms responsible for development of laminitis remain to be determined, the most prominent hypothesis regarding the pathogenesis of the condition focuses on the development of local inflammatory changes in the foot and alterations in digital hemodynamics.

The studies described in this dissertation were performed to determine the vasoactive properties of prostanoids and isoprostanes in order to determine the possible role of these inflammatory mediators in the selective venoconstriction that occurs during onset of the condition, and to evaluate alterations in gene expression in laminar tissues at three time points during the onset of the disease. Vascular constrictive effects of thromboxane, prostaglandin $F_{2\alpha}$, prostaglandin E_2 and the isoprostanes were compared using laminar arteries and veins from healthy horses and horses with the earliest signs of acute laminitis induced by administration of black walnut heartwood extract (BWHE).

The results of these studies provide convincing evidence that genes associated with inflammation; activation and extravasation of leukocytes; antimicrobial activities; and

destruction of the lamellar basement membrane, are induced in response to administration of BWHE. Using microarray techniques, proinflammatory genes were up-regulated as early as 1.5 hours after BWHE administration, and transcripts for endogenous antioxidants were expressed at Obel grade 1 laminitis. Furthermore, increases in plasma concentrations of prostanoids and laminar tissue concentrations of isoprostanes were identified after administration of BWHE, and were associated with venoconstriction and impairment of vasodilatory pathways identified in the *in vitro* studies performed with the laminar vessels. Collectively, these findings add credence to the results of recent studies indicating that inflammation and venoconstriction occur concurrently during the prodromal stages of equine laminitis, and suggest that prostanoids and isoprostanes contribute to the development of laminitis by increasing post-capillary resistance in the laminar dermis.

INDEX WORDS: Equine Laminitis, Prostaglandins, Isoprostanes, Equine laminar veins, Arteries, Microvessels, Microarrays, Gene expression, Inflammation

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Dedicated to my loving wife,
Anna,
for her continued, unquestioned support,
encouragement and trust

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CHAPTER 1

INTRODUCTION

The studies reported in this dissertation were undertaken to add to our knowledge concerning the pathogenesis of equine laminitis. Chapter 2 provides a comprehensive review of the relevant scientific literature and is divided into 3 sections. Section I describes the anatomy of horse's foot, and the clinical disease of laminitis, specifically taking into account what is known regarding its pathophysiology. Section II summarizes the primary mechanisms regulating tissue perfusion and vascular tone, microvascular function, and the endogenous vasoactive substances implicated in the development of acute laminitis. The primary focus of this section is on the prostanoids and isoprostanes, the different types of prostanoid receptors and their distribution, and currently available prostanoids receptor agonists and antagonists. Section III contains a detailed description of gene expression profiling and the microarray technique. This section also reviews the principal genes involved in the control of vascular function, methods for monitoring gene expression, and microarray analysis. The results of the principal studies comprising this dissertation project are described in Chapters 3 through 6. Chapter 3 examines the roles of thromboxane and isoprostanoids in laminitis. Chapter 4 examines the role of prostaglandin $F_{2\alpha}$. Chapter 5 examines the role of prostaglandin E_2. Chapter 6 summarizes changes in laminar gene expression identified at three time points during the developmental stages of laminitis. Finally, Chapter 7 provides a general discussion of the whole project with a special emphasis on how it

provides the first integrating data to link the vascular and inflammatory hypotheses of the pathogenesis of laminitis.

CHAPTER 2

LITERATURE REVIEW

SECTION I: EQUINE LAMINITIS

INTRODUCTION

The interdigitating primary and secondary laminae bond the hoof wall, distal phalanx and the sole of the equine foot into a single unit, allowing the horse to walk and suspend the distal phalanx from the inner surface of the hoof capsule.[1] The inner hoof wall (stratum internum) is comprised of 550 to 600 primary epidermal lamellae, each of which consisted of 150 to 200 secondary lamellae that form longitudinal grooves for interdigitating with the laminae of the laminar dermis. The interface between the lamellar epidermis and the dermis (corium) is formed by the basement membrane, a tough and unbroken three-dimensional sheet of fine, anastomosing cords of filaments forming the extracellular matrix.[2]

The highly vascular corium (dermis) consists of a dense matrix of tough connective tissue containing a network of arteries, veins and capillaries, and sensory and vasomotor nerves. All parts of the corium, except for the lamellar corium, have papillae that fit tightly into spaces in the adjacent hoof. The lamellar corium has dermal lamellae that interlock with the epidermal lamellae of the inner hoof wall and bars. The vascular system of the corium provides the hoof wall with nourishment. The dense matrix of connective tissue in the corium connects the basement membrane of the dermal–epidermal junction to the surface of the distal phalanx, thereby suspending the distal phalanx from the inner wall of the hoof capsule.[2]

LAMINITIS

Aristotle described an acute febrile condition affecting the horse and referred to it as "barley disease".[3] It is now accepted that Aristotle was referring to the syndrome that was given the name "laminitis" in the 1700s. Laminitis is a debilitating, potentially career-ending and life-threatening disease of the sensitive and insensitive laminae of the digit in all breeds of adult horses. Laminitis causes structural changes in the digit that lead to the breakdown of the lamellar structure of the inner hoof wall. Without the proper attachment of the distal phalanx to the inside of the hoof, the weight of the horse and the forces of locomotion drive the bone into the hoof capsule, damaging and often shearing arteries and veins and crushing the corium of the sole and coronet. These events cause unrelenting pain and a characteristic lameness. Therefore, a clinical definition of laminitis is "failure of attachment between the distal phalanx and inner hoof wall".[4]

Several risk factors are associated with the development of equine laminitis. For example, hormonal factors appear to play a role in the pathogenesis of the disease since there is a higher incidence rate in geldings than in intact males,[5] and in mares compared to geldings or intact males.[6] In addition, horses from 5 to 7 years of age and from 13 to 31 years of age appear to be at increased risk for developing the disease. However, there is evidence that age, breed, sex, or seasonality are not key underlying factors in the development of acute laminitis.[7,8] Laminitis is often associated with diseases such as colic, strangulating obstructions, colitis and inflammatory bowel disease, grain overload, retained fetal membranes and subsequent to metritis, pleuro-pneumonia, and diseases associated with endotoxemia.[9,10] In a study performed in seven private practices and at a university veterinary hospital, gastrointestinal tract disease was found to be the most common primary disease in 54% of horses that developed acute laminitis.[8]

GRADING OF THE STAGES OF LAMINITIS

Although laminitis can affect all four feet, it most commonly affects the forelimbs presumably because the forelimbs bear approximately 60% of the horse's body mass.[1] Furthermore, laminitis commonly occurs in the contralateral limb of horses that have a severe non-weight bearing lameness in the opposite limb.[11] To better define the severity of clinical signs exhibited by affected horses, a grading system was established by Obel in 1948.[12] Grade 1 is the least severe and is characterized by alternately lifting the feet; lameness is not evident at a walk, but at a trot a short stilted gait is noted. Horses that walk with a stilted gait but can still have a foot lifted are classified as having Obel grade 2 laminitis. Horses with grade 3 move very reluctantly and vigorously resist lifting of a foot. The most severe classification is grade 4 noted by the horse refusing to move unless forced.[1,8] Other clinical signs characteristic of laminitis in the forelimbs are heat present over the dorsal surface of the hoof wall, strong digital pulses (*i.e.*, increase in the difference between systolic and diastolic digital arterial pressures), sensitivity to hoof testers, swelling of the coronary band, and redistribution of weight to the hind limbs ("sawhorse stance" or rocking of weight to the hind limbs). More severe signs are a dropped sole or palpation of a depression located at the level of the coronary band, both indications of rotation or sinking of the distal phalanx within the hoof capsule.[13]

HYPOTHESES OF THE CAUSES OF LAMINITIS

In 1937, Backus[3] hypothesized that laminitis was due to the effects of a toxic substance entering the blood stream, causing digital vasoconstriction and altering hemodynamics. Backus[3] also suggested that an increased body temperature, increased heart rate, increased respiratory rate, muscle tremors, and injection of mucous membranes were precursors to the development of

laminitis. In those earlier times, the developmental phase of laminitis was difficult to distinguish from enteritis, pneumonia and peritonitis. Over the past 20 years, the leading hypothesis regarding the initiating factor in the cascade of events leading to necrosis and structural failure of the interdigitating sensitive and insensitive laminae has focused on alterations in digital hemodynamics. However, the initiating event that triggers these vascular alterations has yet to be determined. The results of *in vitro* and *in vivo* studies indicate that the intestinal mucosal barrier becomes dysfunctional in horses with experimentally-induced laminitis, potentially allowing for the entry of endotoxin into the circulation.[14-16] However, administration of *Escherichia coli* 055:B5 endotoxin did not induce changes in equine digital hemodynamics or Starling forces that occur in horses in the developmental stages of acute laminitis induced with either the black walnut extract (BWE) or carbohydrate overload (CHO).[17] In contrast to the venoconstriction that characterized the developmental stage of laminitis, administration of endotoxin elicited arterial vasoconstriction and digital hypoperfusion.[17] Circulating concentrations of endotoxin have been reported to be increased in horses with CHO-induced laminitis and in horses with naturally-occurring gastrointestinal tract disease.[18,19] Endothelial damage has been found after a single infusion of endotoxin including endothelial cell retraction and pyknosis, a loss of barrier function, and cell lysis. Although endotoxin administration did not induce acute laminitis in horses, substantial evidence exists to support a role for endotoxin in the pathophysiology of acute laminitis secondary to gastrointestinal tract diseases, metritis, and pleuropneumonia. [9,10,20,21,

EXPERIMENTAL MODELS OF LAMINITIS

Carbohydrate Model of Laminitis

Garner *et al*[22] developed in 1975 a consistent and clinically relevant induction model of acute equine laminitis involving CHO of the gastrointestinal tract. The laminitis-inducing ration, a combination of 85% corn starch and 15% wood cellulose flour, was administered via a nasogastric tube at a dose of 17.6 g/kg. Although the exact cascade of events linking CHO administration and the development of acute laminitis remain to be determined, alterations in cecal flora, lactic acidosis, and endotoxemia after CHO administration have been documented.[14-16,18,23,24] In these studies, horses developed clinical signs of laminitis and progressed to Obel grade 3 by approximately 40 hours post-CHO administration. The CHO model became the standard for laminitis studies due to its similarity to grain overload, a common cause of naturally-occurring laminitis cases, and one with high morbidity.

Black Walnut Model of Laminitis

A second model for inducing laminitis involving intragastric administration of an aqueous extract of the heartwood of black walnut (Juglans nigra) trees was introduced in 1987.[22,25,26] This model arose when it was determined that horses developed acute laminitis after exposure to fresh shavings from black walnut trees.[26] To use this model, the heartwood from living trees felled in the fall of the year were planed to produce shavings, that were then soaked in water over night. The BWE was then administered via nasogastric tube.[26] The BWE model was considered an improvement over the CHO model, as diarrhea did not occur, there was no evidence of endotoxemia, and signs associated with acute laminitis were recognized far sooner than with the CHO model.[25,27-29] Other differences between these models may reflect a

divergence of mechanisms leading to the onset of laminitis. Indeed, the results of one study indicate that the magnitude of venoconstriction within the digit was less in the BWE model than with the CHO model.[25]

Oligofructose Model of Laminitis

A third model for inducing acute laminitis was described in Y2002.[30] In this model, oligofructose, a fructan derived from chicory roots, was administered via a nasogastric tube to 6 horses. Although all horses developed clinical signs of laminitis, they developed diarrhea, pyrexia, tachycardia, and had hematological alterations that were similar to the CHO model.[30,31]

POTENTIAL MECHANISMS RESPONSIBLE FOR DEVELOPMENT OF LAMINITIS

At present, there are 4 principal theories as to the mechanisms responsible for the development of equine laminitis, namely (1) the ischemic/vascular, (2) mechanical/traumatic, (3) inflammatory, and (4) metabolic/enzymatic, theories.[13] Since the mechanisms involved in the pathogenesis of laminitis are most likely numerous and interrelated, there are probably multiple factors from each theory that participate in the pathogenesis of the disease.

The Ischemic/Vascular Theory

The "ischemic/vascular theory" proposes that altered digital perfusion is the initiating factor in the cascade of events that leads to local metabolic dysfunction and structural failure of the laminae.[32] Although the pathogenesis of laminitis is not fully understood, the initial vascular mechanisms are characterized by hypoperfusion due to venoconstriction, laminar edema formation, and opening of arteriovenous shunts that allow blood to bypass the laminar tissues.

These deleterious changes in local blood flow lead to tissue ischemia, necrosis of the interdigitating laminae, and ultimately mechanical failure with displacement of the distal phalanx away from the hoof wall.[22,30,33,34] Venoconstriction is considered to be the initiating factor causing decreased laminar perfusion,[35] as it causes increased vascular resistance and capillary hydrostatic pressure. The increased capillary hydrostatic pressure forces fluid out of the capillaries and into the interstitium thereby increasing laminar interstitial pressure. When tissue pressure exceeds capillary critical closing pressure, the capillaries collapse leading to tissue ischemia. Increased pressure in a confined anatomical space reduces blood flow of those tissues and can lead to ischemia; this condition is referred to as "compartment syndrome". Allen et al[33] hypothesized that horses develop compartment syndrome in the digit during the developmental stages of laminitis, leading to laminar ischemia.

The toxic (enzymatic) theory

The "toxic (enzymatic) theory" of laminitis, states that the fundamental event leading to the failure of the laminar interdigitations is the delivery of blood-borne toxins to the epidermal laminae, which results in weakening and loss of cellular attachments.[36] As such, the loss of cellular attachments is the precursor to the vascular and inflammatory alterations described within the ischemic theory. Proponents of the toxic/enzymatic theory propose that hyperperfusion, rather than hypoperfusion, of the digit delivers the toxins to the laminar tissues.[29] Pollitt[36,37] proposes that the targets of blood-borne toxins are mediators of enzymatic remodeling that is part of the processes involved in the normal movement of the proliferating hoof wall across the surface of the distal phalanx. Excessive activation of enzymes leads to uncontrolled dissolution of the basement membrane components resulting in separation of the epidermal

laminae from the dermal laminae. Proponents of this theory suggest that the loss of basement membrane attachments might be due to increased activation of extracellular metalloproteinases[37,38] by exotoxins from Streptococcus bacteria in the cecum.[23,36,39] Using the CHO model, researchers have identified changes in bacterial populations in the cecum, excessive production of lactic acid, a rapid decline in intracecal pH, and death of cecal bacteria including Streptococcus species.[23,40] Based on these data, prevention of laminitis should be aimed toward abolishing the activation of enzymes responsible for the dissolution of the basement membrane.

The Inflammatory Theory

The inflammatory theory is adapted from the results of recent studies that revealed a marked increase in pro-inflammatory cytokines, mediators and enzymes in the laminar tissue during the onset of laminitis. While showing consistent presence of inflammatory products in the laminae, the inflammatory hypothesis is based on a systemic activation of inflammatory processes that distribute activated inflammatory cells into many tissues (as shown by parallel skin and laminar infiltration by neutrophils) of the body. Investigators demonstrated increased expression of interleukin-1β (IL-1β),[28,41] IL-6,[41] and the Molecule possessing Ankyrin-repeats Induced by Lipopolysaccharide (MAIL),[41] which has a key role in cellular regulation of inflammatory cytokine expression. Other immunohistochemical studies found increases in leukocyte numbers around the dermal vasculature of the laminae. This theory and recent findings of a significant up-regulation of COX-2 mRNA[42] and protein[43] in laminar tissues during the developmental stages of laminitis, supports the aggressive use of non-steroidal anti-inflammatory drugs in horses at risk for developing laminitis. A more recent study found that the lamellar inflammation is characterized by a strong innate immune response in the developmental stages of

laminitis suggests that drugs that reduce lamellar cytokine expression[44] should also be considered, perhaps in combination with drugs that target prostanoid activity, in the treatment of horses at this stage of laminitis or in horses at risk of laminitis.

The "Mechanical (Traumatic) Theory

The "mechanical (traumatic) theory" is based on cases of laminitis that result from direct trauma to the digit and not a primary systemic disease leading to the development of laminitis.[13] Common examples of traumatically-linked laminitis are road founder, laminitis secondary to unilateral lameness of the opposite foot ("support limb laminitis"), and development of laminitis after long trailer rides.[11] The exact mechanisms that lead to structural failure of the laminae are unknown, but several hypotheses have been suggested. For example, excessive force applied to the dermal and epidermal laminar interdigitations may initiate an inflammatory response with vasospasm, thereby increasing capillary hydrostatic pressure, leading to edema formation ultimately resulting in a compartment-like syndrome much like that proposed by the "ischemic/vascular theory".[13] Another hypothesis is that application of excessive force results in tearing of the dermal and epidermal laminar interdigitations, which is followed by an inflammatory response and/or vasospasm that leads to ischemic damage of the laminar interdigitations.[13]

CHANGES IN VASCULATURE PERFUSION IN THE FOOT IN LAMINITIS

Researchers have indirectly demonstrated reduced perfusion in the vasculature of the foot in laminitic horses using contrast radiography, and hoof wall surface temperature.[13,27,45,46] Using contrast radiography, researchers demonstrated reduced perfusion in the terminal vasculature of

the foot after CHO induced laminitis.[45] Hood *et al*[45] used hoof wall surface temperature to indirectly monitor laminar perfusion during the developmental stages of CHO-induced laminitis, and reported that surface temperature decreased 8 to 12 hours before the onset of lameness. The drop in temperature could obviously have resulted from decreased laminar perfusion and/or decreased metabolic activity. Hood *et al*[45] also found that hoof wall surface temperature actually increased as clinical signs of laminitis became evident. More recently, Pollitt and colleagues demonstrated increases in hoof temperature (an indicator of increased lamellar blood flow) 16 to 40 h after CHO overload.[22,33,46,47]

Although many mediators may contribute to the above mentioned vascular alterations, the principal mediators have yet to be determined. Following CHO-induced laminitis, microvascular thrombi formation occurs in the laminae, in addition to hemodynamic alterations, and likely contribute to the decreases in laminar perfusion due to microvascular obstruction.[48,49] Using laser Doppler flow probes to measure laminar capillary perfusion, Adair *et al*[27] found that laminar microvascular blood flow decreased in the first 1-2 hours after BWE administration. This initial decrease was then followed by a return of laminar microvascular blood flow to near baseline values. Then, approximately 8 hours into the disease, laminar blood flow again decreased, which corresponded temporally with development of the clinical signs of laminitis. Although a different method for examining laminar perfusion was used, these findings are similar to those reported by Hood *et al*[27,45,46] using hoof wall surface temperature as an indication of laminar perfusion after CHO administration. Using gamma scintigraphy of regionally infused 99mTc-labeled macroaggregated albumin before and 12 hours after BWE administration, Galey *et al*[50] demonstrated that the administration of BWE elicited reductions in digital perfusion, which were greater in the dorsal laminar and coronary dermis than the remainder of the forelimb.

SECTION II: VASCULAR FUNCTION AND EICOSANOIDS

MICROVASCULAR BIOLOGY OF THE LAMINAR MICROCIRCULATION

Introduction

In the 19th century, direct measurements of arterial and venous blood pressure by Jean Leonard Marie Poiseuille revealed that decreases in pressure in the circulation takes place mainly in small arteries of peripheral vascular beds. Collectively, these small arteries, which have a diameter of 100 μm or less, are collectively referred to as the microcirculation, which is the site of most of the resistance to blood flow. This resistance to blood flow depends on the architecture of the microvascular network and on the rheological behavior of blood flowing through it. The resistance arteries also interconnect to form networks in tissues, and directly feed capillaries, which are responsible for exchange of materials between blood and the surrounding tissues. Therefore, the mechanics of blood flow in the microcirculation controls the distribution of blood to the tissues and largely influences material exchange in the circulatory system.

Anatomy and physiologic function of the laminar microvasculature

The lamellar corium derives most of its blood supply from the branches of the terminal arterial arch, which perforate the distal phalanx. Numerous anastomoses form an arterial network beneath and between the epidermal lamellae, allowing blood to flow proximally to the coronary circumflex artery and distally to the solar circumflex artery.[51] The parallel veins join to form the terminal vein, which is joined by branches from the venous plexus to form the palmar digital vein. Veins within the foot are in majority lack valves, a feature that can be exploited clinically in performing contrast radiography (venograms) or administering drugs into the foot. The direction of flow within the veins is dependent upon weight bearing forces.[51]

A large number (500/cm^2)[52] of arteriovenous anastomoses connect the axial arteries to axial veins in the lamellar dermal microcirculation.[2] These anastomoses occur in the dermis of the coronary band, in the neurovascular structures within the dermal laminae, and at the entrance to and along the length of the dermal laminae.[52,53] There are several hypotheses regarding the physiological function of these anastomoses. One hypothesis is that during long periods of cold exposure, the anastomoses open to allow for warming of the feet by increasing blood flow.[52] Another hypothesis is that high pressure fluctuations that occur in the digit during galloping or jumping cause the anastomoses to open to diffuse the tremendous increases in pressure and may act as a "safety valve" for the vasculature.[52] Due to the anatomy of the distal limb and the forces required to return venous blood to the heart, the digital veins are quite muscular compared with veins in other tissues and other species.[54] The nerve supply to the foot is principally derived from the medial and lateral palmar digital nerves. A dorsal branch, and in approximately 30% of horses an intermediate branch, supply sensory and vasomotor innervation to the dorsal aspect of the distal interphalangeal joint and the perioplic and laminar coria.[51] The palmar digital nerve continues distally to supply the laminar and solar coria.[51,53,55-60]

Bifurcation of arteries results in the formation of small muscular arterioles, the function of which is to reduce blood flow coming from the larger arteries and thereby to prevent damage to the fragile capillaries that connect the arterial and venous vascular systems. Through a complex interaction with autacoids, hormones, and neurotransmitters released at sympathetic synapses, flow through the microcirculation is regulated by altering the tone of the arteriolar smooth muscle. The capillary is characterized by a lack of smooth muscle, a single layer of endothelial cells (approx. 0.25 μm), a basement membrane, and is enveloped in a population of pericytes or "Rouget" cells that varies in distribution within vascular beds.[61,62] It is across this

large surface area of the capillary bed that the majority of nutrients, solutes, water and oxygen are exchanged between the blood and surrounding tissues. The exchange of solutes and water usually results from simple diffusion according to solute concentration gradients and hydrostatic and osmotic pressures (*i.e.,* Starling forces). There are several types of capillaries (continuous, fenestrated, and discontinuous), with continuous capillaries being most common. These capillaries have a continuous endothelial and basement cell membrane layer and are present in many organs such as the heart, lung, kidneys, brain, and laminar tissue.

Capillary walls consist mainly of endothelial cells, which serve as the main barrier to, and regulator of, material exchange between the circulating blood and tissues. The mechanisms by which endothelial cells and the clefts between these cells modulate permeability to water and solutes have been studied for more than 50 years. To bridge the gap between the structural data obtained from serial electron microscopic sections of the cleft between endothelial cells and functional *in vivo* data regarding capillary permeability to water and solutes, a series of mathematical models have been developed.[63] These models suggest that the surface glycocalyx layer at the luminal entrance of the cleft serves as a molecular filter and is crucial for the ability of the capillary wall to maintain a low permeability to macromolecules. Endothelial cells provide the rate-limiting barrier to extravasation of plasma components from electrolytes to proteins.

There are four primary exchange pathways that have been observed in the capillary wall by electron microscopy: intercellular clefts, transcellular pores, vesicles and fenestrae. Different pathways may be utilized in different types of capillaries, in different tissues, and under different physiological and pathological conditions. The cleft between adjacent endothelial cells is widely believed to be the principal pathway for transport of water and hydrophilic solutes through the microvessel wall under normal physiological conditions. This interendothelial cleft is also

purported to be the pathway for the transport of high molecular weight plasma proteins and for the movement of leukocytes out of the microvasculature in disease.

The mechanisms used by the endothelium to modulate smooth muscle function have not been fully elucidated in either normal or diseased conditions. The vascular endothelium, the largest endocrine, paracrine, and autocrine regulatory tissue in the body, participates in numerous processes related to the functioning of the vessel wall. In response to various stimuli, endothelial cells synthesize and release numerous biologically active factors such as nitric oxide, endothelins, prostanoids, endothelium-derived hyperpolarizing factor, oxygen free radicals, growth factors, tissue plasminogen activator and inhibitor, and adhesion molecules. The stimuli for release of these factors include hemodynamic forces (shear stress, cyclical strain) and circulating and locally derived vasoactive substances (*e.g.,* angiotensin II, catecholamines, vasopressin, arachidonic acid, thrombin), as well as interactions among endothelial cells, leukocytes and platelets. Short-term interactions between these factors contribute to the regulation of vascular tone, while the longer-term interplay of such factors modulates smooth-muscle cell proliferation, extracellular matrix production, hypertrophic growth, and apoptosis to regulate vascular structure and remodelling. Endothelium-derived factors also regulate platelet aggregation, coagulation, and thrombosis, as well as leukocyte adhesion and inflammation via the expression of chemotactic and adhesion molecules.[64]

In the standing horse, digital blood flow is relatively stable and minor shifts in weight do not significantly change flow.[65] Blood flow decreases as the horse shifts weight bearing between the feet completely onto the digit and *vice versa*.[65] The digital arteries and veins supplying the hoof have unique vasoactive properties, when compared to vessels of similar size from other tissue beds or species. The veins are muscular, have a relatively inelastic vascular wall, and are

situated in a noncompliant compartment between the hoof wall and distal phalanx, resulting in a vasculature having low compliance.[54] Digital vessels are very sensitive to vasoconstrictor substances, particularly to norepinephrine and endothelin.[66] Furthermore, the digital veins are more sensitive than arteries *in vitro* to the vasoconstrictor substances as angiotensin II, thromboxane, norepinephrine, serotonin, and endothelin.[66] Similarly, the results of an *in vitro* study of palmar digital arteries and veins indicate that incubation of digital veins with either acepromazine or isoxsuprine resulted in a greater degree of dilation than occurred when digital arteries were treated in the same manne.[67] In further studies, incubation of digital vessels with acetylcholine, prostaglandin E_2, and prostaglandin I_2 (prostacyclin) were found to elicit vasodilator responses, most likely via endothelium-derived nitric oxide since these responses were abolished by the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester.[67-69] Furthermore, the palmar digital vessels were determined to be more responsive to 5-hydroxytryptamine (serotonin) than either facial or tail arteries.[70]

Using a technique that allowed *in vitro* study of small laminar arteries and veins, Robertson *et al*[61,62] reported that induction of capacitative calcium entry elicits vasoconstriction in equine laminar veins but not in laminar arteries. They proposed that capacitative calcium should be considered as a potential mechanism by which selective venoconstriction occurs in horses during the development of acute laminitis.[71] In other studies, Peroni *et al,*[72,73] they determined that induction of the prodromal stages of laminitis resulted in a consistent and selective dysfunction of the laminar veins, and that there may be a predisposition for venoconstriction within the vasculature of the equine digit. These findings might help to explain why laminitis can result from a variety of pathological systemic conditions.[72]

The combined effects of low compliance and high sensitivity to vasoactive substances predispose the equine digit to increased venous and hydrostatic pressures, thereby increasing the likelihood for laminar edema formation. Unfortunately for the horse, the microcirculation of the equine foot is poorly adapted to handling edema. For example, the main safety factors that counteract edema formation under normal circumstances are capillary permeability, pre-to-post capillary resistance, and lymphatic drainage. For those tissues in which capillary permeability is low, the endothelium serves as a barrier to movement of fluid and protein, resulting in a high gradient between capillary and tissue oncotic pressures. This situation favors movement of fluid from the tissues into the capillary lumen. However, the results of studies performed to measure Starling forces within the equine digit indicate that the normal equine digital capillary bed is highly permeable to fluid and macromolecules.[54] In fact, this capillary bed retains only 67% of the macromolecules within the vasculature, and thus is as "leaky" as the capillary bed in the lung.[74] The end result is a high concentration of protein in the interstitial space, which would favor edema formation.

Under normal circumstances, far more of the resistance to blood flow in the equine digit is due to precapillary resistance (92%) than to postcapillary resistance (8%), which reduces capillary pressure and the hydrostatic pressure for transcapillary fluid filtration. The pre-to-postcapillary resistance ratio in healthy horses is comparable to that in other musculoskeletal beds in other species.[54] In studies performed using the same techniques, Allen *et al*[25,33,34] found that induction of laminitis with either the CHO or BWE model resulted in increased post-capillary resistance to flow, increased tissue pressure and a reduction in the pre-to-post capillary resistance ratio, all of which favor the formation of edema within the digit.

The third factor that protects vascular beds from edema formation is provided by lymphatic drainage. The lymphatics of the equine digit are few in number and small in diameter; therefore, it is unlikely that the lymphatic circulation can effectively protect the foot against edema when capillary hydrostatic forces are increased.[33]

The earliest detected histological changes in the laminae after onset of lameness induced using the CHO model include swelling of endothelial cells and mild edema formation.[32] Subsequently, laminar capillaries become congested with erythrocytes, perivascular leukocyte infiltration occurs that then dissipates as the inflammatory cells migrate into the epidermal layer, and arteriolar endothelial cells become deformed as a result of cytoplasmic processes that extend into the lumen. Lesions detected later include microvascular thrombi, severe edema formation, and hemorrhage occurs within the primary dermal laminae. Induction of laminitis also results in structural changes in the laminae, the earliest reported changes being thinning and lengthening of the lamellar structures accompanied by reduction, flattening and displacement of epithelial cells. The orientation of the secondary lamina is altered such that lamina nearer the base of the dermal lamina are directed toward the distal phalanx, and those nearer the laminar tips are directed toward the hoof wall. Morphologic alterations that have been detected later include swelling, vacuolization, nuclear swelling and/or pyknosis, and leukocytic infiltration of the secondary epidermal lamina.

Alterations in endothelial cell function during the onset of acute laminitis may affect endothelium-dependent regulation of vascular tone and alter the responsiveness of the digital vasculature to vasoactive agents. *In vitro* studies have provided evidence that endothelium-derived nitric oxide is a potent vasodilator in normal equine digital vessels. Indeed, the release of nitric oxide appears to account for approximately 70% to 85% of the maximal relaxation induced

by acetylcholine.[66,75] In horses with CHO-induced laminitis, acetylcholine-mediated relaxations of digital vessels *in vitro* are reduced, suggesting that the nitric oxide producing capacity of the digital vascular endothelium is reduced. The loss of nitric oxide-dependent vasodilation renders the vessels more sensitive or vulnerable to vasoconstrictive agents.[69]

CONTROL OF PERFUSION AND VASCULAR TONE

In general, each body tissue controls its own local blood flow to match its metabolic needs. These needs include oxygen and nutrients (*e.g.*, glucose, amino acids, fatty acids) in addition to the removal of CO_2 and H^+ ions, maintenance of proper ion concentrations, and transport of hormones and other substances to the tissue. The local control mechanisms respond rapidly to maintain appropriate local tissue blood flow via vasodilation or vasoconstriction, and can adapt in the long term by decreasing or increasing the physical sizes and numbers of blood vessels supplying the tissue.

The organization of vasomotor responses within and among resistance vessels can be explained by the spread of electrical, chemical, and physical signals between endothelial and smooth muscle cells. These signals can be triggered by vasoactive substances released locally or systemically, or released by nerve terminals, and by changes in transmural pressure and luminal flow. Thus, from several perspectives, cell-to-cell communication coordinates the local control of blood flow in accord with the metabolic demands of the perfused tissue. This humoral regulation of perfusion is achieved via substances secreted or absorbed into the body fluid such as hormones, ions and other vasoactive chemicals. These substances may be formed by special glands (*e.g.,* adrenal gland) and then transported in the blood throughout the entire body, or may be generated in local tissues areas and cause only local circulatory effects. Norepinephrine and

epinephrine are examples of hormones released systemically after sympathetic nerve stimulation of the adrenal medulla; these hormones cause vasoconstriction in most vascular beds although epinephrine, and to a lesser degree norepinephrine, elicit hindlimb vasodilation (as part of the hemodynamic adjustments of the fright and flight response). Additional examples of vasoconstricting substances are angiotensin II, arginine vasopressin and endothelin. Vasodilating substances such as bradykinin and histamine are usually produced locally and are linked with inflammatory or allergic reactions.[76] Some substances can lead to either vasoconstriction or vasodilation depending on the receptors and receptor distribution among the tissue.

In summary, beside the most likely blood-borne factors such as the catecholamines, there are tissue released factors signaling hypoxia or ischemia such as adenosine, endothelial cell mediating factors such as nitric oxide and the endothelins, as well as flow induced effects and pressure induced effects (also known as the myogenic response) involved in the concert of controlling vascular tone. At any one location down the vascular bed, these factors may interact to produce the final response. It has been hypothesized that the balance of these mechanisms varies down the vascular bed, and between different regions of the vasculature.

VASCULAR FUNCTION MEASUREMENTS

Techniques – small vessel myography

One of the most commonly used methods to investigate blood vessel function is to isolate the blood vessel and study its properties in an tissue bath. While this is a difficult task to accomplish for small veins and arteries, it is a relatively simple task for larger vessles. Vessels may be removed as rings or strips with intact or denuded endothelium and their mechanical, electrophysiological, or biochemical properties investigated. The function of the blood vessel is

normally assessed in terms of contraction or relaxation. Usually, contractile activity of a vessel can be recorded by use of a force or displacement transducer under a given resting tension. Changes are recorded as the tissue either contracts or relaxes under the experimental conditions established by the investigator. These preparations are advantageous as they allow for the assessment of function independent of variations in either nervous system activity or blood flow.[77] Furthermore, morphological characteristics of the vessels can be assessed under *in vitro* conditions without introducing artifacts inevitably introduced by fixation for histology. With the advent of wire myographs,[77] vessel and wall dimensions, including measurements of adventitial, medial, and intimal thicknesses can now be determined. The dimensions may be normalized on the basis of the resting wall tension-internal circumference characteristic using the Laplace relation to determine the dimensions corresponding to a specific transmural pressure.[77]

To isolate laminar arteries and veins, the equine digit is placed in ice-cold physiological salt solution (PSS, containing in mM: NaCl 118; $NaHCO_3$ 24; $MgSO_4$ 1; NaH_2PO_4 0.435; glucose 5.56; $CaCl_2$ 1.8 and KCl 4, gassed with 95% O_2/5% CO_2, pH 7.40). The hoof wall is removed to the level of the laminar dermis with a large scalpel blade. The laminar dermis is carefully shaved away with a mounted razor blade until only a thin layer of tissue remains above the laminar vascular bed. The tissue is then transferred to the stage of a high-powered dissecting microscope and micro-fine instruments used to dissect laminar microvascular arteries and veins free of connective tissue. These small arteries and veins with 100-500 μm internal diameters are carefully dissected from the laminar tissue for mounting on 40 μm diameter stainless steel wires in a small vessel myograph. As with large arteries and veins, the tissue is pinned down in a silastic-based dish, which is bathed with PSS solution. With the aid of a dissecting microscope

(4-100x) and cool light source, 2-mm long vessel segments free of branches are removed from the tissue taking care to remove the connecting tissue without traumatizing the vessel wall.

The essential components of the myograph are the dual set of stainless steel jaws; one driven by a micrometer, the other attached to a very sensitive force transducer, which allows for the measurement of developed tension. Each 2-mm long segment of artery or vein is then mounted on 40-µm diameter stainless steel wires that are secured tight by a screws set into the jaws[78] of a dual chamber myograph.[79,80] After warming, all vessels are subjected to a normalization routine. The resistance arteries may be under a different length and axial tension than *in vivo*. For example, isolated mesenteric resistance arteries may retract in length from their tethered *in vivo* length, and their length is transmural pressure-dependent *in vitro*.[81]

Quality assessments (ensuring vessels are functional)

The importance of performing a "normalization" is three-fold. First, the results of experiments with elastic preparations, such as blood vessels, can only have meaning if the experiments are performed under conditions in which the size of the vessel is clearly defined. Second, the stretch of the vessels at the initiation of the experiment must be clearly defined as it can alter the sensitivity of the vessels to agonists and antagonists. Third, in order to identify the active responses of vessels, they must be set at the internal circumference that yields the maximal response. The aim of the "normalization procedure" is to stretch the mounted vessel segment to an internal circumference (IC_1) defined as a set fraction of the internal circumference (IC_{100}) that a fully relaxed segment would have at a specified transmural pressure. For a number of reasons, the vessel size in such studies is defined at the point at which the arterie is fully relaxed and is under a transmural pressure of 100mm Hg.[82]

In practice, the normalization procedure performed for small vessels follows the protocol used for large ring segment myography by distending the segment stepwise and measuring sets of micrometer and force readings. These data are converted into values of internal circumference (μm) and wall tension (mN/mm). Plotting wall tension against internal circumference reveals an exponential curve and by applying the isobar curve corresponding to 100mm Hg, IC_{100} is calculated from the point of intersection using the Laplace relation. Using the law of Laplace (Tension = riP, where ri is internal radius and P is the transmural pressure), the effective pressure Pi is calculated for each pair of readings. The effective pressure is an estimate of the internal pressure, which is needed to extend the vessel to the measured internal circumference. IC_1 is calculated from IC_{100}, yielding the internal circumference at which the active force production and the sensitivity to agonists are maximal. For rat mesenteric arteries the factor is 0.9, but this factor and the transmural pressure have to be optimized for each vascular segment. The normalized internal diameter is calculated by dividing IC_1 by π. The stepwise stretching is continued until the calculated effective pressure exceeds the target transmural pressure. For rat mesenteric arteries the target transmural pressure is normally 100mm Hg (13.3 kPa), but the value needs to be optimized for individual tissues. An exponential curve then is fitted to the internal circumference pressure data.[83]

In the next step, the vessels are partially relaxed by adjusting the micrometer to reduce the circumference. This micrometer reading is used as the standardized internal diameter and the optimal point of passive stretch for maximal development of active tension. The vessels are then suspended passively until they are maximally dilated. Active force measured as a contraction is then generated by applying a depolarizing solution of 80 mM K^+ (KPSS: isotonic replacement of Na^+ by K^+ in PSS). Having set the vessel to a normalized passive stretch, the active force

developed by a constrictor stimulus is measured as the increase in pressure against which the vessel will contract. A increase in wall tension on stimulation is used to demonstrate that the vessel is viable.[78]

Types of data generated

The tension developed by the vessels is measured by a transducer and then relayed to a computer where data can be continuously recorded in real-time using Powerlab software (Chart). This software acts as a digital chart recorder with the addition of calculation, statistical, display and analysis options. Contractile responses are calculated as a percentage of the maximal contractile response to KPSS (% T_K) for each vessel. Drug potencies are calculated using the graded dose response curve, with the EC_{50} representing the concentration of the tested compound at which 50% of its maximal effect is observed.

Procedures used to evaluate vasoactive agents (*e.g.*, removing endothelium, antagonists)

The endothelium plays a crucial role in the regulation of cardiovascular function by releasing several mediators in response to biochemical and physical stimuli. By exposing endothelial receptors to certain agonists, dose-dependent dilation or relaxation of the vessel occurs. Depending on the vessel size and type, endothelium-dependent dilation can be elicited by a number of agonists, including acetylcholine, bradykinin, substance P, adenosine triphosphate, vasopressin, and histamine. The endothelial-mediated dilations elicited by theses agonists often involve the production and release of nitric oxide, PGI_2, or a combination of both. The endothelium is the largest autocrine, paracrine, and endocrine organ that regulates vessel tone, leukocyte adhesion, platelet activation, thrombogenesis, inflammation, lipid metabolism, vessel

growth, and remodeling.[84,85] Because endothelial injury is an important risk factor for vascular diseases and to study physiological function of endothelial-smooth muscle interactions, numerous models of endothelial injury have been used. Previous investigations have induced endothelial injury mechanically (via balloon distension, microsurgical instrument, or air desiccation) or chemically (via application of hydrochloric acid or Triton X-100)[86,87] to scrape off or digest the endothelium. The most widely used and simplest technique to mechanically denude the endothelium from small vessels uses a human hair that does not damage smooth muscle cells and elastin fibers. This technique can be used to study the roles of receptors and pathways in their relation to endothelial or smooth muscle distribution.

Vessel studies conducted in the presence of specific receptor antagonists are widely applied using small vessels myographs. After the vessel has equilibrated and the normalization procedure has been completed, the inhibitor is added to the solution bathing the vessel. A pre-incubation time of approximately 10 minutes is given to allow for equilibration of the antagonist and receptor binding before concentration response curves are established for the appropriate agonist. This approach can reveal the specific receptor influence on smooth muscle contraction, and can identify antagonists that might be used in future clinical studies to test their potential as therapeutics.

Limitations of the technique

Small vessel myographs yield a combined measure of tension generated by the vessels, regardless of whether the drug exerts its effect intra- or extra-luminally. In contrast, studies of isolated perfused vessel preparations allow these factors to be differentiated, may be characterized by less endothelial cells damage, and thus might correspond more closely to the *in*

vivo situation. Several studies have demonstrated differential effects of intra- and extra-luminal administration of vasoactive substances such as noradrenaline, adrenaline, adenosine, and endothelin-1, with the difference depending on the particular drug.[88,89] For example, endothelin-1 and adenosine were more effective when administered extraluminally, whereas noradrenaline, adrenaline and K^+ were more effective when administered intraluminally. It is clear that the endothelium, at least in the porcine and human retinal arterioles, presents a diffusion barrier for those vasoactive substances with differential effects of intra- and extra-luminal administration. The mechanisms responsible for these differential effects are unknown[64] but possible differences in the types, affinity and numbers of receptors on the intra- and extra-luminal sides of the vascular smooth muscle and endothelial cells might account for this.[89,90] Controlled perfusion techniques have a known flow rate where the intraluminal pressure and vessel diameter can be measured and drug-induced changes in perfusion pressure provide information about vascular resistance. It also is possible that the 2-wire set up used in the small vessel myographs may reduce exposure of the endothelium to the drugs.

A general limitation that applies to many *in vitro* preparations is the lack of drug or compound interaction with circulating blood cells, plasma proteins, and factors released from the tissues adjacent to the vessel. *Ex vivo* preparations also exclude the role played *in vivo* by the innervation of the vessel,[91,92] and the small vessel myography technique does not account for the effects exerted by blood flow *in vivo*.

ENDOGENOUS VASOACTIVE SUBSTANCES IMPLICATED IN LAMINITIS

Prostanoids and Isoprostanes

The term *prostanoids* includes two classes of eicosanoids: prostaglandins (PGs) and thromboxanes (TXs).[93] Eicosanoids are oxygenated hydrophobic compounds that largely function as paracrine mediators. Eicosa (Greek for twenty) denotes the number of carbon atoms in arachidonic acid,[94] the precursor of all eicosanoids. Arachidonic acid is a fatty acid derivative that is hydrolyzed from membrane phospholipids by phospholipases A_2 and C.[95] PGs and TXs are hormone-like fatty acids and include several subtypes. They are ubiquitously distributed in most mammalian tissues and organs, and have an equally wide and diverse range of biological effects. For example, PGs and TXs initiate physiological and pathological activities, including smooth muscle contraction, inflammation, and blood clotting.[96] They are generated in the membranes of cells throughout the body, especially during times of illness, stress, or injury. PGs affect the nervous, reproductive, gastrointestinal, and renal systems, as well as the regulation of body fluids and temperature, and have profound effects on the body's defense mechanisms.[97]

Isoprostanes, discovered more than a decade ago, are a family of PG-like compounds generated nonenzymatically in vivo by reactive oxygen species and free radical catalyzed-peroxidation of arachidonic acid in cell-membrane phospholipids.[98,99] Several of the isoprostanes possess potent biological activity and thus may be mediators of oxidant injury. Isoprostanes are not only biological markers of oxidant stress in vitro and in vivo,[100] but they also evoke important biological responses on most types of cells.[101] The potent vascular properties of isoprostanes, together with their increased release in many vascular diseases, serve as reasons to investigate their pathophysiological roles in laminitis.

PROSTANOIDS AND ISOPROSTANES

Introduction

The history of prostaglandins began in 1930 with the observation by Kurzrok and Lieb, gynecologists at Columbia University, that human semen causes marked contractions and relaxation of human myometrium after artificial insemination.[102] These investigators proposed that the activities they were measuring were due to acetylcholine. However, Ratner[103] soon discovered a low molecular mass compound in human semen that induced uterine contractions, and later noted that this work "constituted the first recorded description of the uterine contracting properties of a prostaglandin". Ulf S. von Euler (Karolinska Institute, Stockholm, Sweden) first realized that this bioactivity was not due to any known mediator or catalyst.[104] In 1935, von Euler showed that the mysterious lipid soluble substance in semen affected numerous types of smooth muscles, and lowered the blood pressure of laboratory animals.[105,106] Because von Euler presumed that the substance was produced by the prostate, he coined the term "prostaglandin".[106] Others later determined that the prostate was not the source of this vasoactive substance. In 1970 von Euler received the Nobel prize for his seminal work on PGs.

Subsequently, Bergstrom demonstrated that the active principle in PGs was a new type of highly active, lipid-soluble, unsaturated fatty acid.[107] Later known as arachidonic acid, this chain of hydrocarbon molecules forms a part of the structure of cellular membranes. In the 1960s, work by Samuelsson revealed the processes by which PGs are metabolized and demonstrated the existence of two distinct pathways originating from arachidonic acid, one pathway leading to prostanoid formation, the other to the formation of leukotrienes.[108] Over the ensuing decade, Bergstrom and his colleagues isolated pure crystals of the two types of PG,[109-112] and in 1962

reported the chemical structure of three PGs.[113] It was this crucial breakthrough for which Bergstrom later was recognized by the Nobel committee.[114]

In 1967, Sergio H. Ferreira and John R. Vane reported that PGs were inactivated in a few seconds upon passage through the pulmonary circulation.[115]. Vane and Piper also reported that anti-inflammatory agents, such as aspirin, inhibited the production of PGs.[116] Hamberg *et al*[117] then reported that ingestion of either aspirin or indomethacin, an aspirin-like drug, almost completely inhibited PG synthesis at the endoperoxide stage. In 1971, Sir John Vane proposed that increased activity of an enzyme now known as cyclooxygenase (COX) leads to the generation of PGs that cause inflammation, swelling, pain, and fever.[118] Vane further proposed that the therapeutic effects of aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) are due to their ability to bind directly to COX to inhibit PG production. Since then, there has been considerable interest in understanding how COX signaling functions in physiological and pathophysiological conditions. Bergström, Samuelsson, and Vane shared the Nobel prize in Medicine in 1982 "for their discoveries concerning PGs and related biologically active substances".[119]

In 1975, Samuelsson and colleagues reported the results of additional studies regarding the mechanism of action of endoperoxides in platelet aggregation.[120] In the same year Hamberg *et al*[121] published a breakthrough paper entitled "Thromboxanes: a new group of biologically active compounds derived from prostaglandin endoperoxides". This paper reported the discovery of thromboxane A_2, the unstable intermediate formed during the conversion of PGH_2 into thromboxane B_2. Thromboxane A_2 proved to be the mysterious substance that so powerfully contracted rabbit aorta tissue and caused blood platelets to aggregate in the 1974 paper on the isolation and structure of two new endoperoxides.[122]

Evidence for *i*n vitro generation of autooxidation products derived from polyunsaturated fatty acids was published more than 30 years ago.[123,124] However, the first demonstration that these compounds were produced in humans was reported in 1990 by Morrow et al.[99] That landmark paper reported the discovery of PGF_2-like compounds, that they termed F_2-isoprostanes. These compounds were generated by free radical-induced peroxidation of arachidonic acid that occurred independent of COX (this enzyme had previously been considered to be an obligatory component of endogenous prostanoid synthesis). Since then, F_2-isoprostanes have been used extensively as clinical markers of lipid peroxidation in cardiovascular disorders, and both F_2- and E_2-isoprostanes have been proven to have vasoactive properties. Although it has long been known that inappropriate storage of unsaturated fatty acids leads to the formation of several PG-like compounds, the biological relevance of these non-enzymatic PG derivatives was not determined until 1990, when other $PGF_{2\alpha}$ isomers were characterized.[99] Subsequent evidence suggests that quantification of these products of lipid peroxidation, now termed isoprostanes (IsoPs), provides a reliable marker of oxidant injury both in vitro and in vivo.[101]

Subtypes, Structure and Biosynthesis

Arachidonic acid is the main precursor of the eicosanoids that regulate the function of various organs and systems. Arachidonic acid is released from cellular membrane phospholipids by phospholipase A2 or indirectly by phospholipases C and D, and then metabolized by prostaglandin H2 synthase activity, also called as cyclooxygenase (COX).[95] Prostaglandins, prostacyclin (PGI2), and thromboxane A2 (TxA2) are enzymatically generated from arachidonic acid as active lipid molecules. Aside from the prostanoid pathway, arachidonic acid can also serve as a substrate for the formation of hydroxyeicosatatraenoic acids (HETEs), leukotrienes,

and lipoxins by the action of lipoxygenase enzymes.[93] Of the 12 PGs, the most potent are PGD2, PGE2, and PGF2. Other PGs are synthesized by transformation of arachidonic acid (PGG2, PGH2), by degradation of the above-mentioned PGs, or are not synthesized physiologically but are generated chemically (PGK2, PGL2). Three primary COX isoenzymes have been identified; COX-1 (considered to be constitutively expressed and responsible for basal production of prostanoids); COX-2 (inducible and regulated by NF-κB in response to inflammatory stimuli such as endotoxin, interleukin-1β and tumor necrosis factor-α), and COX-3, which is detected mainly in the central nervous system.[125,126] COX enzymes reside in the endoplasmic reticulum and nuclear membranes with the substrate-binding pocket precisely orientated to take up the released arachidonic acid. Although the three isoenzymes differ in their gene sequences, transcription factors, primary protein structure, reactivity to substrates, and are inhibited by different drugs, the crystal structures of the three isoenzymes are remarkably similar, with a single amino acid difference providing for a larger ‘‘side-pocket’’ for substrate access in COX-2.[127] In contrast to the COX-1 gene, that is constitutively expressed by most cells, COX-2 expression is detected in the placenta and fetal tissue in late pregnancy. In the postnatal period, COX-2 expression decreases rapidly, and is observed in kidney, forebrain, spinal cord, as well as some other organs, but to a minor degree. It increases in inflammatory, degenerative, and neoplastic processes.

TxA2 and PGs (PGD2, PGF2, and PGH2) have the subscript 2 (the “series-2” PGs) due to the presence of two double bonds in the side chains attached to the cyclopentane ring. These products contain a cyclic ring and have very similar chemical structures. PGH2 is an unstable intermediate formed from PGG2 by the action of PGH2 hydroperoxidase in the arachidonate cascade.[96] In mammalian systems, PGH2 is efficiently converted into more stable structurally

related PGs, including PGE2, PGD2, PGF2a, PGI2 (also known as prostacyclin), and TxA2, by the action of specific PG synthases.[128]

The coupling of PGH2 synthesis to metabolism by downstream enzymes is orchestrated in a cell-specific fashion. For example, thromboxane synthase is present mainly in platelets and macrophages, and TxA2 stimulates platelet aggregation and vessel constriction. Prostacyclin synthase is expressed by endothelial cells, and its product, PGI2, has cardioprotective properties that cause platelet de-aggregation and vessel dilation. PGF synthase is present in the uterus, and two types of PGD synthase are in brain and mast cells.[93] Two PGE synthases have been described: microsomal PGE synthase (mPGES), a member of the MAPEG (Membrane-Associated Proteins in Eicosanoid and Glutathione metabolism) family,[129] and cytosolic PGE synthase (cPGES). These two PGE synthases largely account for PGE2 synthesis in mammals.

A parallel a nonenzymatic pathway for prostanoid formation has been described. Specifically, isoprostanes are generated by peroxidation of membrane phospholipids by free radicals and reactive oxygen species.[130,131] Hydrogen peroxide (H2O2), superoxide radical (O2 −), and oxygen-centered radicals react with the unsaturated bonds in arachidonic acid to form different bicycloendoperoxides intermediates.[132] Reduction of these intermediates leads to four different classes of isoprostane regioisomers, all of which are composed of a cyclopentane ring with two alkyl chains positioned *cis* to one another; prostanoids have the two side chains in a *trans* position.[131] Isoprostanes that contain the F-type prostane ring are isomers of PGF2α and are termed F2-isoprostanes. They constitute the most studied class of isoprostanes due in part to their stability.[133] Unlike PGs, isoprostanes do not require microsomal cyclooxygenases for their biosynthesis. Another distinction between free radical-generated isoprostanes and COX-derived PGs is that cis side chains to the cyclopentane ring are predominant in the former whereas the

trans orientation exists in the latter.[134] Isoprostanes are formed in situ as phospholipids are affected by free radicals. Once released from cell membranes by phospholipases, isoprostanes circulate in the plasma in free forms and, therefore, may activate membrane receptors.

Receptors

In the signaling cascade downstream of cyclooxygenase in mammals, there are at least 9 known cell surface PG receptors, as well as several additional splice variants [135]. Prostanoid receptor nomenclature is based on the ligand that binds to the receptor with the greatest affinity [136]. Thus, D prostanoid (DP) receptor binds PGD2, E prostanoid (EP) receptor binds PGE2, F prostanoid (FP) receptors bind PGF2α, I prostanoid (IP) receptors bind PGI2 and thromboxane prostanoid (TP) receptors bind TxA2. Four of the receptor subtypes bind PGE2 (EP1–EP4); two bind PGD2 (DP1 and DP2). The FP, IP, and TP receptors bind $PGF2_{\alpha}$, PGI2, and TxA2, respectively. Except for DP2, a member of the chemoattractant receptor subgroup, all PG receptors are categorized as three clusters of a distinct subfamily in the rhodopsin-like G protein-coupled receptor superfamily of seven hydrophobic transmembrane-spanning proteins. Both extracellular and transmembrane regions of these receptors are involved in ligand binding.[137]

The biological activities of PGE2 are mediated by 4 receptors designated as EP1 to EP4 that are coupled to several intracellular signaling pathways; TP receptors can be subclassified into two subtypes. All of these receptors can be categorized based on the implied activated G protein.[138] The ‘‘stimulatory’’ receptors including IP, DP1, EP2, and EP4 that signal through Gs-mediated increases in intracellular cyclic adenosine monophosphate (cAMP) form one group. The EP1, FP, and TP receptors form a second group that signals through Gq-mediated increases in intracellular calcium.[139-141] Finally, the EP3 receptor is regarded as an ‘‘inhibitory’’ receptor

as it couples to Gi proteins to decrease cAMP formation.[142] Mechanisms other than ligand–receptor binding on the plasma membrane, have been implicated in PG signaling. For example, there is evidence that EP receptors can be internalized into the nucleus,[143] and EP3 and EP4 receptors regulate gene transcription by modulating the release of calcium from nuclear calcium pools or by activating calcium channels. It should be noted that alternative spliced messenger RNA variants have been described for TP, FP, EP1 and EP3. Because of its physiological significance, PG signaling is an especially important area of research.[132] Two isoforms of the TP receptor, TPα and TPβ, are generated by alternative splicing occurring in the carboxy-terminal region after the seventh transmembrane domain.[144] Vasoconstriction induced by PGE_2 and $PGF_{2\alpha}$ in vascular systems is also mediated via TP-receptors,[145,146] leading Sametz *et al*[147] to propose that the TP-receptor is the only prostanoid receptor in vascular smooth muscles, and is responsible for vascular constriction induced by eicosanoids.

The biological actions of isoprostanes were first described in renal vascular smooth muscle, where vasoconstriction was observed. This effect purportedly involved prostanoid TP (TxA2) receptors because it was antagonized by a selective TP receptor antagonist.[148] Other findings suggest that TP receptors are involved in the excitatory effect of isoprostanes.[149-153] The existence of a specific isoprostane receptor with close homology to the prostanoid TP receptor has been suggested but not proven.[154] Habib and Badr argue that the near-absence of agonist activity of isoprostanes in platelets indicates clearly that isoprostanes do not act through the TxA2 receptor.[155] While isoprostanes may activate thromboxane receptors on vascular smooth muscle, they clearly act through sites that are specific for isoprostanes.[155] Ligand-receptor interactions, signal transduction pathways, cellular context and receptor expression profiles are

responsible for the key roles played by prostanoid receptors in physiologic and pathologic settings.[132]

VASCULAR EFFECTS AND PROSTANOID RECEPTOR DISTRIBUTION

Introduction

Prostanoids contract or relax smooth muscle by activating different prostanoid receptors.[136] [135,156] Results of isolated tissues have shown that prostanoid activation of TP-, FP-, EP_1- or EP_3-receptors produces smooth muscle contraction by increasing Ca^{2+} or reducing intracellular concentrations of cyclic AMP.[157]

PGF2α

F series PGs are widely distributed in various organs of mammals[158,159] and induce a variety of biological activities, including contraction of pulmonary arteries.[160-163] $PGF_{2\alpha}$ plays a major role in reproduction,[164,165] renal function,[166] and regulation of intraocular pressure,[167] and has been implicated in proliferative states such as endometrial carcinoma [168] and cardiac hypertrophy.[169] Consistent with these functions, FP receptor expression occurs in the corpus luteum, kidney, ocular tissues, and ventricular myocytes. No expression of FP receptors has been demonstrated in the spleen or thymus, nor has FP receptor expression by immune cells been reported.[170] According to Tilley and colleagues, there is very little evidence to support a role for $PGF_{2\alpha}$-FP receptor signaling in inflammatory and immunological processes.[132,170,171] In contrast, other investigators reported after the infusion of Salmonella typhimurium endotoxin into pregnant mares a biphasic release pattern of $PGF_{2\alpha}$ and fetal death.[172] Activation of FP-receptors by prostanoids induces contraction of cat iris sphincter,[173] bovine ciliary muscle,[174] rabbit

uterus,[175] and ewe myometrium.[176] $PGF_{2\alpha}$ is a potent constrictor of the uterus and is a luteolytic factor in several mammalian species.[177] Bider *et al*[178] suggested that intra-umbilical vein injection of $PGF_2\alpha$ might be a beneficial, non-surgical method for treating retained placenta.[178] $PGF_{2\alpha}$ analogues are used clinically to treat ocular hypertension and glaucoma.[179]

PGE2

Prostaglandin E2 (PGE2), generated by the PGE synthase that catalyses the isomerization of PGH2 to PGE2, plays a key role in numerous physiological and physiopathological states, including inflammation. PGE2 induces constriction or relaxation of both vascular and nonvascular smooth muscle, depending on tissue and receptor distribution.[180-182] This prostanoid also modulates immune responses by regulating the function of cells such as macrophages, T and B lymphocytes leading to pro- and anti-inflammatory effects.[132] PGE2 signaling also acts on vascular smooth muscle cells to regulate blood pressure.[93,127] In studies involving corpora lutea of rats, PGE causes dose-dependent increases in expression of vascular endothelial growth factor (VEGF) mRNA.[183] PGE2 is thought to promote isthmic transport of the ovum and embryo by relaxing the associated circular smooth muscle fibers, and inhibits isthmic smooth muscle contractility near the time of isthmic transport in rabbits,[184] pigs,[185] and women.[186] Allen *et al*[187] recently reported that application of PGE2 to the external surface of the oviduct in mares stimulates relaxation and contractility of the isthmic musculature.

One of the first reported effects of PGE_2 was relaxation of both arterial and venous vascular beds.[188-190] This effect is mediated, in part, by a direct relaxant effect of PGE_2 on smooth muscle that is purported to be coupled to increased generation of cAMP.[189,190] Selected structural analogs of PGE_2 that reproduce the dilator effects of PGE_2, are completely inactive in tissues in

which PGE_2 is a constrictor.[191] Conversely, analogs that initiate the constrictor effects of PGE_2, fail to affect tissues in which PGE_2 is a dilator.[192] These differential effects of PGE_2 analogs provided important evidence for the existence of multiple PGE_2 (EP) receptors.[136] The literature is confusing regarding the nomenclature pertaining to EP_2 receptors. Prior to 1995, when the human EP_2 receptor was cloned, the EP_4 receptor was misclassified as the EP_2 receptor.[193] EP_2 receptors are selectively activated by butaprost,[194] and may be distinguished from the EP_4 receptor, the other major relaxant EP receptor, by its relative insensitivity to the EP_4 agonist PGE_1-OH and to the weak EP_4 antagonist AH-23848.[188] Interestingly, a single point mutation in the seventh transmembrane domain of the EP_2 receptor allows it to respond to prostacyclin analogs.[195] The precise tissue distribution of EP_2 receptors has been only partially characterized via measurement of mRNA distribution, although EP_2 mRNA is expressed at much lower levels than EP_4 mRNA.[196,197] This technique has been used to determine that EP_2 receptors are most abundant in the uterus, lung, and spleen.[194,197,198] Functional studies suggest that the EP_2 receptor plays a role in uterine implantation[199] and a relaxant role in trachea and the vasculature.[188,192]

Similar to the EP_2 receptor, the EP_4 receptor signals by increasing cAMP concentrations intracellularly.[200] EP_4 receptors may be pharmacologically distinguished from EP_1 and EP_3 receptors by their insensitivity to sulprostone, and from EP_2 receptors by their insensitivity to butaprost and relatively selective activation by PGE_1-OH.[194,201] Furthermore, [^{3}H]PGE_2 binds to the EP_4 receptor with at least 10-fold higher affinity than to the EP_2 receptor. Structurally, the EP_4 receptor has a much longer COOH-terminal sequence than the EP_2 receptor[202] and undergoes short-term agonist-induced desensitization, which is absent in the EP_2 receptor.[203] EP_4 receptor mRNA is relatively highly expressed compared with mRNA for the EP_2 receptor, and is widely distributed in thymus,[204] ileum, lung, spleen,[205] adrenal gland,[206] and kidney.[200] Vasodilator

effects of EP_4 receptor activation in venous and arterial beds have been described.[136,188] A particular role for the EP_4 receptor in regulating closure of the pulmonary ductus arteriosus has also been suggested based on studies in mice.[207,208] Other roles for the EP_4 receptor in controlling blood pressure have been suggested, including their ability to stimulate aldosterone release from zona glomerulosa cells.[209] In the kidney, EP_4 receptor mRNA is expressed primarily in the glomerulus, where it might contribute to regulation of the renal microcirculation as well as to renin release.[206]

The involvement of EP_1- or EP_3-receptors in contraction of numerous mammalian smooth muscle preparations is well recognized.[210] In mammals, activation of EP_3-receptor induces contraction of smooth muscles in ileum,[211] colon,[176] myometrium and corpus luteum.[212] PGE_2-induced contractions of preparations derived from either the guinea-pig trachea[213] or gastrointestinal tract[214] via the EP_1-receptor has been described using the selective EP_1-receptor antagonist (SC19220). PGE2 dilates and constricts systemic vascular beds,[210] and it is an important and ubiquitous vasoactive eicosanoid. PGE2 is a potent vasodilator in most peripheral [136,189,190] and cerebral vascular beds in humans and laboratory animals,[215] where it directly relaxes vascular smooth muscle cells.[189] Prostaglandin E2 relaxes circular smooth muscle of the rabbit jugular vein at very low concentrations, most likely via EP2-receptors.[189]

TxA_2

TxA_2, a potent activator of platelets, vasoconstrictor and smooth-muscle-cell mitogen, is produced mainly in blood platelets but can also be synthesized by monocytes, neutrophils, lung parenchyma[121,216-218] and vascular smooth muscle cells.[219] Due to its vascular effects, this prostanoid is the physiological counterpart of prostacyclin. Prostanoid TP receptors, localized to

plasma membrane and cytosolic compartments, are mainly distributed in highly vascularized tissues, such as the lung, heart and kidney. These receptors are involved in a multitude of physiological and pathological processes. TxA_2 is a potent stimulator of platelet shape change and aggregation as well as a potent stimulator of smooth muscle constriction and proliferation and bronchial hyperresponsiveness.[220] Due to its biological properties, TxA_2 has been linked to cardiovascular diseases, such as heart failure and myocardial ischemia, in which its synthesis is increased.[221,222] TxA_2 also plays a crucial role in the pathogenesis of bronchial asthma, primarily due to its potent constrictive effects on bronchial smooth muscles and stimulation of airway smooth muscle cells proliferation.[223,224] TxA_2 is also implicated in COPD.[132]

The important roles played by TxA_2 in cardiovascular diseases, renal diseases, pulmonary diseases make TP receptor antagonists and TxA_2 synthase inhibitors potential therapeutic agents for such diseases.[132] Walch reported in 2001 that contractions induced by prostanoids involved TP- and EP(1)-receptors in human pulmonary venous smooth muscle.[182] In most human arteries, the TxA_2 mimetic, U46619, induced contraction via TP receptor stimulation.[146] Although there are few reported studies of TP receptor stimulation in veins, venoconstriction via TP receptor stimulation has been documented in the hand,[225] placenta,[226] and legs[227] of humans and dogs.

Isoprostanes

Isoprostanes are formed by the action of free radicals on phospholipids.[228] Once released from cell membranes by phospholipases, the isoprostanes may induce vasoconstriction, platelet aggregation and cellular proliferation. F2-isoprostanes are released at sites of free radical-induced tissue injury and then enter the circulation. Local concentrations of isprostanes may be sufficiently high to induce regional vasoconstriction, but their effects depend on the vascular

beds, species of mammal, and blood vessel type.[153] In addition to their vasomotor properties, F2-isoprostanes may promote DNA synthesis and endothelial cell proliferation,[229,230] although the pathophysiological role of such processes remains unclear. Whether E2-isoprostanes induce systemic or local vasoconstrictor effects in a clinical setting cannot be determined. However, E2-isoprostanes also may cause pronounced vasomotor effects in vivo, via activation of TP receptors. Consequently, E2- and F2-isoprostanes may play similar roles in the pathogenesis of vascular diseases.[153] In addition to the F2 and E2-isoprostanes, isothromboxanes have recently been described.[130] Although the mechanism involved in the conversion of isoprostane endoperoxides to isothromboxanes remains undetermined, A2-isothromboxanes are formed and become rapidly metabolized to stable B2-isothromboxanes. Because thromboxane A_2 is a very potent vasoconstrictor,[231] isothromboxanes may possess similar potent biological activities.

Isoprostanes exert their effects on platelet function and vascular tone in vivo by acting as incidental ligands at membrane TP receptors rather than via a distinct isoprostane receptor.[232] Activation of TP receptors by isoprostanes may be of importance in syndromes in which COX activation and oxidant stress coincide, such as in atherosclerosis and reperfusion after tissue ischemia. Due to their profound vasoconstrictor actions and ability to elicit robust intracellular responses in vascular smooth muscle and endothelium,[155] the isoprostanes are emerging as important mediators in vascular pathology.

PROSTANOID RECEPTOR AGONISTS

U-46619

U46619is a stable analog of the endoperoxide PGH_2, and a TP receptor agonist.[233] It has properties similar to TxA_2, causing platelet shape change and aggregation, and contraction of

vascular smooth muscle.[234,235] Mean EC_{50} values for shape change in human, rat, and rabbit platelets are 4.8, 6.0, and 7.3 nM respectively, and for aggregation are 82, 145, and 65 nM, respectively.[236]

11-d-PGE2

11-deoxy PGE_2 is a stable, synthetic analog of PGE_2. In contrast to PGE_2 which has bronchodilatory effects, 11-deoxy PGE_2 is a powerful bronchoconstrictor and contracts human respiratory tract smooth muscle with potencies ranging from 5 to 30 times greater than $PGF_{2\alpha}$.[237]

17-pt-PGE2

17-phenyl trinor 8-iso PGE_2 (17-phenyl trinor 8-iso PGE_2) is the C-8 epimer of 17-phenyl trinor PGE_2, a synthetic analog of PGE_2. 17-phenyl trinor PGE_2 is an EP1 and EP3 receptor agonist that causes contraction of the guinea pig ileum at a concentration of 11 μM.[189] There are no published studies of the pharmacological properties of 17-phenyl trinor 8-iso PGE_2.

PROSTANOID RECEPTOR ANTAGONISTS

AH 6809

6-Isopropoxy-9-oxoxanthene-2-carboxylic acid (AH 6809) has been used successfully to characterize EP receptor subtypes. AH 6809 is regarded as an antagonist at human EP_1, EP_2, and DP_1 receptors.[238-240] The ability of AH 6809 to behave as an antagonist at the EP_1 receptor is widely accepted and is an important facet of EP receptor pharmacology in laboratory animal preparations.[238] Although AH 6809 exhibits weak antagonism in these animals at DP and TP

receptors,[192] within the EP receptor family AH 6809 appears to possess a high degree of selectivity for the EP_1 receptor.

SC-19220

The dibenzoxazepine, SC-19220, is a selective antagonist of PGE_2 at the EP1 receptor.[241] At concentrations between 0.3-300 μM, SC-19220 competitively blocks PGE_2-induced smooth muscle contractions of guinea pig ileum and stomach.[242,243] SC-19220 also antagonizes PGE_2-induced contraction of the guinea pig trachea via the EP1 receptor.[213] In contrast, SC-19220 binds weakly and with no selectivity to the mouse EP1 receptor, suggesting the presence of important species differences in EP1 receptors.[244]

SQ 29,584

SQ 29,548 is a selective TP receptor antagonist that binds to recombinant human TP receptors,[233] inhibits U46619-induced aggregation of human platelets,[245] and antagonizes U-46619-induced contraction of rat and guinea pig tracheal, arterial, and venous smooth muscles.[246] SQ 29,548 also inhibits 8-iso $PGF_{2\alpha}$-induced contractions of rat vascular smooth muscle.[247]

PROSTANOIDS / ISOPROSTANES AND EQUINE LAMINITIS

Baxter *et al*[66] provided the first evidence that prostanoids may be mediators of equine laminitis. In *in vitro* studies examining the sensitivity of digital arteries and veins to vasoconstrictor agents, Baxter *et al*[66] found that the digital vasculature of horses with early-stage laminitis were less responsive to $PGF_{2\alpha}$ and U46619 than vessels from healthy horses. Similarly, maximal contractions of digital arteries and veins from horses with early laminitis were less than

those for healthy horses. Baxter *et al*[66] suggested that the different responses in the horses with acute laminitis may be due to receptor down regulation, desensitization or endothelial damage.

Owens reported that plasma concentrations of PGE_2 and leukotriene B_4 (LTB_4) in blood collected from digital veins of healthy and chronic laminitic horses were not significantly different from one another, suggesting that PGE_2 and LTB_4 do not mediate the signs of pain and pathologic features of chronic laminitis.[248] Rodgerson *et al*[249] reported that COX-2 may have an important role in endotoxin-related vascular disease processes in horses, including laminitis. Rodgerson *et al*[249] detected an increase in COX-2 expression in isolated vascular smooth muscle after incubation with a low dose of endotoxin. Because COX-2 mRNA levels usually correlate well with protein concentrations, their finding suggest that a similar increase in functional COX-2 protein concentrations would occur. Rodgerson *et al*[249] suggested that the ischemic events purported to occur in the equine digit during acute laminitis may be an important component in the up-regulation of the inducible isoform of COX. Alterations observed/proposed to occur within the digital circulation during laminitis (i.e., thrombosis, dilation of arteriovenous anastomoses, and venular constriction) are consistent with the vascular effects of certain prostanoids and thromboxanes. Due to findings that endotoxin caused greater increases in COX-2 expression in human venous smooth muscle cells than in arterial smooth muscle cells, Rodgerson *et al*[249] suggested that such an differential effect may account for the development of post-capillary constriction in the equine digit during the onset of acute laminitis.

Peroni *et al*[72] recently reported that $PGF_{2\alpha}$ elicits a more profound contractile response in laminar veins than in laminar arteries. Peroni *et al*[72] suggested that this effect coupled to a generalized increase in sensitivity of laminar veins to $PGF_{2\alpha}$ might help to explain why diverse conditions/humoral factors initiate equine laminitis by eliciting venoconstriction. Peroni *et al*[72]

argued that systemic conditions characterized by endotoxemia and increased circulating levels of inflammatory mediators, such as PGF2, may lead to venoconstriction in the laminar vascular bed of the horse, thereby predisposing the development of laminitis via an increase in post-capillary resistance. There are no published reports on the role of isoprostanes on the effects of isoprotanes on microvascular function in horses or in the development of laminitis.

GENES INVOLVED IN CONTROLLING VASCULAR FUNCTION

Introduction

To investigate cellular mechanisms and identify genes associated with vascular physiology and pathophysiology, distinct stimuli must be applied to endothelial and smooth muscle cells.[250] For example, a mechanical stimulus, such as extracellular injury, triggers an inflammatory response by inducing expression of genes and synthesis of a variety of pro and anti-inflammatory proteins.[251-253] Microarray studies have verified the results of earlier findings using Northern and polymerase chain reaction analyses, and have detected the expression of previously unidentified genes, leading to new hypotheses regarding how cells and vascular tissues respond to biochemical and mechanical stimuli. These techniques are now used in many different applications including drug discovery, studies on the effects of mechanical forces on vascular cell phenotype, and elucidation of patterns of gene expression underlying cardiovascular function. This new information has enabled the formulation of new hypotheses about the mechanisms responsible for responses to different stimuli. Many investigators use microarray studies to provide insight into changes in gene expression in vascular tissue. This technique is particularly suitable for sifting through the spectrum of candidate genes in an attempt to focus on specific culprits and identify novel pathways associated with vascular function and dysfunction.

The mechanical forces on vascular cells, which accompany blood flow and pressure changes, may modulate vascular disease processes via changes in gene expression.[254] Endothelial cells, at the interface with the flowing blood, are constantly subjected to shear stress due to the viscous drag forces produced as blood flow along the vessel wall. Endothelial cells and the underlying smooth muscle cells experience changes in circumferential strain with pulsatile blood flow. Extensive research has described similar changes in cultured endothelial cells and smooth muscle cells subjected to shear stress and cyclic strain in vitro.[255,256]

SECTION III: GENE EXPRESSION AND MICROARRAY TECHNOLOGY

METHODS FOR MONITORING GENE EXPRESSION

Amplification versus hybridization (qPCR *versus* Northern blots)

Northern blot analysis remains a standard method for detecting and quantifying mRNA despite the advent of powerful techniques, such as RT-PCR and microarray analysis. Northern analysis was developed by Alwine and Stark at Stanford University to analyze RNA by electrophoresis and detect transcripts with a hybridization probe.[257] Northern analysis provides a direct relative comparison of message abundance between samples on a single membrane film. It is a favored method to determine transcript sizes and to detect alternatively spliced transcripts within RNA samples. This straightforward technique provides the opportunity to evaluate progress at various points within the process, as assessment of the RNA sample integrity and how efficiently it has transferred to the membrane. RNA samples are first separated by size via electrophoresis in an agarose gel under denaturing conditions. The RNA is then transferred to a membrane and hybridized with a labeled single probe in solution under low stringency. After post washes of increasing stringency used to remove non-specific signal, a signal for the gene

under examination is detected, which is proportional to the concentration of the mRNA in the sample. Thus, this technique allows for the monitoring of expression of a single gene under a limited number of conditions (*e.g.* different stimuli used for different time intervals).

Another molecular biology technique for the enzymatic replication of an nearly unlimited number of copies of any piece of DNA, called the polymerase chain reaction (PCR) was developed more than two decades ago by Mullis,[258] who in 1993 the Nobel Prize in Chemistry with Michael Smith for his invention. The PCR method exponentially amplifies within hours millions of DNA molecule copies depending on the number of initial templates in the sample. Therefore, this method can be used to quantify gene expression after the mRNA template has been converted to cDNA by reverse transcription. The key feature of PCR is a heat stable polymerase enzyme that amplifies a specific region of a DNA strand bracketed by two primers. Primers are short, artificial pieces of complementary DNA that bind to the DNA strands and determine the beginning and end of the region to be amplified; using these primers polymerase copies the region between primer pairs.

The amplification process is achieved in a thermal cycler, an instrument that heats and cools within the precise temperature required for each step of the reaction. Several cycles DNA strand denaturation followed by primer annealing and primer extension allow DNA replication to occur. As a result, the DNA between the primers is doubled within each cycle, resulting in an exponential growth of DNA copies.

In order to determine the original number of transcripts in a sample a modification of polymerase chain reaction called the quantitative polymerase chain reaction (Q-PCR) can be used as an indirect method and performed in real-time. This technique enables one to determine whether a genetic sequence is present and to determine the number of transcripts in a sample.

Most often a double stranded DNA dye, such as SYBR Green, is used while the number of amplification cycles needed before the dye can be detected are counted.

When using a combination of reverse transcription followed by PCR the whole process is called RT-PCR (reverse transcription - polymerase chain reaction). Today, quantitative RT-PCR[259] is routinely applied to measure mRNA abundance. The assay is cheap, versatile, and requires only small amounts of starting mRNA.[260] Similar to Northern blots RT-PCR allows quantification of the change in expression of a single gene in a sample.

Most commonly, gene expression analysis using Northern Blots or RT-PCR is reported relative to the concentration of m-RNA for a housekeeping gene in the sample. Housekeeping genes (*e.g.* GAPGH or β-actin) are defined as gene transcripts expressed constitutively and that show little change in expression as a consequence of cellular activation.

Limitations and advantages

Northern hybridization is exceptionally adaptable in that labeled DNA, *in vitro* transcribed RNA and oligonucleotides can all be used as hybridization probes. Also, sequences with only partial homology, as cDNA from a different species or genomic DNA fragments containing an intron, may be used as probes. Despite these advantages, there are limitations associated with Northern analysis. First, if RNA samples are slightly degraded, the quality of the data and the ability to quantify expression are severely compromised. Thus, RNase-free reagents and techniques are essential. Second, a standard Northern procedure is, in general, less sensitive than RT-PCR, although improvements in sensitivity can be achieved by using high specific activity antisense RNA probes, optimized hybridization buffers and positively charged nylon membranes. Sensitivity can be further improved with oligo dT selection for enrichment of

mRNA. Physical constraints of gel electrophoresis and membrane transfer limit the amount of RNA that can be analyzed without loss of resolution and saturation of the transfer membrane. A third limitation of Northern blotting is the difficulty associated with multiple probe analysis. To detect more than one message, it is usually necessary to strip the initial probe before hybridizing with a second probe. This process can be time consuming and problematic, since harsh treatment is required to strip conventional probes from blots.

The PCR revolutionized molecular biology to a similar extent as the discovery of plasmids. However, there are several advantages and limitations to real-time PCR[261]. Advantages includes: wide dynamic range over 7 to 8 log10 nucleic acid copies/mL, high sensitivity to <5-10 copies/reaction, high precision with < 2% CV for accurate identification of transcripts, no post-PCR analysis, and minimized risk for amplicon contamination because amplification and detection occur in a sealed reaction container. High throughput and simultaneous detection of a limited number of different targets through multiplexing is also possible. Limitations of PCR include the use of external standards for calibration, the increased risk of false-negative values without use of an internal standard, carry-over contamination of amplicons, and failure to amplify the expected amplicon due to PCR inhibitors that may be present in samples or in transport medium. Because specific knowledge of target oligonucleotide sequence is required, false-negative results may arise with a high variability in the expected target sequence. In order to avoid false-positive PCR results, reactions should be set up in duplicate along with appropriate positive and negative controls. A limitation common to both methods is the small amount of different probes that can be analyzed at the same time.

MICROARRAY TECHNIQUE

Introduction

DNA microarrays are miniature devices containing complementary deoxyribonucleic acid (cDNA) fragments that are either synthesized directly or by the use of a robotics system spotted in ordered arrays onto glass slides or other matrices. Thousands of genes can be represented in a single microarray slide or "gene chip", which is designed to concurrently measure the expression levels of all these genes in a particular tissue or cell type. The fundamental concept of microarray technology is to hybridize preprocessed mRNA called target sequences to the cDNA sequences called probes bound to a solid surface and to quantify the amount of specifically hybridized target, typically by fluorescence detection. Various technologies are available for this purpose, and can be categorized based on the chip design, chemistry, manufacturing or signal detection. In general, microarrays are divided into cDNA arrays and oligonucleotide arrays based on the probe material used, whether the probes are synthesized *in situ* or are spotted (contact or non-contact) onto the slides, and whether they are one-color or multi-color arrays based on the staining and detection techniques used. Many reviews and books extensively describe microarray technologies, applications and analysis methods.[262-265]

History of Microarrays

More than a decade ago, microarrays or "gene chips" of DNA probes were introduced and there has been a rapid evolution of the technology since then. Schena *et al.* pioneered the first solid-surface DNA microarray in 1995 with 45 cDNA probes spotted and immobilized on a glass slide to assess gene expression analysis[266] as part of a Stanford University research team

using mRNA samples of *Arabidopsis thaliana* (mustard plant). Due to rapid technological progress, one year later 1000 probes were arrayed.[267-269] An alternative technology based on *in situ* synthesis of DNA on solid support directed by photolithography, was developed by the pioneering company in the field, Affymetrix.[270] By 1996, this company had developed microarrays with 135,000 probes.[271-273] These high-density oligonucleotide microarrays were manufactured using light-directed synthesis to bind modified nucleotides to the chip surface. Currently the GeneChip® (Affymetrix) expression microarrays are capable of containing millions of probes on a 1.28 cm^2 surface of quartz, and mass produced whole genome chips are available for several organisms, including human, mouse, rat and dog among others.

By 1995, considerable improvements had been made in the first simple DNA arrays called "dot blots" developed in the 1970's. In the dot blot technique, DNA is immobilized on membranes and usually probed using radioactively labeled DNA.[262,274,275] The major improvements to the original dot blot technique included miniaturization of the spots, which allowed better sensitivity and more genes to be analyzed from smaller amounts of samples;[276] the use of fluorescence[277] for detection; and the introduction of rigid solid glass supports[278,279] facilitating a faster and more precise analysis of signals. This novel microarray technique allowed for the analysis of a vast number of genes simultaneously including entire transcriptomes (i.e., all expressed genes in a given genome) in a single experiment due largely to miniaturization. As opposed to Nothern blots and RT-PCR, microarrays allow for monitoring of expression of 1000's of genes in samples prepared from cells or tissue treated under a single condition. The hybridizations performed on a single microarray are equivalent to performing tens of thousands of Northern blots in 1 day.

Early articles clearly identified the power of microarray analysis, and many laboratories decided to add this technology to their repertoire. In a footnote in one article,[280] the authors suggested it would take a well-organized laboratory only 6 months to set up yeast array experiments. This would include everything from building an array printer, to generating oligonucleotide probes for all 6,400 yeast genes, to printing and utilizing the arrays. DeRisi and Brown developed and posted a "how to" manual for building an array printer from scratch, known as the Mguide,[281] and laboratories throughout the world began to create these printers. Today, commercial array printers and scanners are widely available, as are commercial prespotted slides. As a result, the use of microarrays in basic and applied research is growing at an extraordinary rate. Currently, on a single glass based microarray with a surface of 1 to 8 cm^2, the expression of 40,000 or more different mRNA molecules or 100,000 different single nucleotide polymorphisms (SNP) can be analyzed. These large microarrays, which are used primarily in research of genomes and transcriptomes, can be produced by spotting or in situ synthesis, while diagnostic microarrays have quality controlled cDNA probes spotted onto the surface of the slides.

Other types of microarray-based assays have been proven to be powerful tools as antibody microarrays now are used as a substitute for ELISA,[282-284] small molecule measurements,[285] high throughput transfections[286] and DNA-protein interaction analysis for genome wide promoter studies[287] and to measure protein-protein interactions.[288] Microarrays have also been proven to be useful as a diagnostic tool where low numbers of probes are used.

Types of Microarrays and Generation of Microarrays

Microarrays can be classified into two types, the open standardized platform, based on the microscope glass slide format, and all others. The open platform can be customized to specific requirements and budgets and is generated by many suppliers. There are several different types of hardware available that accept these slides for arraying, scanners, washing stations and hybridization stations. Other types of microarrays (*e.g.* protein microarrays) printed on microscope slides can be analyzed using the same instruments as DNA microarrays. In contrast, non-standardized systems, such as those produced by Affymetrix, consist of a "kit" containing a washing-station, hybridization stations and scanners that fit specifically the Affymetrix DNA microarray chips.

Based on different probe materials used, microarrays can be further subdivided. The other two major microarray systems used are the cDNA and oligonucleotide arrays. cDNA array probes are typically PCR products, generated from clone collections or cDNA libraries. The probes are printed from a microtiter plate onto nylon membranes or glass microscope slides coated with poly-L-lysine or aminosilane as spots at defined locations, commonly 100-300 μm in size and an equal distance from each other.[266,289-291] In contrast, the shorter oligonucleotide probes, representing only the most unique part of a given gene transcript, are prone to less specific hybridization, reduced sensitivity and make detection of closely related genes or splice variants possible. After printing, the cDNA or oligonucleotide probes are covalently cross-linked to the glass slide.

The *in situ* synthesis method used by Affymetrix to generate oligonucleotide microarrays produces high-density microarrays, containing many more probes than traditional cDNA arrays. These arrays can be further classified by the use of contact or noncontact (ink-jet, developed by

Boehringer Mannheim) printing methods of presynthesized oligonucleotide probes. Among the multiple microarray systems available, inconsistencies and varying degrees of correlation in the measured gene expression levels have been reported across the different platforms.[292-297] However, the gene expression data correlation between commercial and custom-made platforms has been reported to be insignificant.[292-295] Affymetrix arrays compare control and experimental gene expression profiles using two separate arrays, which are scanned separately. The signal from the reference or control array is then compared with that of an experimental sample using appropriate software.

To manufacture *in situ* synthesized high-density oligonucleotide microarrays, a photolithographic procedure is used by Affymetrix for the GeneChip® microarrays. Using this method, a series of masks are used to synthesize 25-mer oligonucleotide probes (probe lengths are limited to 25 bases) onto a silicon wafer in such a manner that a large number of different sequences can be produced in parallel in a small number of steps.[270,272,273,298] The probes are synthesized in repeated cycles using modified nucleotides and ultraviolet light. Photolithographic masks are used to selectively de-protect probes at given locations. Due to the achievement of extremely high spot densities, this synthesis is a very powerful method.

Using the inkjet printing method, much longer probe sequences (60-100 bp) can be synthesized *in situ* and printed on the microarrays by the use of digital sequence files. The probes are synthesized using oxidative chemistry and ink-jet printing, where the nucleotides are fired from chambers containing a given nucleotide. While these longer probe sequences guarantee better accuracy and precision for target hybridization, the spot densities, however, are much lower than in light-directed synthesis.

The alternative for *in situ* synthesis is the use of presynthesized oligonucleotides or cDNA that can be printed onto coated glass sides either with contact pins or by non-contact method using ink-jet technology. These spotted microarrays can be custom made but are nowadays in increasing amounts commercially manufactured by numerous vendors. Microarray fabrication using contact printing is based on computer controlled robotic arms linked with a head of high definition pins or capillary devices.[266] The pins pick up small drops of the probe solution from multiwell plates and carry them to the microarray surface. Upon contact with the substrate the small amounts of probe solution are released and deposited to the surface. In non-contact printing, small dispensing devices mounted on robotic arms use ink-jet, bubble-jet[299] or piezo-electric propulsion[300-303] to transfer the oligonucleotides or cDNA to the microarray surface. The ink-jetting technique does not require direct surface contact, but operates on a "drop-on-demand" basis, firing small droplets of probe solution from miniature nozzles to precise locations on the substrate surface. Many factors, such as immobilization chemistry, spotting buffer, probe concentration and physical factors like spotter type, environmental conditions and the utilized pins influence the fabrication of DNA microarrays and have to be accounted for in the array design. Thus, whether using in situ synthesized or spotted arrays, the choice between platforms is an important decision, as this will affect the downstream operations of microarray sample processing and data analysis.

In contact printing, high-definition pins deposit small volumes of probe solution directly on the array surface. In non-contact printing, the probe is delivered by propelling a small droplet of the solution from a miniature nozzle into the array surface. By repeating the cycles, large amounts of oligonucleotides or cDNA can be printed with high definition.

Probe Preparation, Kinetics and Hybridization

The first and most critical step in probe preparation is isolation of total or poly(A)$^+$ enriched RNA from control and experimental sources. The purified RNA should always be visualized by denaturing gel electrophoresis to verify the integrity of the ribosomal RNA bands. If the RNA is degraded, it will not be useful for labeling. Successful RNA isolation is facilitated through the use of supplies and reagents that are certified RNase-free. Once extracted from the two sources, the RNA is labeled with fluorescent dyes in order to generate probes. The commercial cyanine dyes Cy3 and Cy5 are commonly used in these labeling reactions. Other dyes, such as Alexa Fluor® 546 and Alexa Fluor® 647 (Molecular Probes, Eugene, OR), are becoming more popular because the cyanine dyes, especially Cy5, are unstable and susceptible to degradation by ozone, light, and the lasers used in slide scanning.

Fluorescently labeled probes can be prepared by several different methods including direct or indirect cDNA labeling, cDNA labeling with fluorescent dendrimers, direct mRNA labeling, and direct or indirect labeling of amplified RNA[265,281,289,304,305]. RNA amplification may be the method of choice when isolating RNA from small samples, such as those from tissue biopsies. For use in microarray protocols requiring cDNA synthesis, SuperScript II reverse transcriptase from Invitrogen Life Technologies (Carlsbad, CA) is recommended because it generates high cDNA yields. An alternative enzyme is M-MLV reverse transcriptase from Promega Corporation (Madison, WI). In the direct cDNA labeling method, fluorescently modified deoxynucleotides are incorporated during the first strand cDNA synthesis from an RNA template using reverse transcriptase.[281,289,304] Although this procedure is relatively straightforward, fluorescently modified nucleotides are bulky and incorporate the labeled deoxynucleotides less efficiently than unmodified nucleotides. In the indirect cDNA labeling

method, aminoallyl-modified nucleotides are incorporated during the reverse transcription reaction, and fluorescent dyes are subsequently coupled to the reactive amino groups in the cDNA. Because the amine-modified nucleotides resemble unmodified nucleotides more than the fluorescently labeled nucleotides used for direct labeling, the reverse transcription reaction is more efficient. One disadvantage of the indirect labeling method is that the procedure takes more time to perform.

A newer method uses fluorescent dendrimer complexes to label cDNA.[265,304] After cDNA is synthesized, a fluorescent dendrimer with hundreds (~850) of dye molecules per complex is hybridized to the cDNA. Genisphere offers the 3DNA (dendrimer) sample kits with Cy3/Cy5 or Alexa 546/647 dyes to GCAT members, and protocols using 3DNA products. The differently labeled probes prepared from the two RNA sources are co-hybridized to the same cDNA microarray, which results in competitive binding of the target to the arrayed sequences. The conditions during this step must be optimized to promote specific binding of labeled probe to its target and reduce nonspecific binding. Important parameters include hybridization temperature, duration of hybridization, concentration of salts, pH of the solution, and the presence or absence of denaturants such as formaldehyde in the hybridization buffer. Microarrays are often prehybridized with a solution containing bovine serum albumin to block nonspecific binding of labeled probe to the surface. Hybridization and wash solutions must be evenly distributed over the arrays to maximize interactions between probe and target sequences and minimize background fluorescence. During the hybridization process, the arrays are stored in a humidified, temperature controlled, darkened environment. Small, affordable, aluminum chambers housing one or two microarrays work well, and can simply be placed in a standard incubator or water bath during the hybridization steps.

As with Northern blot hybridizations, specificity of probe/target binding is controlled through a series of post-washes of increasing stringency (*e.g.* increased temperature and decreasing slat concentration) that precludes non-specific or weak interactions between non-complementary sequences.

Microarray data analysis: Scanning and Normalization

Microarray analysis has become a key experimental tool, enabling the analysis of genome-wide patterns of gene expression. One of the most powerful new technologies to emerge from the age of genome sequencing comes from this tiny microarray slide, carrying the capacity to comparatively scan genome-wide patterns of gene expression for any organism with a sequenced genome. Array technologies now are being adapted to the diagnosis of disease predisposition in people, rapid identification of viruses, and protein analysis through the growing field of proteomics. Analysis of microarray data is a multi-step process that must be considered when designing experiments. The complex processes of sample preparation and hybridization account for part of the variability in results obtained. The intensities of the signals generated by the probes must be adjusted, analyzed and normalized to correct for the non-biological variability (or noise) inherent in the system. These procedures are referred to as "data normalization".

The hybridized array is classically scanned with a system that uses lasers to excite the probes, and either photomultiplier tubes or phospho-imagers to detect the probes.[265] For cDNA microarrays, fluorescence images are captured by the photomultiplier tubes using narrow spectrum band pass filters at specific wavelengths that correspond to the fluorescent dyes used and merged to produce a composite image of the microarray. The image contains the measurements of the transcript levels ratios for each gene represented on the array. Most

commercially available array scanners scan sequentially, acquiring one image at a time at both fluorescence excitation wavelengths, and then build the final ratio image. Other scanners use dual laser scanning to acquire both images at the same time, thereby reducing scan times and eliminating potential errors associated with aligning two separately generated images. The only disadvantage associated with this technique is the possible overlap of the excitation and/or emission wavelengths used to generate the signals.

The final result of microarray scanning is an image file that contains the recorded intensity values of each spot on the entire array, representing the emitted signal intensities that are relative to the amount of hybridized cDNA on the microarray. Following the scanning, in the semi-automated image analysis, a computer-aligned grid must be placed over the image surface and a GenePix Array List (GAL) file, which identifies the spots representing the arrayed genes, must be applied. Image analysis software is then used to calculate the intensity of each spot or probe on the array and to store these intensities as numeric values in a text file. During image analysis, the array surface is also evaluated for spatial hybridization biases and poorly hybridized spots to assess the hybridization quality and to flag any "bad spots". Signal adjustment, also known as background correction[306,307] or within-array normalization, calculates the background fluorescence locally for the pixels surrounding each spot and subtracts that value from the hybridization signal intensities. Using this technique, adjustments to compensate for background noise and variation in form of fanning, processing effects and cross hybridization caused by the binding of non-specific target (DNA or RNA) to the array can be made.[306,307] The normalization procedure also corrects for non-biological variations between different arrays in a dataset and removes non-biological variations within a single array to minimize the variance of the intensity values across the dataset and to make the distribution more symmetric. To achieve that

normalization, house-keeping genes (i.e., genes that are believed to be constantly expressed and not influenced by the different experimental conditions) or external control sequences from another organism (*i.e.*, Array Control™ of Ambion®), referred to as spike-in controls, can be used. Biological and technical sample replicates are also utilized to diminish the effect of outlier samples and in enhancing the confidence of the data.[308-313]

Differentially expressed genes are identified by comparing the fluorescence intensity of control and experimental probes hybridized to each spot.[265,290,291] Classically, the experimental target sequences are labeled with Cy5, which fluoresces red light (~667 nm), and control targets are labeled with Cy3, which fluoresces green light (~568 nm). The ratio of the red to green signals is then used as a measure of the effect of the experimental treatment on the expression of each gene. A ratio of 1 (yellow spot) indicates no change in the expression level between experimental and control samples, while a ratio greater than 1 (red spot) indicates increased transcription in the experimental sample, and a ratio less than 1 (green spot) indicates decreased transcription in the experimental sample.[314] A widely used representation of the expression data is the scatter plot, wherein the signal intensities of the experimental and control samples are plotted along the *x*- and *y*-axes, and the ratio values are plotted as a distance from a diagonal line.[265] This diagonal line separates spots with higher activity than the control sample from spots with lower activity than the control sample, and thereby provides a visual representation of the fluorescence ratios obtained from the experimental and control samples. This way of representing the data also makes it easy to choose points that represent several fold increases or decreases in gene expression to focus additional analyses on these genes. In order to ensure quality and comparability of microarray analysis results, guidelines for microarray data reporting

and standards for minimum information about microarray experiments (MIAME)[315] have been developed.

In order to compare separate arrays, normalization of the data must occur. With several experimental conditions (*e.g.*, different time-points or drug doses), the genes are often grouped into clusters that behave similarly under the different conditions. Complex computational methods such as hierarchical clustering or k-means are used to analyze the massive amounts of data generated by these experiments. Gene clusters are visualized with trees or color-coded matrices by placing genes with similar patterns of expression into a clustered group. Image processing and analysis software is commercially available, and several packages are available as freeware.

Microarray analysis of gene expression has limitations that researchers must consider. In experiments involving multiple arrays, inter-chip variation due to non-biological factors, such as dye effects, sample or scanner differences, and unequal quantities of starting RNA, always exists. Normalization is a method that attempts to remove some of this variation. It is important to recognize that changes in gene expression do not necessarily correlate with production of the specific proteins. Translational and post-translational regulatory mechanisms that impact the activity of various cellular proteins are not examined by DNA microarrays, though the emerging field of proteomics is beginning to address this issue. Other limitations of microarray analysis include the impact of alternative splicing during transcript processing and the limited detection of unstable mRNAs. Differential gene expression results must be confirmed through direct examination of selected genes. These analyses are typically at the level of RNA blotting or quantitative RT-PCR to examine the transcripts of a specific gene,[257] and/or detection of protein concentration using immunoblots.

Statistical analysis of microarray data

After preprocessing, normalized gene expression data can be analyzed using statistical tools and exploratory methods to extract genes or patterns having biological significance. The analysis of one-color and two color microarray data is more or less similar, the main difference between these methods lying in the normalization of the data. The investigation of biological processes through microarray analysis can be approached in various ways. Main interests can lie in finding single changes in gene expression that may be a key to a given alteration in phenotype or to look at overall patterns of gene expression in order to understand the architecture of genetic regulatory networks. With data analysis software, complex problems and datasets generated by microarray experiments can be assessed and the structure of the experimental data can be characterized by extracting statistically significant patterns. Several commercial and non-commercial software tools for statistical analysis and visualization of gene expression data are available, including GeneCluster,[316] GeneSpring (Agilent Technologies), SAM,[317] dCHIP,[318] Cluster and Treeview,[319] and "R" (Bioconductor).[320]

There is considerable variation within the methods used to analyze data, in part, because the analysis of microarray data is by nature exploratory. Generally used tools comprise filters[321] to remove redundant genes from the experiment, statistical tests to identify differentially expressed genes, and classification methods to discover pathway level expression patterns and detect specific expression profile signatures. In most cases, addition of large numbers of genes that are irrelevant or unchanged increase the degree of noise and uncertainty to the data when expressed and compromise classical statistical testing (i.e., reducing the number of genes being analyzed, increases the power of statistical testing procedures). Gene filtering helps to remove redundant genes and to highlight genes that are differentially expressed. Typically, Student's t-

test[322] or analysis of variance (ANOVA)[323] tests are used in microarray analysis, which assume normal distribution of the data.

The fact that microarray experiments typically have large numbers of observations but only few samples leads to testing of multiple hypotheses. As some of the observed differences happen by chance alone, the significance values of statistical tests must be adjusted to take multiple testing into consideration. These adjustments include corrections such as the Bonferroni method and False Discovery rate.[324,325] The most widely used method to identify differential expression of genes is by calculating the fold change.[326] This method has its shortcomings, especially when used alone. It also lacks the ability to account for the variance and the lack of associated levels of confidence.

Interpreting microarray data and generating biologically meaningful knowledge can be challenging. Inevitably, the goal of each experiment is to generate data in order to test hypotheses or to develop new ones. Frequently the output of such an analysis is a list of significant genes or a ranked list of genes. In DNA microarray studies, data analysis often leads to lists of hundreds of differentially expressed genes. Many methods are available to extract significantly regulated genes, which commonly are then represented in spreadsheet-like lists. Functional properties of these genes then must be gathered (if not already provided in the local database) and common functional properties of genes must be identified. While this might prove feasible for smaller number of genes in few experimental conditions, the list of regulated genes in a typical microarray experiment can easily exceed hundreds of genes. These numbers render non-computer based data analysis not only tedious and time-consuming, but also prone to errors. This bottleneck in data analysis has led to the development of various tools to analyze microarray data in the context of gene annotations.

Classification, also known as clustering, of gene expression data may result in clusters of tens to hundreds of genes. However, these data are of little use if one is not able to interpret the results in a biological context. Thus, clustering is widely used either to discover new categories within a dataset or to assign genes to a given category. The two major methods used for clustering are the unsupervised[326,327] and supervised[328,329] methods. In the supervised clustering (or class prediction) methods, algorithms are developed to assign objects, such as genes or samples, to predetermined categories. In the unsupervised clustering (or class discovery), objects are grouped into classes based on some sort of similarity metric that is computed for one or more variables. Typically, genes are grouped into classes on the basis of similarities in their expression profiles across tissues or conditions. Unsupervised clustering can further be split into non-hierarchical clustering methods such as self-organizing maps or k-means clustering.[330] Typically, these approaches divide the genes into a predetermined number of groups in a manner that maximizes a specific function and hierarchical clustering methods,[328] and also produces a tree diagram (dendrogram). This hierarchical clustering method provides via the Gene Ontology Consortium a controlled vocabulary to annotate the biological knowledge that exists or is predicted for a given gene. Gene Ontologies (GOs) are organized as a hierarchy of annotation terms that facilitate analysis and interpretation at different levels. The top-level ontologies are molecular function, biological process, and cellular component. Tools that focus on analysis of individual properties, such as sequence information (*e.g.,* prediction of transcription factor binding sites), identification of homologies or prediction of 3-dimensional protein structures, are readily available. Several annotation databases for genes of different organisms exist. GO helps to biologically interpret the lists of genes resulting from high-throughput microarray experiments.[331]

Although the existence and direction of changes in gene expression can be reliably detected for the majority of genes in appropriate sensitivity ranges, accurate measurements of absolute expression levels and reliable detection of low-level abundance genes are presently beyond microarray technology.[297] The detection limit of current microarray technology is estimated to be between 1 and 10 copies of mRNA per cell, depending on the microarray platform and target material used.[332] Sources of inaccuracy and inconsistencies in microarray measurement/analysis include probe sequence design, redundant annotations, splice variant effects, folding of target transcripts and cross-hybridization.[297,333] Due to the high variability in analysis methods and increased probability of false positive findings in microarray data analysis, data validation is generally regarded as an integral part of the analysis process. Data validation can be either internal or external. In internal validation, the existing data are typically re-sampled, and probabilities are calculated on permutated sets of the data to evaluate the original findings. In external validation, the results are typically validated by using another method on a new sample (or data) set, such as mRNA measurement of a number of selected genes by quantitative reverse-transcriptase RT-PCR, which is believed to protect against erroneous inferences due to measurement quality problems. However, this type of validation procedure has recently been questioned,[309] and the requirements for acceptable microarray results remains to be determined in the future.

EQUINE MICROARRAYS AND GENOME

3076 gene microarray at UGA

Our laboratory developed an equine-specific 3076-gene cDNA microarray in Y2003 using cDNA inserts from an equine expressed sequence tag (EST) unigene set as templates for

the PCR-amplified, immobilized target genes on glass slides. Because relatively little is known about the equine genome, with only a limited number of equine genome sequences (~4,300) having been deposited in GenBank, this equine EST project provides a starting point for studies involving many, but certainly not all, equine genes. In this project ~14,000 equine-specific ESTs were generated by sequencing cDNAs (sequenced from both 5' and 3' ends) obtained from a number of equine cDNA libraries prepared from different tissues. These sequences were analyzed for single nucleotide polymorphisms (SNPs) and simple sequence repeats (SSRs) with the aim to archive all unique cDNA sequences and to establish this information together with the corresponding bacterial clones as a public resource for other investigators. Unique equine ESTs were then incorporated into an equine-specific microarray that is used to identify changes in gene expression profiles.

In collaboration with the Drs. Sumio Sugano and Yutaka Suzuki (University of Tokyo), full-length transcript enriched cDNA libraries were prepared from equine leucocyte. To increase the yield of information generated during these studies, cells were obtained from 6 different horse breeds (Thoroughbred, Quarter Horse, Tennessee Walking Horse, Dartmoor Pony, Belgian Draft and Arabian). This was done to increase the likelihood of polymorphic marker detection (*e.g*. SNPs and SSRs) during the sequencing and sequence analysis phase of the study so that each constructed tissue library contains genetic information from different horse breeds.

REASON FOR SELECTING GENE POPULATION

Many of the most severe equine disease have an inflammatory component, consequently 14 of the cDNA libraries used for EST generation were prepared from equine leukocytes and the fiteenth from acute phase liver.

In a collaboration between laboratories at the University of Georgia, more than 14,000 high quality equine gene sequences were generated from equine cDNA libraries from leukocytes and liver . These quality sequences, defined as those having a contiguous region of >100 base

Table 2.1. Summary of our leukocyte EST data.

Cell treatment	**None**		**LPS 4 hrs**	
Library name	**Leuko N1-N6**		**Leuko S1-S6**	
Primer	5'	3'	5'	3'
Seq. Total (attempts)	4,224	4,512	3,840	3,840
Q16 (NV≤ 100 bp)	2,769	2,737	2,936	2,594
% good sequence	66	61	77	68
Ave Q16 length (bp)	560	535	560	538
Ave Q20 length (bp)	542	496	542	499

pairs (bp) exceeding an overall PHRED score of Q16 (97.5% accuracy) *after* removal of vector and polyT, averaged after trimming ~600 nucleotides (nt). Clustering analysis of the 3' sequences reveled 3,040 unique genes, for a gene discovery rate of 42%. Table 2.1 summarizes the data obtained from our leukocyte cDNA libraries. All sequences have been assigned a provisional annotation by BLAST against the PIR database. These quality sequences have been deposited in GenBank and can be viewed without trimming in the Horse Project area on our website: http://fungen.org. These equine cDNAs cover a wide range of function, including secreted proteins (*e.g.* IL-8 GCSF and matrix metalloprotease 9), cellular receptors (*e.g.* IL-4 receptor, Angiotensin II receptor,CD44 and Toll-like receptors 2, 4 and 8), housekeeping genes (*e.g* GAPDH), nuclear proteins (*e.g.* the NF-κB subunits, p65 and p50), and signal transducers (*e.g.* TRAF-2 and fra-2). All of these cDNAs were archived in duplicate at -80°C and as such can

serve as template fro the generation of cDNA microarrays or be used to directly study gene function through heterologous expression systems. Of the cDNAs with an apparent match to known genes, the majority included those involved in cell signaling/cell communication. The use of a bioinformatic pipeline allowed the detection of polymorphic markers, such as SNPs and SSRs, that might be useful in gene mapping studies or for studies associated with specific QTLs such as susceptibility to disease.

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CHAPTER 3

EICOSANOIDS AND BLACK WALNUT HEARTWOOD EXTRACT INDUCED LAMINITIS: I. THROMBOXANE AND ISOPROSTANES

[1] E. Noschka, J. N. Moore, J. F. Peroni, S. J. Lewis, J. D. Morrow, T. P. Robertson. Submitted for publication in the *Journal of Veterinary Immunology and Immunopathology*

ABSTRACT

Inflammation and vascular dysfunction occur during the prodromal stages of equine laminitis. The aim of this study was to provide initial insights into the possible role that eicosanoids, and specifically thromboxane and isoprostanes, may play in the development of black walnut heartwood extract (BWHE)–induced laminitis. Horses were randomly divided into two groups, either control or BWHE-administered horses. At 12 hours (for the control group) or Obel grade 1 laminitis (for BWHE group) the horses were euthanized and laminar tissue taken for the isolation of laminar arteries and veins (200-800 micron internal diameter) and for the determination of laminar iso-$PGF_{2\alpha}$ concentrations. Laminar vessel constrictor responses to either a thromboxane mimetic (U46619), iso-PGE_2 or iso-$PGF_{2\alpha}$ were determined using small vessel myographs. In some vessels, the effects of putative prostanoid and thromboxane receptor antagonists, SQ 29,548, SC-19220 and AH 6809 upon contractile responses were determined. Plasma concentrations of thromboxane increased transiently and coincided with the nadir in white blood cell counts. Plasma concentrations of iso-$PGF_{2\alpha}$ did not change in control or BWHE-administered horses, whereas laminar tissue concentrations of iso-$PGF_{2\alpha}$ were significantly greater in BWHE horses when compared to control horses. In control horses, U46619, iso-$PGF_{2\alpha}$ and isoPGE$_2$ more potently and efficaciously constricted laminar veins when compared to laminar arteries. Responses of laminar veins from BWHE horses to iso-PGE_2 were similar to those of laminar veins from control horses, whereas iso-$PGF_{2\alpha}$ elicited significantly greater responses in laminar veins from BWHE horses when compared to controls. In contrast, responses to U46619 were smaller in laminar veins isolated from BWHE horses when compared to those in laminar veins from control horses. In the presence of the thromboxane receptor

antagonist, SQ 29,548, iso-$PGF_{2\alpha}$ elicited a small dilation in laminar veins from control horses, which was not apparent in laminar veins from BWHE horses. The results of this study are consistent with both systemic and local inflammatory events occurring during the prodromal stages of BWHE-induced laminitis. Because laminar veins are exquisitely sensitive to thromboxane and isoprostanes, these substances may act as conduits between the inflammatory and vascular events occurring in laminitis and, as such, may serve as viable therapeutic targets for this crippling condition.

INTRODUCTION

Equine laminitis is a debilitating and potentially life-threatening condition that affects the sensitive laminae of the digit. Although the precise cascade of events involved in the pathogenesis of this condition remain unresolved, it is apparent that vascular dysfunction occurs during the developmental stages of laminitis. This dysfunction, which occurs at the level of the laminar dermis, is manifested as a selective venoconstriction and resultant increase in post-capillary resistance (Allen et al., 1990; Eaton et al., 1995; Galey et al., 1990; Hood, 1999; Hood et al., 1993; Moore et al., 2004; Peroni et al., 2005).

The initiating event(s) that triggers the vascular dysfunction in laminitis has yet to be determined. However, recent advances led to the first studies of the contractile responses of laminar arteries and veins isolated from the equine digit. These studies demonstrated that the equine digit may be pre-disposed to venoconstriction at the level of the laminar dermis, (Peroni et al., 2006) as laminar veins were far more sensitive than laminar arteries and constricted to a greater degree, when exposed to a variety of vasoconstrictors. For example, small laminar veins

respond with robust and sustained contractile responses when exposed to the eicosanoid prostaglandin $F_{2\alpha}$ whereas the responses of laminar arteries were negligible. Because the prodromal stages of laminitis are also associated with a marked inflammatory response in the laminae (Blikslager et al., 2006; Waguespack et al., 2004), it is possible that this local inflammation may result in increased production of vasoactive prostanoids that, in turn, result in venoconstriction, increased post-capillary resistance and edema formation, all of which appear to contribute to the development of laminitis. Indeed, this possibility is supported by recent reports that have demonstrated that cylco-oxygenase 2, an enzyme responsible for prostanoid production, is up-regulated during the early stages of laminitis (Blikslager et al., 2006).

Another potential consequence of inflammation within the digit is the possibility that the oxidative stress associated with the inflammatory response could result in the production of isoprostanes, which are formed by the non-enzymatic free radical-catalyzed peroxidation of arachidonic acid in cell-membrane phospholipids (Liu et al., 1999; Morrow et al., 1990). Isoprostanes, such as, and increased concentrations of isoprostanes have been associated with several pathological conditions (Cracowski et al., 2001; Minuz et al., 2006). However, isoprostanes have also been reported to exert potent biological actions and, therefore, may also participate in the disease process rather than merely being biological indicators. One of the biological effects of isoprostanes that may be pertinent to the development of equine laminitis is vasoconstriction. Currently, however, there is a lack of consensus regarding the mechanism of action of isoprostanes, primarily because data support both a role for a discrete isoprostane receptor and also for these compounds acting via activation of the thromboxane receptor.

There are several known cell surface prostaglandin receptors, as well as additional splice variants (Narumiya et al., 1999). Prostanoid receptor nomenclature is based on the ligand that

binds to the receptor with the greatest affinity (Coleman et al., 1994). For example, E prostanoid (EP) receptors bind PGE_2, though it is apparent that these receptors can also be activated by other members of the prostaglandin family. There are four subtypes of the EP receptor (EP_1, EP_2, EP_3 and EP_4) that are known to have vasoactive effects. Specifically, EP_1 and EP_3 receptors are responsible for the vasoconstrictor properties of prostaglandins, whereas activation of EP_2 and EP_4 receptors results in vasodilation.

The aim of the present study was to gain insights into the possible role of isoprostane and thromboxane production in the development of laminitis. Specifically, we aimed to determine whether isoprostane and thromboxane concentrations increase during the prodromal stages of laminitis and to compare the vasoactive effects of these prostanoids in laminar arteries and veins isolated from control horses and horses with experimentally-induced Obel grade 1 laminitis. We also report here, for the first time, the effects of currently available thromboxane and prostanoid receptor antagonists, namely the thromboxane receptor antagonist, SQ 29,548, prostaglandin EP_1 receptor antagonist, SC-19220 and the prostaglandin EP_1, EP_2, EP_3 and DP_1 receptor antagonist, AH 6809, on the responses of equine laminar vessels.

MATERIALS AND METHODS

Mixed breed horses ranging in age from 4 to 12 years old (mean 9 years) were used in this study. Each horse lacked clinical evidence of lameness and survey radiographs of the forelimb digits were within normal limits. Horses were randomly assigned to control or black walnut heartwood extract (BWHE) groups. Horses in the control group (5 horses) received 6 L of water via nasogastric tube, and were euthanatized after the collection of the final (12 hour) blood

sample. Horses in the BWHE group (5 horses) received BWHE via nasogastric tube and were euthanatized at the onset of Obel grade 1 laminitis (clinical signs consisting of weight shifting and bounding digital pulses without evidence of lameness at a walk) or at 12 hours after intubation, if signs of Obel grade 1 laminitis had not developed by that time. Each horse was evaluated prior to intubation and every hour thereafter for attitude, heart rate, respiratory rate, capillary refill time, hoof temperature, digital pulses, and evidence of lameness consistent with Obel grade 1 laminitis. Blood samples were obtained via a jugular catheter at 0, 1, 2, 3, 4, 6, 8, and 10 hours. Catheter patency was maintained by flushing the catheter with 20 ml heparinized saline (5 IU heparin/ml) after each blood collection. In a parallel study, laminar tissue samples were also obtained from a further control group of horses (samples collected 3 hours after nasogastric administration of 6L of water) and two further groups of horses administered BWHE (samples collected 1.5 or 3 hours after BWHE administration). Horses in these latter 3 groups were studied at The Ohio State University as previously described (Loftus et al., 2007). All protocols were approved by the Institutional Animal Care and Use Committees of The University of Georgia and Ohio State University. The horses were euthanatized using a penetrating captive bolt, as approved by the Report of the American Veterinary Medical Association's Panel on Euthanasia (AVMA, 2001).

The blood samples were aliquoted into three ice chilled vacuum-evacuated tubes containing EDTA, one of which was used for determination of the peripheral WBC count. The other samples were placed on ice for 10 minutes and then immediately centrifuged at 400 x g for 10 minutes at 4 ºC. Plasma from the latter tubes was frozen at -80ºC until assayed for thromboxane B_2 (TxB_2) and isoprostane 8-iso $PGF_{2\alpha}$ (iso-$PGF_{2\alpha}$) concentrations. Plasma concentrations of TxB_2 were determined using a commercially available enzyme immunoassay

(EIA) kit (Cayman Chemical, Ann Arbor, MI). Plasma and tissue iso-$PGF_{2\alpha}$ concentrations were determined by gas chromatography/negative ion chemical ionization mass spectrometry using stable isotope dilution methodology.

Collection of laminar tissue

Both forelimbs were disarticulated at the level of the metacarpophalangeal joint, and the hooves cut into sections with a band saw. During the procedure, thermal damage was minimized by constant irrigation of the tissue with ice-cold physiologic salt solution containing the following: 118mM NaCl, 24mM $NaHCO_3$, 1mM $MgSO_4$, 0.435mM NaH_2PO_4, 5.56mM glucose, 1.8mM $CaCl_2$, and 4mM KCl. Two full-thickness segments from each forelimb foot were placed in the ice-cold physiologic salt solution, and the keratinized portion of the hoof and distal phalanx were removed. Laminar specimens were rapidly frozen in liquid nitrogen and stored at −80ºC and one gram of each frozen sample was used for determination of iso-$PGF_{2\alpha}$ concentrations as detailed below.

Isolation of laminar vessels

Laminar arteries and veins were isolated as previously described (Peroni et al., 2005; Peroni et al., 2006; Robertson et al., 2005). Briefly, two full thickness segments of the dorsal hoof were placed in ice-cold physiological salt solution (PSS) containing (in mM): NaCl 118; $NaHCO_3$ 24; $MgSO_4$ 1; NaH_2PO_4 0.435; glucose 5.56; $CaCl_2$ 1.8, and KCl 4; gassed with 21% O_2 and 5% CO_2 (pH = 7.40 ± 0.01). On the stage of a high-powered microscope, the lamellar portion of the dermis was shaved until only a thin layer covered the laminar vascular bed. Laminar arteries and veins (2 - 3 cm distal to the coronary band, 200 - 800 μm internal diameter,

1-2 mm in length) were isolated using micro-fine surgical instruments and mounted on small vessel myographs (Model 500A, Danish Myo Technology, Denmark). The vessels were bathed in PSS and the bath temperature was raised to, and maintained at, 37^{o}C for one hour while the vessels equilibrated. Laminar arteries and veins were then stretched to produce equivalent transmural pressures of 3.1 kPa and 1.9 kPA, respectively (Peroni et al., 2005). Data were collected for each agonist and, where appropriate, in the presence of specific antagonists, from at least 2 arteries and veins from all 10 horses. The numbers of vessels and horses used to obtain the data in this study were based on our previous experience with isolated laminar arteries and veins (Peroni et al., 2005; Peroni et al., 2006; Robertson et al., 2005).

Experimental vessel protocols

All vessels were given three 2-minute exposures to 80 mM KCl-PSS (isotonic replacement of NaCl with KCl, KPSS), 15 minutes apart to establish the maximal contractile response to a depolarizing stimulus. Concentration response curves were then obtained for either the thromboxane receptor agonist U46619 (U46619, 1 nM – 10 μM), 8-iso prostaglandin PGE_2 (iso-PGE_2, 1 nM – 10 μM) or 8-iso prostaglandin $PGF_{2\alpha}$ (iso-$PGF_{2\alpha}$, 1 nM – 100 μM) by cumulative addition of each agonist.

Similar experiments were performed in which vessels were pre-incubated with each of the following antagonists: SQ 29,548 (thromboxane receptor antagonist, 50 μM), SC-19220 (prostaglandin EP_1 receptor antagonist, 50 μM), or AH 6809 (prostaglandin EP_1, EP_2, EP_3 / DP_1 receptor antagonist, 50 μM) 10 minutes prior to commencement of the appropriate concentration response curve. The concentration of each antagonist was based upon published reports of their efficacy in isolated blood vessels (Daray et al., 2004; Sametz et al., 2000; Walch et al., 2001).

Sample preparation for enzyme immunoassay (EIA)

Prior to determining concentrations of TxB_2, the stable metabolite of TxA_2, plasma was subjected to methanol extraction with one ml of plasma added to 9 ml methanol. The samples were then centrifuged for 10 min at 400 x g, and the supernatant was decanted and concentrated by vacuum centrifugation (Hetovac Vacuum Rotator model VR-1, Heto-Holten, Denmark). The concentrated supernatants were stored at −80ºC until reconstituted to a 2 times concentration in the EIA buffer immediately before performing the assay according to the manufacturer's instructions.

EIA for TxB_2

Plasma TxB_2 concentrations were determined using a commercially available EIA kit (Cayman Chemical, Ann Arbor, MI). Briefly, 50 µl of standard or extracted plasma samples were placed in a 96-well plate pre-coated with mouse monoclonal antibody. Thereafter, 50 µl of TxB_2 tracer and TxB_2 antiserum were added into each well and incubated for 2 h at room temperature. After washing with wash buffer 200 µl of Ellman's reagent containing acetylcholinesterase was added. The plates were read at 412 nm and concentrations of TxB_2 were calculated from a standard curve generated with known concentrations of TxB_2. The detection limit of the assay was 11 pg/ml.

Assay for isoprostanes

Free F_2-isoprostanes in plasma and laminar tissue were quantified after purification and derivatization by selected ion monitoring gas chromatography negative ion chemical ionization/mass spectrometry employing $[^2H_4]$8-iso-$PGF_{2\alpha}$ as an internal standard (Morrow and Roberts, 1999). Compounds were analyzed as pentafluorobenzyl ester, trimethylsilyl ether

derivates monitoring the M-181 ions, m/z 569 for endogenous F_2-isoprosanes and m/z 573 for 8-iso-$PGF_{2\alpha}$. The F_2-isoprostanes elute as a series of chromatographic peaks during a 20-second interval and quantification is based on the primary peak eluting at the same time as the internal standard. Data were expressed in pmol/L and the detection limit of the assay was ~ 2 pg.

Data and statistical analyses

Data are presented as mean ± SEM. Contractile responses were calculated as a percentage of the maximal response to KPSS (% T_K) for each vessel. The data were analyzed by repeated measures analysis of variance (ANOVA). Differences between individual means were identified by Student's modified *t*-test using the Bonferroni correction for multiple comparisons between means using the error mean square term from the ANOVA. TxB_2 and isoprostane data were analyzed using an unpaired Student's t test to compare mean values for the control and BWHE horses. A value of $P < 0.05$ was deemed to be significant (Wallenstein et al., 1980; Winer, 1971).

RESULTS

Responses of laminar arteries and veins to vasoconstrictor agonists

Laminar veins were more sensitive to all three agonists, with respect to the concentration of agonist required to initiate contractile responses, and also constricted to a greater degree (when expressed as %T_K) than laminar arteries (Figures 3.1-3.2 and Tables 3.1-3.3). Of the three agonists, U46619 elicited significantly larger contractile responses than either of the isoprostanes. Laminar veins isolated from horses administered BWHE were less sensitive to U46619 than laminar veins from control horses. In contrast, laminar veins from BWHE-

administered horses were more sensitive to iso-$PGF_{2\alpha}$ than laminar veins from control horses; contractile responses of veins from control and BWHE horses to iso-PGE_2 were not significantly different. Contractile responses of laminar arteries from control and BWHE horses to the three agonists were not significantly different.

Modulation of contractile responses to U46619, isoPGE2 and isoPGF2α

Effects of SQ 29,548 on laminar veins: Pre-incubation of laminar veins from control and BWHE horses with the thromboxane receptor antagonist SQ 29,548 (50μM) ablated contractile responses to U46619, iso-PGE_2 and iso-$PGF_{2\alpha}$. Moreover, in the presence of SQ 29,548, high concentrations (above 1μM) of iso-$PGF_{2\alpha}$ elicited a slight vasodilator response in laminar veins isolated from control horses. This vasodilation to iso-$PGF_{2\alpha}$ in the presence of SQ 29,548 was not evident in laminar veins isolated from BWHE-administered horses (Figure 3.3 and Tables 3.1-3.3).

Effects of SC-19220 on laminar veins: Pre-incubation of laminar veins from control and BWHE horses with the prostaglandin EP_1 receptor antagonist SC-19220 (50μM) caused rightward shifts in the concentration response curves to U44619, though the maximal contractile responses were similar at the highest concentration of U46619. Contractile responses of laminar veins, isolated from either control or BWHE-administered horses, to iso-PGE_2 were reduced in the presence of SC-19220, but this effect was more pronounced in laminar veins isolated from control horses than in laminar veins isolated from BWHE-administered horses. Pre-incubation with SC-19220 had contrasting effects upon subsequent contractile responses to iso-$PGF_{2\alpha}$ in laminar veins isolated from control or BWHE-administered horses. In the presence of SC-19220, the responses of laminar veins isolated from control horses were increased when compared to the

responses of laminar veins in the absence of the antagonist. In contrast, SC-19220 pre-incubation reduced the contractile responses of laminar veins isolated from BWHE-administered horses (Figure 3.4 and Tables 3.1-3.3).

Effects of AH 6809 on laminar veins: Pre-incubation of laminar veins from control and BWHE horses with AH 6809 (50μM) reduced contractile responses to all three agonists when compared to responses of laminar veins incubated with the agonists alone. The inhibitory effect of AH 6809 upon subsequent contractile responses to U46619 was more evident in laminar veins isolated from BWHE-administered horses than in laminar veins isolated from control horses. In contrast, AH 6809 inhibited responses to iso-PGE_2 to a lesser degree in laminar veins from BWHE-administered horses than in laminar veins isolated from control horses. Similar to the results obtained with SQ 29,548, pre-incubation of laminar veins isolated from control horses elicited a small vasodilator response upon exposure to iso-$PGF_{2\alpha}$. The dilator response to iso-$PGF_{2\alpha}$ in the presence of AH 6089 was not evident in laminar veins isolated from BWHE-administered horses (Figure 3.5 and Tables 3.1-3.3).

Plasma concentrations of TxB_2 and iso-PGF_2

Plasma concentrations of TxB_2 were significantly greater in the BWHE horses at the time when the peripheral leukocyte count was at its lowest point (Hurley et al., 2006), 3-4 hours after administration of BWHE, when compared to the value for time 0. In contrast, there were no differences in plasma concentrations of iso-$PGF_{2\alpha}$ concentrations within or between the control and BWHE groups at any time point (Figure 3.6).

Laminar tissue concentrations of iso-$PGF_{2\alpha}$

Concentrations of iso-$PGF_{2\alpha}$ were greater in laminar tissue samples obtained from BWHE-administered horses when compared to values for the control horses. In parallel studies, we also obtained laminar tissue samples from horses 1.5 and 3 hours after administration of BWHE and determined that iso-$PGF_{2\alpha}$ were similar to those for control horses at 12 hours and control horses at 3 hours (Figure 3.7).

DISCUSSION

This is the first study comparing the vasoconstrictor responses of equine laminar arteries and veins to isoprostanes and to thromboxane receptor stimulation, via application of the stable thromboxane mimetic, U46619. Isoprostanes are non-enzymatic products resulting from free radical–catalyzed peroxidation of arachidonic acid and are potent vasoconstrictors in a variety of vascular preparations (Daray et al., 2004; Morrow, 2006; Tazzeo et al., 2003). The isoprostanes exert their effects via activation of thromboxane receptors and/or specific isoprostane receptors (Fukunaga et al., 1993; Fukunaga et al., 1997; Longmire et al., 1994). All three agonists used in the present study induced constriction of the laminar vessels, though laminar veins were significantly more sensitive, and constricted to a greater degree, than laminar arteries. These findings are consistent with the results of previous studies in which laminar veins were generally more sensitive to vasoconstrictor agents than laminar arteries (Peroni et al., 2006). It is possible, therefore, that inflammation within the equine digit may result in a preferential constriction of the venous side of the laminar microcirculation via an increase in local production of arachidonic acid metabolites. Because inflammation and venoconstriction occur concurrently during the

prodromal stages of equine laminitis (Moore et al., 2004), it is possible that the local production of prostanoids and isoprostanes, and their associated venoconstriction contribute to the development of this crippling condition. The aims of the present studies were to provide insights into the vasoconstrictor effects of isoprostanes and thromboxane receptor activation, determine the effects of three currently available thromboxane and prostanoid receptor antagonists upon these responses.

Laminar vessel responses to direct thromboxane receptor activation by U46619

Exposure of laminar veins isolated from control horses or BWHE-administered horses to U46619 resulted in robust vasoconstrictor responses. However, the magnitude of the contractile responses was less in laminar veins from BWHE-administered horses than in those from control horses. This reduction in vasoconstrictor efficacy in veins from horses with Obel grade laminitis is similar to that observed in a previous study for phenylephrine (PE) and 5-hydroxytryptamine (5-HT) –induced vasoconstriction (Peroni et al., 2005). Although U46619 elicited much smaller contractile responses in laminar arteries from control and BWHE-administered horses, there were no differences between the two groups. Therefore, it appears that the decrease in thromboxane receptor-mediated vasoconstriction at the time of Obel grade 1 laminitis is confined to laminar veins, which is a pattern similar to that reported for PE and 5-HT (Peroni et al., 2005).

Laminar vessel responses to iso-PGF and iso-PGE

While exposure to either iso-PGE_2 or iso-$PGF_{2\alpha}$ elicited concentration-dependent constrictor responses in laminar veins, these responses were smaller in magnitude when compared to direct thromboxane receptor stimulation by U46619. These observations are

consistent with previous studies reporting the following ranked order of potencies in human vascular tissues: U46619> iso-PGE_2> iso-$PGF_{2\alpha}$ (Oliveira et al., 2000). Similar to the results obtained for U44619, the isoprostanes were far less effective in terms of eliciting contractile responses in laminar arteries. This finding is consistent with the hypothesis that the equine digit may be pre-disposed to venoconstriction at the level of the small laminar vessels (Peroni et al., 2006). In contrast to U46619, the responses to isoprostanes were not reduced in laminar veins isolated from BWHE-administered horses. Indeed, the responses to iso-$PGF_{2\alpha}$ were greater in laminar veins isolated from horses with BWHE-induced Obel grade 1 laminitis than in laminar veins from control horses. The preservation of the isoprostane venoconstrictor responses, coupled with the increased local concentration of iso-$PGF_{2\alpha}$ in the laminar tissue of BWHE-administered horses, is consistent with local production of isoprostanes being a potentially important factor in the venoconstriction observed during the development of laminitis.

Effects of putative prostanoid receptor antagonists on laminar vessel responses to isoprostanes and U46619

SQ 29,548 is purported to be a selective antagonist of the thromboxane receptor (Abramovitz et al., 2000). In the present study, pre-incubation with SQ 29,548 ablated vasoconstrictor responses to all three agonists in laminar veins from control horses or those with BWHE-induced Obel grade 1 laminitis. However, in the presence of SQ 29,548, iso-$PGF_{2\alpha}$ elicited a small vasodilation in laminar veins isolated from control horses but not in from BWHE-administered horses. These observations suggest that; 1) iso-$PGF_{2\alpha}$ possesses vasoactive effects that iso-PGE_2 does not, namely an ability to elicit vasodilation in laminar veins when the thromboxane receptor is blocked, and 2) that the vasodilator pathway activated by iso-$PGF_{2\alpha}$ is

compromised or absent at the onset of Obel grade 1 laminitis. The latter may explain why responses to iso-$PGF_{2\alpha}$, but not to iso-PGE_2, are enhanced in laminar veins isolated from BWHE-administered horses when compared to those in laminar veins isolated from control horses (i.e., the absence of the additional dilator actions of iso-$PGF_{2\alpha}$ during BWHE-induced laminitis results in a net increase in the venoconstrictor effects of iso-$PGF_{2\alpha}$). The finding that an eicosanoid-dependent vasodilator pathway is compromised at Obel grade 1 laminitis are also supported by our recent findings regarding the effects of $PGF_{2\alpha}$, PGE_2 and 11-d-PGE_2 in laminar veins from healthy horses and those with BWHE-induced laminitis (Noschka et al., 2007a; Noschka et al., 2007b).

Pre-incubation of laminar veins from control and BWHE horses with the prostaglandin EP_1 receptor antagonist SC-19220 (50μM) did not significantly change the maximal contractile responses to U46619, although a shift in the concentration-response curve to U46619 to the right was observed. The shift observed in the presence of SC-19220 may be due to non-specific binding of U46619 to the EP_1 receptors but may also be due to antagonism of EP_1 receptors by SC-19220. The inhibitory effect of SC-19220 on U46619-induced contractile responses was qualitatively less in laminar veins isolated from horses administered BWHE, a finding that is consistent with the reduction in contractile responses to U46619, in the absence of receptor blockade, being due to down-regulation or desensitization of EP_1 receptors at Obel grade 1 lameness.

In contrast to the effects of SC-19220 upon U46619-induced contractions, pre-incubation of laminar veins with SC-19220 reduced the maximum contractile responses to both iso-$PGF_{2\alpha}$ and iso-PGE_2. The inhibitory effects of SC-19220 were similarly observable in laminar veins from BWHE-administered horses, which would argue against a change in EP_1 receptor function

during the development of Obel grade 1 laminitis. Unfortunately, due to the paucity of available efficacious EP receptor antagonists, it was not possible to resolve the apparent conflicting effects of SC-19220 upon responses induced by U46619 and isoprostanes. The development of more selective and structurally dissimilar receptor antagonists in the future may provide the necessary tools to delineate any changes in EP receptor subtype expression and function during the development of laminitis.

The putative EP_1, EP_2, EP_3 and DP_1 receptor antagonist, AH 6809, reduced the maximal contractile responses of all three agonists in laminar veins from control horses. However, AH 6809 was much more effective, in terms of the degree of inhibition, in blocking contractile responses to the isoprostanes than U46619. In contrast to the effects in laminar veins from control horses, AH 6809 was efficacious in blocking the responses to U46619 in laminar veins isolated from BWHE-administered horses. The reason for the altered efficacy of AH 6809 in veins from horses with Obel grade 1 laminitis is not readily apparent. Indeed, the lack of available alternative EP receptor antagonists, coupled with a lack of convincing data that would support the purported selectivity of the antagonists available for use in this study confounds interpretation of these current data. In the presence of AH 6809, however, iso-$PGF_{2\alpha}$ elicited a small vasodilation in laminar veins of control horses that was not evident in laminar veins from BWHE-administered horses. These observations are consistent with those for SQ 29,548 and iso-$PGF_{2\alpha}$, and would support the concept that a vasodilator pathway activated by iso-$PGF_{2\alpha}$ is compromised by the onset of Obel grade 1 laminitis. These data are also consistent with the biological activities of various isoprostanes being diverse and peculiar to the isoprostane in question. Specifically, in this study it is apparent that iso-$PGF_{2\alpha}$ possesses additional vasoactive effects to those observable to iso-PGE_2 in laminar veins.

Plasma and laminar tissue concentrations of TxB_2 and iso-$PGF_{2\alpha}$

Plasma concentrations of TxB_2 increased transiently and coincided with the decrease in white blood cell in horses administered BWHE (Hurley et al., 2006). This increase is similar to the change in plasma concentrations of $PGF_{2\alpha}$ and PGE_2 in these horses (Noschka et al., 2007a, Noschka et al., 2007b) and is consistent with systemic inflammatory events being present during the prodromal stages of BWHE-induced laminitis. However, the observations that laminar tissue concentrations of iso-$PGF_{2\alpha}$ were increased in BWHE-administered horses whereas plasma concentrations of iso-$PGF_{2\alpha}$ remained unchanged suggests that lipid peroxidation may in fact be confined to the digit during the development of laminitis. The latter finding may help to explain the relative lack of efficacy of non-steroidal anti-inflammatory drugs in treating laminitis, as these drugs will only inhibit the enzymatic production of eicosanoids and will not affect the lipid peroxidation (enzyme-independent) mediated production of isoprostanes.

In summary, the present study represents the first step in determining the possible links between the inflammatory and vascular alterations that occur in, and may contribute to, the development of equine laminitis. The increases in plasma concentrations of thromboxane and laminar tissue concentrations of iso-$PGF_{2\alpha}$ are consistent with inflammation being a central component of the events leading to laminitis. These observations, coupled with the high degree of selectivity for laminar veins in terms of inducing sustained constrictor responses, are consistent with both enzymatic and non-enzymatic production of eicosanoids being a key axis in the development of this crippling condition. As such, they may represent viable targets for the development of more effective therapeutic strategies for equine laminitis.

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Table 3.1. Effects of SC-19220, AH 6809 or SQ 29,548 on contractile responses to U46619 in equine laminar veins and arteries of Control and BWHE horses.

			Veins				Arteries		
Horses	**Treatment**	**No.**	**Max (%T_K)**	**EC_{50} (nM)**	**Max:EC_{50} x 100**	**No.**	**Max (%T_K)**	**EC_{50} (nM)**	**Max:EC_{50} x 100**
Control									
	Saline	8	223 ± 16	13 ± 2	16.9 ± 1.7	10	54 ± 8	95 ± 8	57 ± 8
	SQ 29,548	10	-10 ± 4*	~0*	~0*	NA	ND	ND	ND
	SC-19220	9	194 ± 18	54 ± 8*	3.6 ± 0.4*	NA	ND	ND	ND
	AH 6809	10	140 ± 14*	214 ± 26*	0.65 ± 0.08*	NA	ND	ND	ND
BWHE									
	Saline	9	162 ± 18†	38 ± 5†	4.3 ± 0.6†	11	63 ± 9	55 ± 8*	115 ± 18*
	SQ 29,548	10	3 ± 1*	~0*	~0*	NA	ND	ND	ND
	SC-19220	10	142 ± 17	72 ± 8*	2.0 ± 0.3*	NA	ND	ND	ND
	AH 6809	10	16 ± 6*	~0*	~0*	NA	ND	ND	ND

Mean ± SEM, within group comparisons: *$P < 0.05$, treatments *versus* saline. Between group comparisons: $^{\dagger}P < 0.05$, BWHE *versus* Control
Max = Maximal contraction. $\%T_K$ = Maximal response expressed as a percentage of the maximal contractile response to KCl-PSS. NA = Not applicable. ND = not determined.

Table 3.2. Effects of SC-19220, AH 6809 or SQ 29,548 on contractile responses to iso-PGE_2 in equine laminar veins and arteries of Control and BWHE horses.

			Veins				Arteries		
Horses	**Treatment**	**No.**	**Max (%T_K)**	**EC_{50} (nM)**	**Max:EC_{50} x 100**	**No.**	**Max (%T_K)**	**EC_{50} (nM)**	**Max:EC_{50} x 100**
Control									
	Saline	8	108 ± 7	288 ± 19	37 ± 4	10	10 ± 4	~356	~2.8
	SQ 29,548	10	-3 ± 2*	~0*	~0*	NA	ND	ND	ND
	SC-19220	9	46 ± 8*	3008 ± 423*	1.5 ± 0.2*	NA	ND	ND	ND
	AH 6809	10	24 ± 6*	3396 ± 672*	0.7 ± 0.2*	NA	ND	ND	ND
BWHE									
	Saline	9	98 ± 9	174 ± 15†	55 ± 7†	11	21 ± 5	832 ± 96	2.4 ± 0.5
	SQ 29,548	10	5 ± 3*	~0*	~0*	NA	ND	ND	ND
	SC-19220	10	69 ± 9*	933 ± 88*	7.2 ± 0.8*	NA	ND	ND	ND
	AH 6809	10	54 ± 6*	3467 ± 468*	0.43 ± 0.06*	NA	ND	ND	ND

Mean ± SEM, within group comparisons: *$P < 0.05$, treatments *versus* saline. Between group comparisons: †$P < 0.05$, BWHE *versus* Control
Max = Maximal contraction. $\%T_K$ = Maximal response expressed as a percentage of the maximal contractile response to KCl-PSS. NA = Not applicable. ND = not determined.

Table 3.3. Effects of SC-19220, AH 6809 or SQ 29,548 on contractile responses to iso-$PGF_{2\alpha}$ in equine laminar veins and arteries of Control and BWHE horses.

			Veins				Arteries		
Horses	**Treatment**	**No.**	**Max (%T_K)**	**EC_{50} (nM)**	**Max:EC_{50} x 100**	**No.**	**Max (%T_K)**	**EC_{50} (nM)**	**Max:EC_{50} x 100**
Control									
	Saline	10	64 ± 8	1413 ± 218	4.3 ± 0.6	10	16 ± 6	2818 ± 546	0.54 ± 0.14
	SQ 29,548	10	-41 ± 7*	794 ± 89*	-5.2 ± 0.9*	NA	ND	ND	ND
	SC-19220	10	111 ± 11*	1432 ± 163	7.5 ± 0.7*	NA	ND	ND	ND
	AH 6809	10	-14 ± 2*	251 ± 36*	-5.1 ± 0.7*	NA	ND	ND	ND
BWHE									
	Saline	10	98 ± 8†	912 ± 89†	11.2 ± 2.2†	10	22 ± 7	2570 ± 644	0.83 ± 0.22
	SQ 29,548	10	6.0 ± 1.9*	6314 ± 1999*	0.10 ± 0.03*	NA	ND	ND	ND
	SC-19220	10	55 ± 8*	3304 ± 361*	1.7 ± 0.2*	NA	ND	ND	ND
	AH 6809	10	4.6 ± 1.2*	9562 ± 948*	0.05 ± 0.01*	NA	ND	ND	ND

Mean ± SEM, within group comparisons: *$P < 0.05$, treatments *versus* saline. Between group comparisons: †$P < 0.05$, BWHE *versus* Control
Max = Maximal contraction. $\%T_K$ = Maximal response expressed as a percentage of the maximal contractile response to KCl-PSS. NA = Not applicable. ND = not determined.

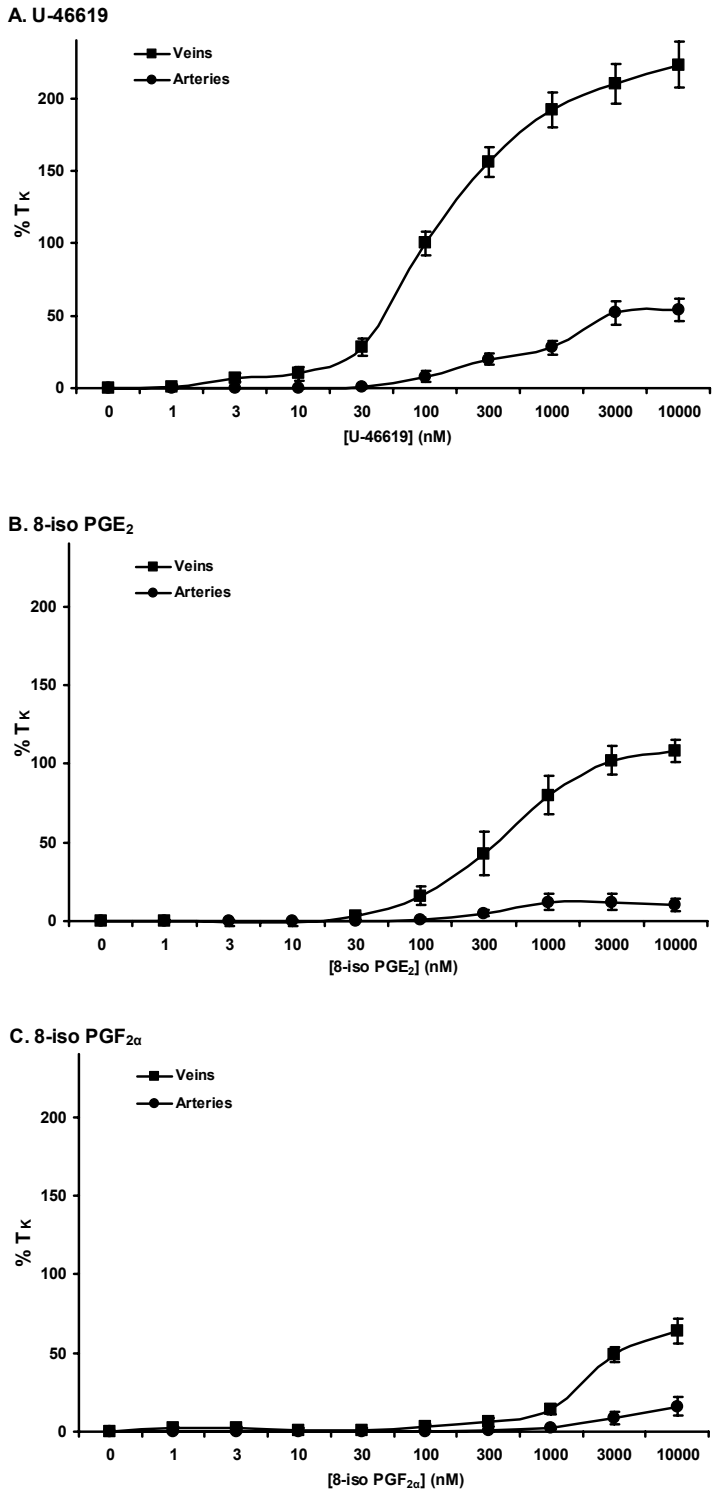

Figure 3.1. Mean ± SEM responses of laminar veins (■) and arteries (●) to increasing concentrations of U46619 (Panel A), iso-PGE_2 (Panel B) or iso-$PGF_{2\alpha}$ (Panel C).

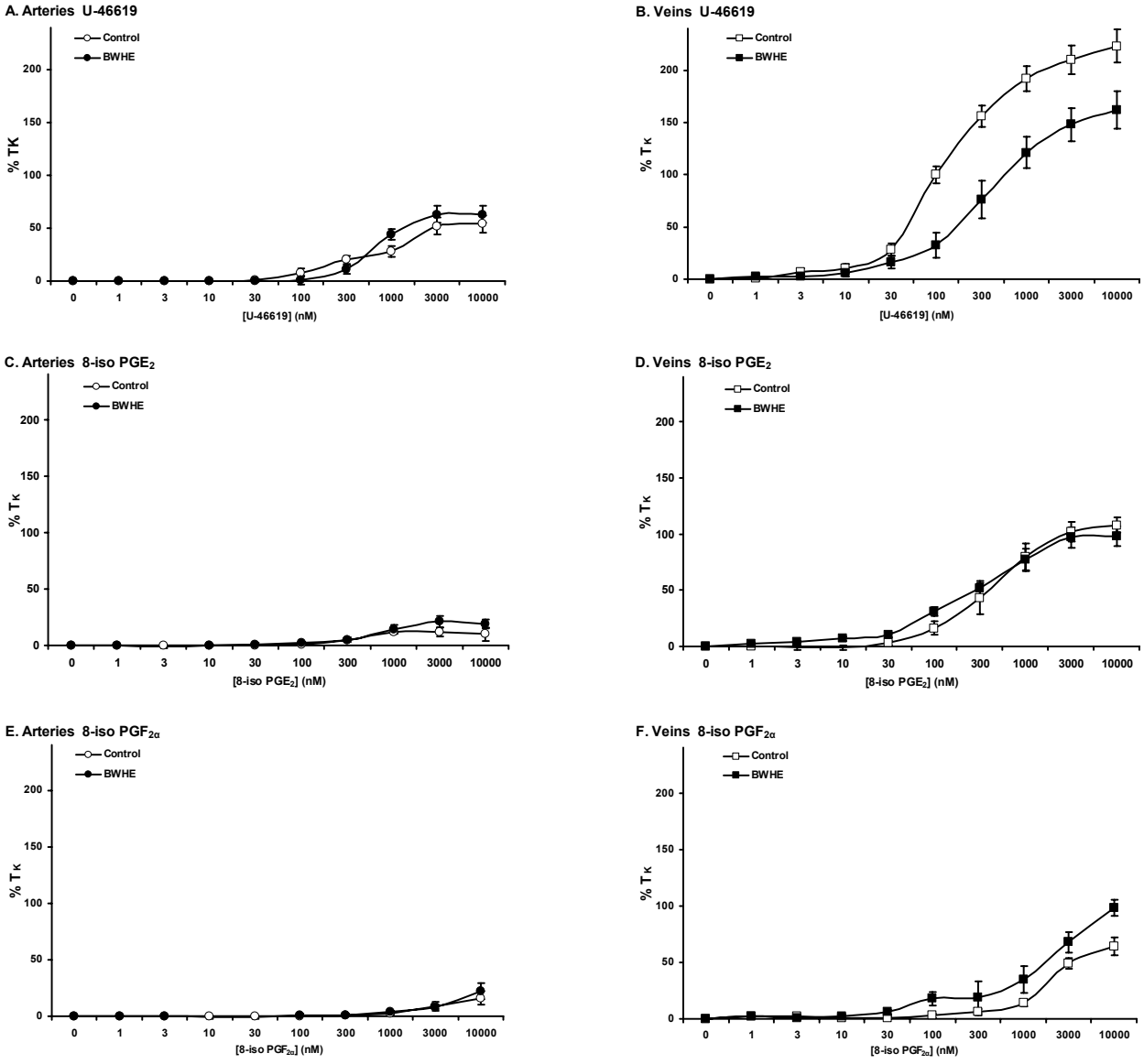

Figure 3.2. Mean ± SEM responses of control laminar veins (□), control laminar arteries (○), BWHE laminar veins (■) and BWHE laminar arteries (●) to increasing concentrations of U46619 (Panels A and B), iso-PGE_2 (Panels C and D) or iso-$PGF_{2\alpha}$ (Panel E and F).

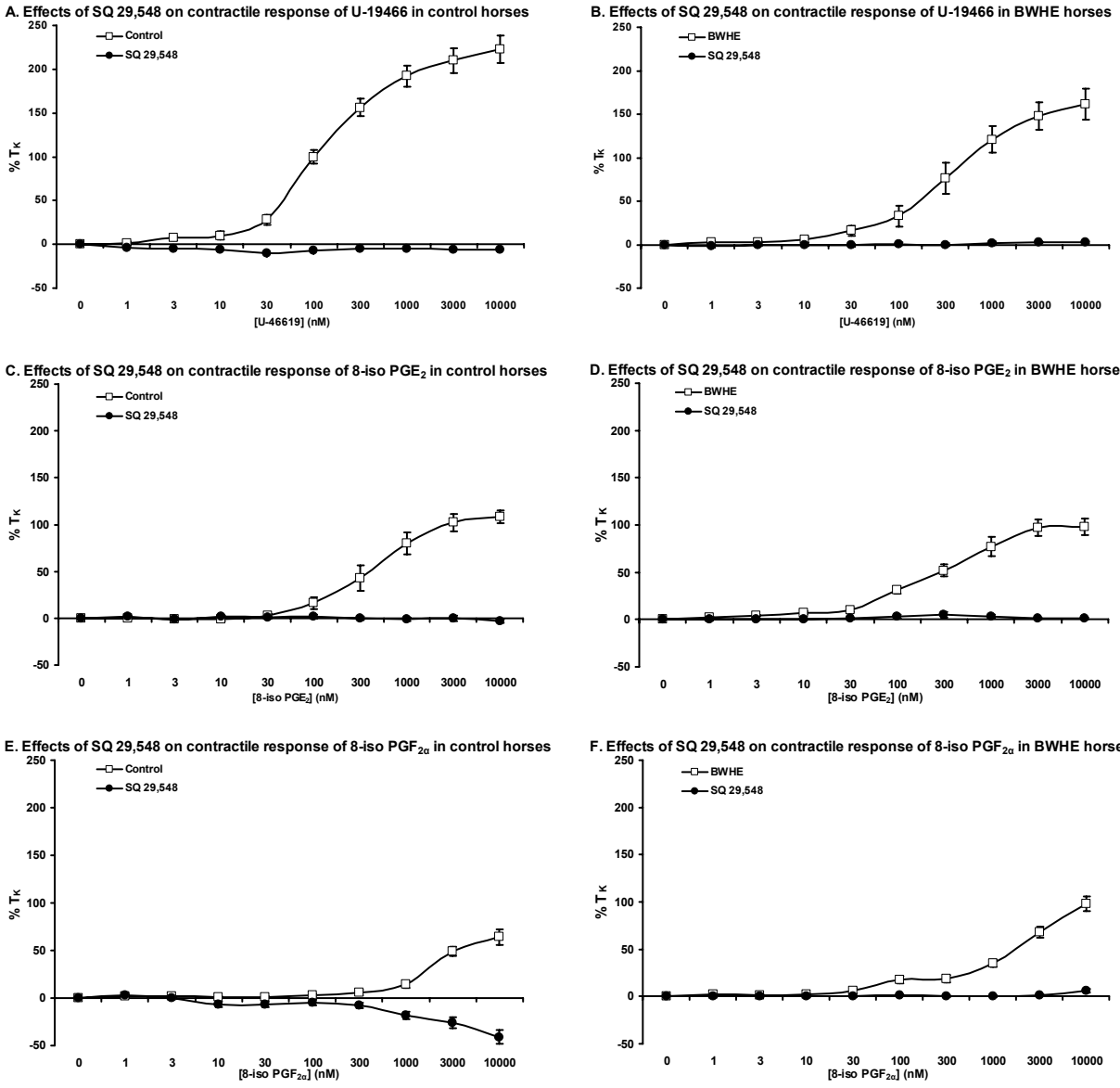

Figure 3.3. Mean ± SEM responses of laminar veins isolated from control (Panels A, C and E) or BWHE horses (Panels B, D and F) to increasing concentrations of U46619 (Panels A and B), iso-PGE_2 (Panels C and D) or iso-$PGF_{2\square}$ (Panel E and F) either in the absence (□) or presence (●) of SQ 29,548.

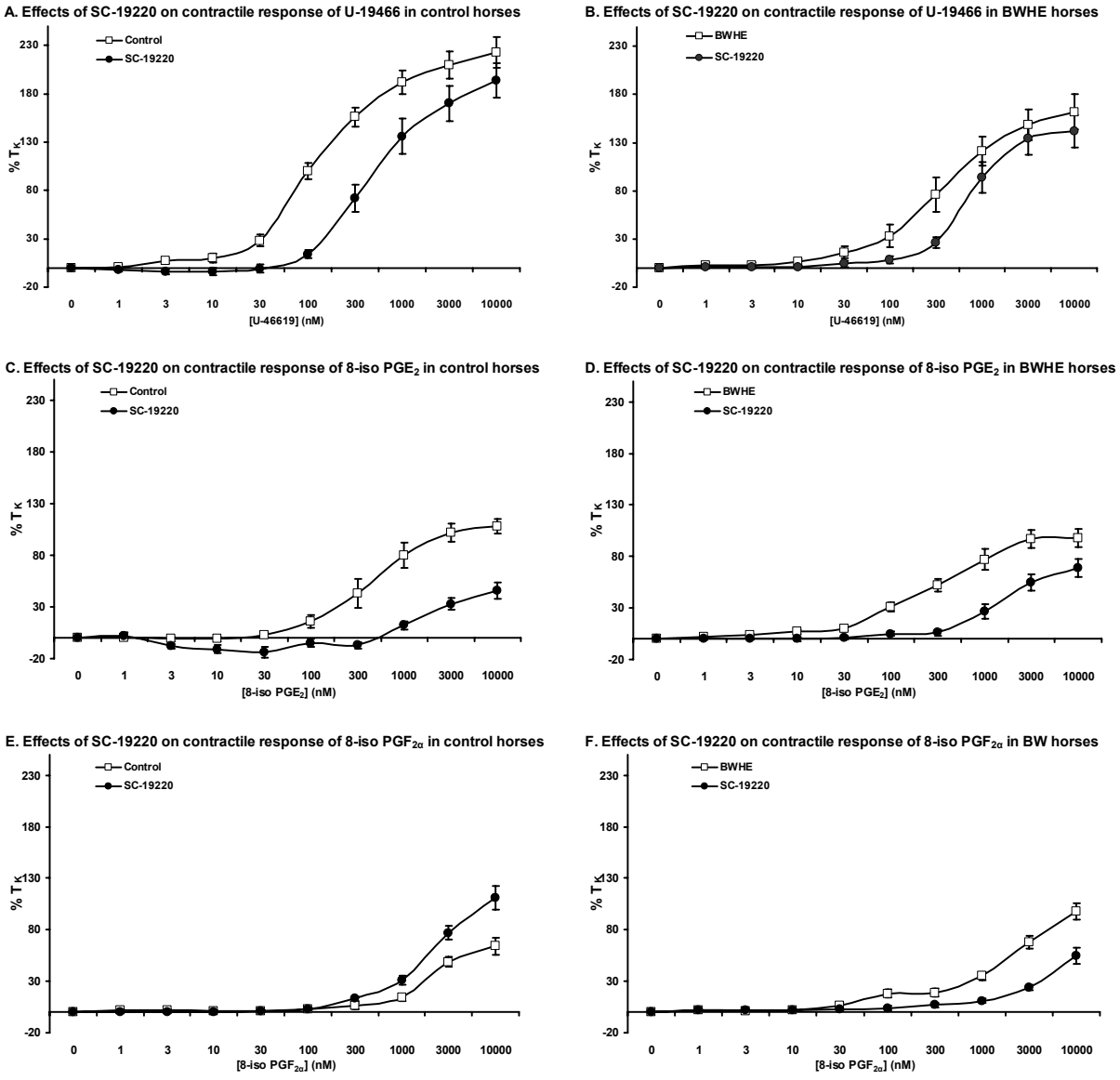

Figure 3.4. Mean ± SEM responses of laminar veins isolated from control (Panels A, C and E) or BWHE horses (Panels B, D and F) to increasing concentrations of U46619 (Panels A and B), iso-PGE_2 (Panels C and D) or iso-$PGF_{2\alpha}$ (Panel E and F) either in the absence (□) or presence (●) of SC19220.

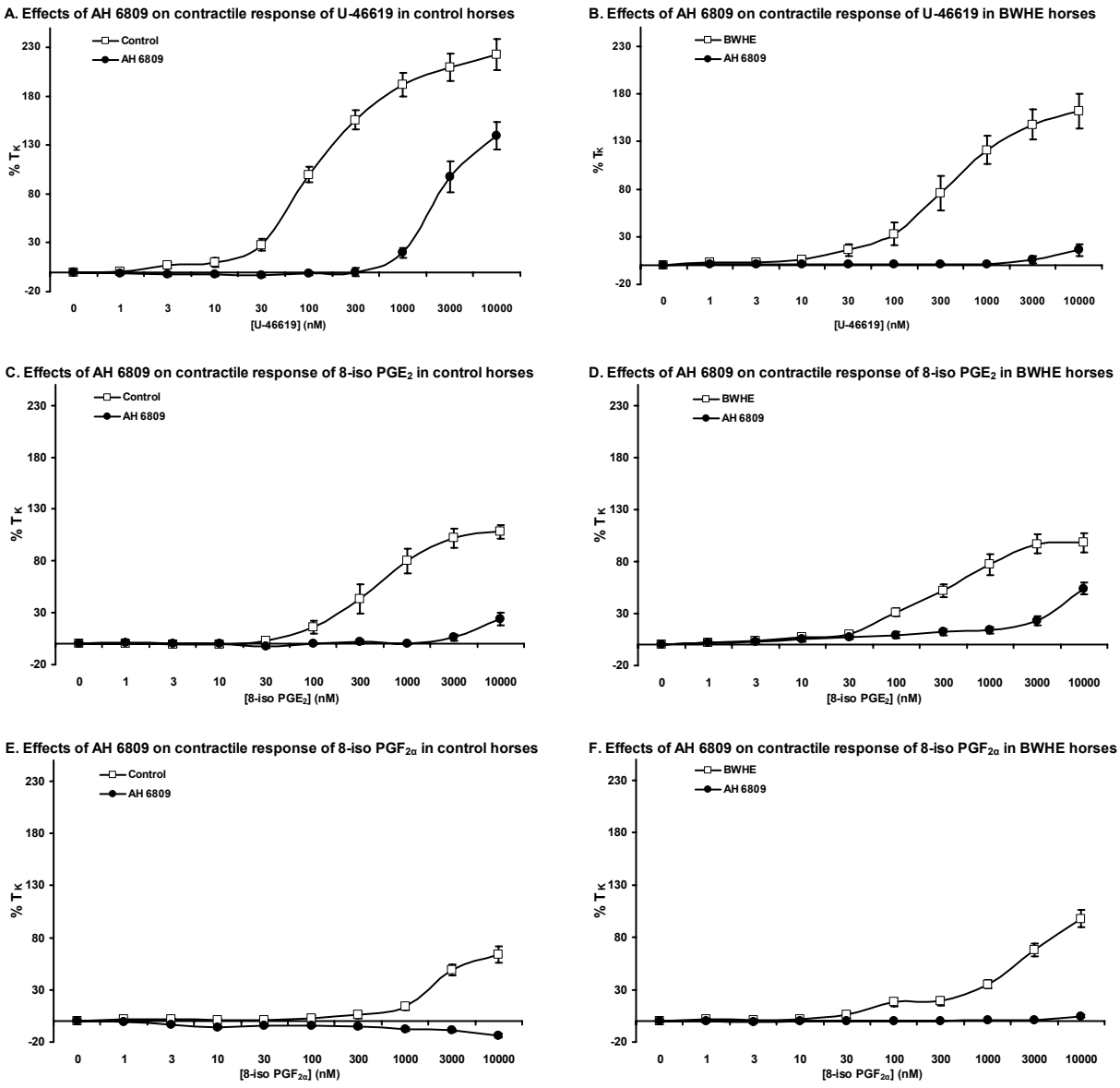

Figure 3.5. Mean ± SEM responses of laminar veins isolated from control (Panels A, C and E) or BWHE horses (Panels B, D and F) to increasing concentrations of U46619 (Panels A and B), iso-PGE_2 (Panels C and D) or iso-$PGF_{2\square}$ (Panel E and F) either in the absence (□) or presence (●) of AH 6809.

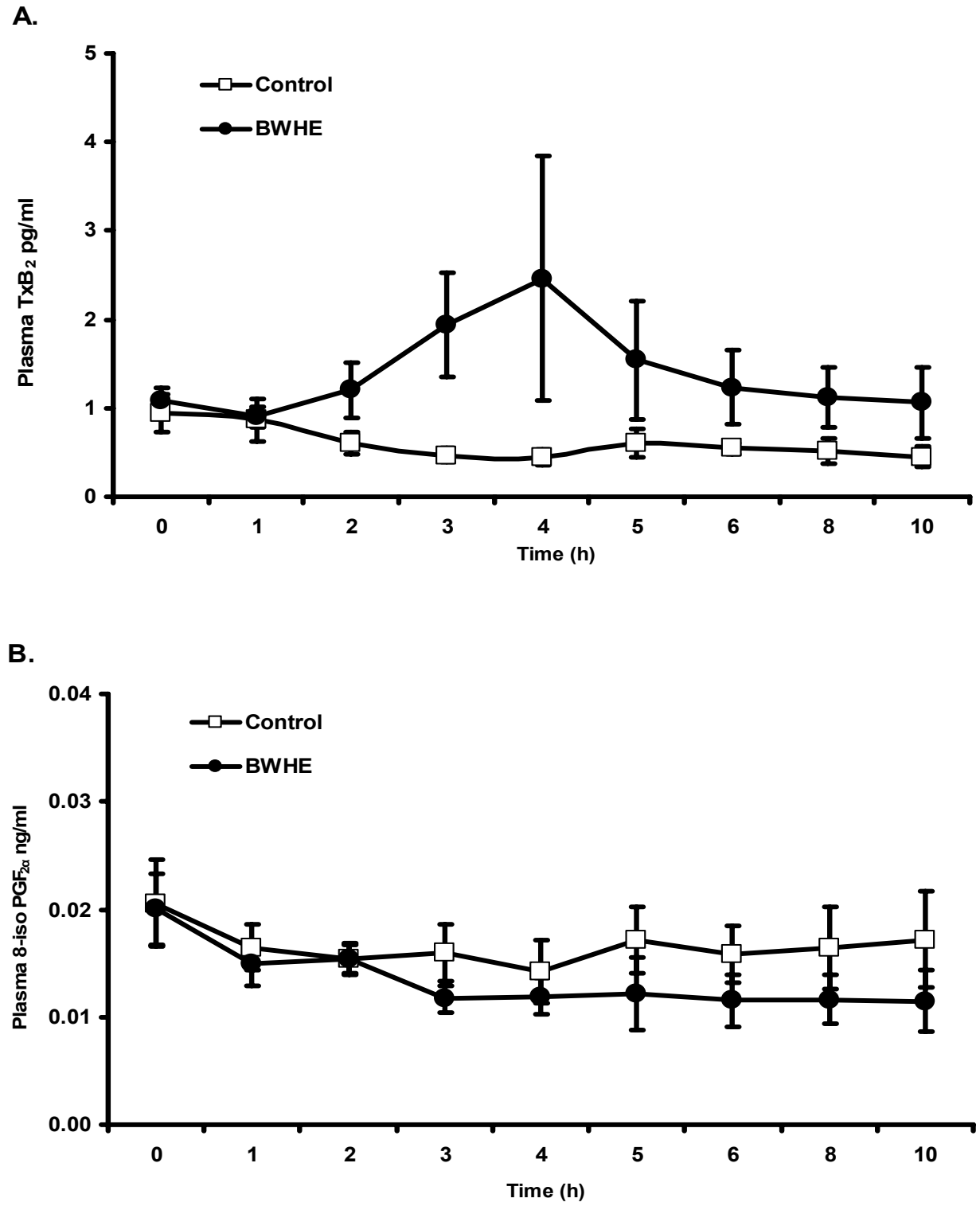

Figure 3.6. Mean ± SEM concentrations of plasma TxB_2 (Panel A) and iso-$PGF_{2\alpha}$ (Panel B) in control (□) or BWHE-administered (●) horses.

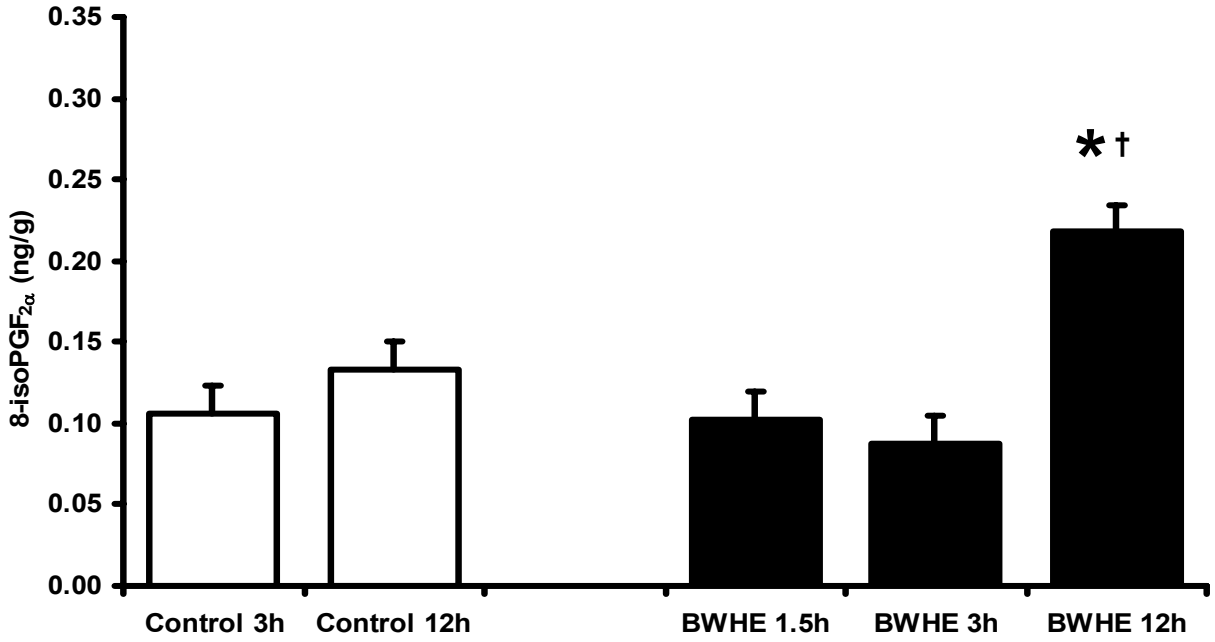

Figure 3.7. Mean ± SEM laminar tissue concentrations iso-$PGF_{2\alpha}$ in control (□) or BWHE-administered (■) horses. * $P<0.05$ vs. control; † $P<0.05$ vs. BWHE 3h and BWHE 1.5h.

CHAPTER 4

EICOSANOIDS AND BLACK WALNUT HEARTWOOD EXTRACT INDUCED LAMINITIS: II. PROSTAGLANDIN $F_{2\alpha}$

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ABSTRACT

The prodromal stages of equine laminitis are associated with perturbations in inflammatory and vascular homeostasis. The aim of this study was to provide initial insights into the possible role that the eicosanoid, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), may play in the developmental stages of equine laminitis induced by ingestion of black walnut heartwood extract (BWHE). Horses were randomly divided into two groups, either control or BWHE-administered groups, and were euthanized at either 12 hours (for the control group) or Obel grade 1 laminitis (for the BWHE group). Laminar tissue was collected for the isolation of laminar arteries and veins (200-800 micron internal diameter) and their function determined using small vessel myographs. Concentration responses to cumulative additions of $PGF_{2\alpha}$ were established either in the absence or presence of putative prostanoid and thromboxane receptor antagonists, SQ 29,548, SC-19220 and AH 6809. Plasma concentrations of $PGF_{2\alpha}$ increased transiently in the horses administered BWHE at approximately the same point at which there was a decrease in white blood cell count. In laminar vessels from control horses, $PGF_{2\alpha}$ was a potent contractile agonist for laminar veins. In contrast, laminar arteries from control horses were virtually unresponsive to $PGF_{2\alpha}$. $PGF_{2\alpha}$ was similarly selective for laminar veins over laminar arteries in BWHE horses, however the magnitude of $PGF_{2\alpha}$-induced venoconstriction was less than that observed in laminar veins from control horses. In the presence of SQ 29,548, laminar veins from control horses responded to $PGF_{2\alpha}$ with a small dilation, whereas laminar veins from BWHE horses did not dilate under these conditions. The results of the present study are consistent with systemic inflammation being a facet of the prodromal stages of BWHE-induced laminitis. Because plasma concentrations of $PGF_{2\alpha}$ increased markedly during the development of BWHE-induced

laminitis and $PGF_{2\alpha}$ selectively constricted laminar veins, $PGF_{2\alpha}$ may play a role in both the inflammatory and vascular dysfunction that is apparent in the prodromal stages of laminitis. It is possible, therefore, that specific prostanoids, such as $PGF_{2\alpha}$, may be viable targets for the development of more effective therapeutic regimens for the treatment of equine laminitis.

INTRODUCTION

The pathogenesis of equine laminitis is multi-factorial and involves perturbations in both inflammatory and vascular homeostasis within the laminar dermis (Eades et al., 2007; Fontaine et al., 2001; Waguespack et al., 2004). Eicosanoids are lipid-derived mediators that are widely distributed in mammalian tissues (Bergstroem, 1966) and have wide-ranging biological actions with respect to inflammatory and cardiovascular regulation (Egan and FitzGerald, 2006; Gerritsen, 1996; Parente and Perretti, 2003). Eicosanoids, therefore, may contribute to etiology of equine laminitis. Indeed, recent evidence suggests that the enzymes responsible for eicosanoid production are up-regulated during the prodromal stages of this condition (Blikslager et al., 2006; Waguespack et al., 2004). The aims of the present study were a) to provide initial insights into the possible roles of eicosanoids in the development of laminitis, b) to provide data regarding the temporal aspects of eicosanoid production, and c) to characterize the vasoactive effects of eicosanoids on the functionally vital small laminar arteries and veins of the equine digit.

In vitro studies, performed by Baxter *et al* (Baxter et al., 1989) determined that conduit digital arteries and veins, isolated from horses with early-stage laminitis, were less responsive to $PGF_{2\alpha}$ and U-46619 than were the corresponding vessels from healthy horses. Similarly,

maximal contractions of digital arteries and veins from horses with early laminitis were less than those for vessels from healthy horses in response to serotonin and norepinephrine, stimulating those authors to suggest that the different responses in the horses with acute laminitis may be due to receptor down regulation, desensitization or endothelial damage (Baxter et al., 1989). However, the significance of conduit vessel function to the local regulation of blood flow within the laminar tissues in the digit may be limited, and it is likely that more pertinent data may be obtained by examining the small arteries and veins that are important in the minute to minute regulation of pre- and post-capillary resistances. Peroni *et al* (Peroni et al., 2006) recently reported that $PGF_{2\alpha}$ elicits profound contractile responses in small laminar veins, whereas similar-sized arteries from the same region of the laminar dermis were virtually unresponsive to $PGF_{2\alpha}$. Furthermore, Peroni *et al* (Peroni et al., 2006) suggested that the veno-selective properties of $PGF_{2\alpha}$ and other vasoactive factors, such as serotonin and endothelin-1, might explain why diverse systemic diseases can result in laminitis. Peroni *et al* (Peroni et al., 2006) argued that systemic conditions characterized by endotoxemia and increased circulating levels of inflammatory mediators, such as $PGF_{2\alpha}$, may lead to venoconstriction in the laminar vascular bed of the horse, thereby predisposing the development of laminitis via an increase in post-capillary resistance.

No information currently exists regarding the expression or function of FP receptors within the laminar dermis. Due to structural similarities among eicosanoids, delineation of specific agonist-receptor interactions in tissues may not be straightforward. For example, PGE_2 and $PGF_{2\alpha}$ are structurally identical except at the C-9 position in the cyclopentane ring where PGE_2 has a keto substituent and $PGF_{2\alpha}$ has a hydroxyl group. It is not surprising, therefore, that PGE_2 activates FP receptors and that $PGF_{2\alpha}$ activates prostaglandin E (EP) receptors (Breyer et

al., 2001). Indeed, based on radioligand binding studies, PGE_2 is only 30-fold less potent than $PGF_{2\alpha}$ at the human FP receptor (Abramovitz et al., 1994), and there is evidence that FP and EP_1 receptors mediate $PGF_{2\alpha}$ and PGE_2 induced expression of IL-1β in a progenitor testicular cell line (Walch et al., 2003). Furthermore, $PGF_{2\alpha}$ binds to EP receptors (Christenson et al., 1994), both $PGF_{2\alpha}$ and PGE_2 constrict isolated rat aortic ring preparations via activation of the thromboxane (TP) receptor (Dorn et al., 1992), and $PGF_{2\alpha}$ also activates EP_1, EP_3 and thromboxane (TP) receptors in the gastrointestinal tract (Okada et al., 2000).

The aim of the present study was to provide insights into the possible role of $PGF_{2\alpha}$ in the development of laminitis. Specifically, we determined whether plasma concentrations of $PGF_{2\alpha}$ are increased during the prodromal stages of laminitis, and compared the vasoactive effects of $PGF_{2\alpha}$ in laminar arteries and veins isolated from control horses or those with Obel grade 1 laminitis induced by administration of BWHE. We also report here the effects of currently available thromboxane and prostanoid receptor antagonists on the responses of equine laminar vessels to $PGF_{2\alpha}$.

MATERIALS AND METHODS

Ten mixed breed horses ranging in age from 4 to 12 years old (mean 9 years) were used in this study. Each horse lacked clinical evidence of lameness and survey radiographs of the forelimb digits were within normal limits. Horses were randomly assigned to control or black walnut heartwood extract (BWHE) groups. Horses in the control group (5 horses) received 6 L of water via nasogastric tube, and were euthanatized after the collection of the final (12 hour) blood sample. Horses in the BWHE group (5 horses) received BWHE via nasogastric tube and were

euthanatized at the onset of Obel grade 1 laminitis (clinical signs consisting of weight shifting and bounding digital pulses without evidence of lameness at a walk) or at 12 hours after intubation, if signs of Obel grade 1 laminitis had not developed by that time. Each horse was evaluated prior to intubation and every hour thereafter for attitude, heart rate, respiratory rate, capillary refill time, hoof temperature, digital pulses, and evidence of lameness consistent with Obel grade 1 laminitis. Blood samples were obtained via a jugular catheter at 0, 1, 2, 3, 4, 6, 8, 10, and 12 hours. Catheter patency was maintained by flushing the catheter with 20 ml heparinized saline (5 IU heparin/ml) after each blood collection. All protocols were approved by the University of Georgia Institutional Animal Care and Use Committee. The horses were euthanatized using a penetrating captive bolt, as approved by the Report of the American Veterinary Medical Association's Panel on Euthanasia (AVMA, 2001).

The blood samples were aliquoted into three ice chilled vacuum-evacuated tubes containing EDTA, one of which was used for determination of the peripheral WBC count. The other samples were placed on ice for 10 minutes and then immediately centrifuged at 400 x g for 10 minutes at 4 ºC. Plasma from the latter tubes was frozen at -80ºC until assayed for $PGF_{2\alpha}$ concentrations. Plasma concentrations of $PGF_{2\alpha}$ were determined using a commercially available enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI).

Isolation of laminar vessels

Laminar arteries and veins were isolated as described previously (Peroni et al., 2005; Peroni et al., 2006; Robertson et al., 2005). Briefly, two full thickness segments of the dorsal hoof were placed in ice-cold physiological salt solution (PSS) containing (in mM): NaCl 118; $NaHCO_3$ 24; $MgSO_4$ 1; NaH_2PO_4 0.435; glucose 5.56; $CaCl_2$ 1.8, and KCl 4; gassed with 21%

O_2 and 5% CO_2 (pH = 7.40 ± 0.01). On the stage of a high-powered microscope, the lamellar portion of the dermis was shaved until only a thin layer covered the laminar vascular bed. Laminar arteries and veins (2 - 3 cm distal to the coronary band, 200 - 800 μm internal diameter, 1-2 mm in length) were isolated using micro-fine surgical instruments and mounted on small vessel myographs (Model 500A, Danish Myo Technology, Denmark). The vessels were bathed in PSS and the bath temperature was raised to, and maintained at, 37°C for one hour while the vessels equilibrated. Laminar arteries and veins were then stretched to produce equivalent transmural pressures of 3.1kPa and 1.9kPA, respectively (Peroni et al., 2005). Data were collected for each agonist and, where appropriate, in the presence of specific antagonists, from at least 7 arteries and veins. The numbers of vessels and horses used to obtain the data in this study were based on our previous experience with isolated laminar arteries and veins (Peroni et al., 2005; Peroni et al., 2006; Robertson et al., 2005).

Experimental vessel protocols

All vessels were given three 2-minute exposures to 80 mM KCl-PSS (isotonic replacement of NaCl with KCl, KPSS), 15 minutes apart to establish the maximal contractile response to a depolarizing stimulus. Concentration response curves were then obtained for either $PGF_{2\alpha}$ (1 nM – 10 μM), or phenylephrine (PE, 1 nM – 100 μM) by cumulative addition of each agonist.

Similar experiments were performed in which vessels were pre-incubated with each of the following antagonists: SQ 29,548 (thromboxane receptor antagonist, 50μM), SC-19220 (prostaglandin EP_1 receptor antagonist, 50μM), or AH 6809 (prostaglandin EP_1, EP_2, EP_3 / DP_1 receptor antagonist, 50μM) 10 minutes prior to commencement of the appropriate concentration

response curve. The concentration of each antagonist was based upon published reports of their efficacy in isolated blood vessels (Daray et al., 2004; Sametz et al., 2000; Walch et al., 2001).

Sample preparation for enzyme immunoassay (EIA)

Prior to determining the concentrations of $PGF_{2\alpha}$, plasma was subjected to methanol extraction with 1 ml of plasma added to 9 ml of methanol. The samples were then centrifuged for 10 minutes at 400 x g, and the supernatant decanted and concentrated by vacuum centrifugation (Hetovac Vacuum Rotator model VR-1, Heto-Holten, Denmark). The concentrated supernatants were stored at −80ºC until reconstituted to a 2 x concentration in the EIA buffer immediately before performing the assay according to the manufacturer's instructions.

EIA for $PGF_{2\alpha}$

Plasma $PGF_{2\alpha}$ concentrations were determined using a commercially available EIA kit (Cayman Chemical, Ann Arbor, MI). Briefly, 50 µl of standard or extracted plasma samples were placed in a 96-well plate pre-coated with mouse monoclonal antibody. Thereafter, 50 µl of $PGF_{2\alpha}$ tracer and $PGF_{2\alpha}$ antiserum were added into each well and incubated for 18 h at room temperature. After washing with wash buffer, 200 µl of Ellman's reagent containing acetylcholinesterase was added. The plates were read at 412 nm and concentrations of $PGF_{2\alpha}$ were calculated from a standard curve generated with known concentrations of $PGF_{2\alpha}$. The detection limit of the assay was 11 pg/ml.

Data and statistical analyses

Data are presented as mean ± SEM. Contractile responses were calculated as a percentage of the maximal response to KPSS (% T_K) for each vessel. The data were analyzed by repeated measures analysis of variance (ANOVA). Differences between individual means were identified by Student's modified *t*-test using the Bonferroni correction for multiple comparisons between means using the error mean square term from the ANOVA. Plasma concentrations of $PGF_{2\alpha}$ were analyzed using an unpaired Student's t test to compare mean values for the control and BWHE horses. A value of $P < 0.05$ was deemed to be significant (Wallenstein et al., 1980; Winer, 1971).

RESULTS

Responses of laminar arteries and veins to $PGF_{2\alpha}$ or PE: Consistent with our previously published data (Peroni et al., 2006), laminar veins were significantly more sensitive to $PGF_{2\alpha}$, with respect to the concentration of agonist required to initiate contractile responses, and also constricted to a greater degree (when expressed as $\%T_K$) than laminar arteries (Figures 4.1-4.2 and Tables 4.1-4.3). Laminar veins of BWHE horses were significantly less sensitive to $PGF_{2\alpha}$ and PE than veins from control horses, while laminar arteries of BWHE horses were significantly more sensitive to $PGF_{2\alpha}$ than arteries from control horses.

Effects of SQ 29,548 on the contractile responses of laminar veins to $PGF_{2\alpha}$: Pre-incubation of laminar veins from control and BWHE horses with the thromboxane receptor antagonist SQ 29,548 (50μM) significantly reduced contractile responses to $PGF_{2\alpha}$ when

compared to responses of laminar veins exposed to $PGF_{2\alpha}$ alone (Figure 4.3). After pre-incubation of laminar veins from control horses with SQ 29,548, addition of $PGF_{2\alpha}$ resulted in slight dilation. In contrast, laminar veins isolated from horses administered BWHE and pre-incubated with SQ 29,548, did not dilate upon subsequent exposure to $PGF_{2\alpha}$. SQ 29,548 had no significant effect on vasoconstrictor responses of laminar veins isolated from either control or BWHE-administered horses to PE (Figure 4.3 and Tables 4.1-4.3).

Effects of SC-19220 on the contractile responses of laminar veins to $PGF_{2\alpha}$: Pre-incubation of laminar veins from control horses with the prostaglandin EP_1 receptor antagonist SC-19220 (50μM) significantly reduced maximal contractile responses to $PGF_{2\alpha}$ when compared to responses of laminar veins exposed to $PGF_{2\alpha}$ alone. The inhibitory effect of SC-19220 was much less pronounced in laminar veins isolated from BWHE-administered horses. SC-19220 had no significant on the vasoconstrictor responses of laminar veins isolated from either control or BWHE-administered horses to PE (Figure 4.4 and Tables 4.1-4.3).

Effects of AH 6809 on the contractile responses of laminar veins to $PGF_{2\alpha}$: Pre-incubation of laminar veins from control and BWHE horses with the prostaglandin EP_1, EP_2, EP_3 / DP_1 receptor antagonist AH 6809 (50μM) significantly reduced contractile responses to $PGF_{2\alpha}$ when compared to responses of laminar veins incubated with $PGF_{2\alpha}$ alone. AH 6809 caused a slight rightward shift in the PE concentration response curve in laminar veins from control horses, though the maximal response was not different from that observed in laminar veins exposed to PE alone. In contrast, AH 6809 significantly reduced the maximal response to PE in laminar veins isolated from BWHE-administered horses. (Figure 4.5 and Tables 4.1-4.3).

Plasma concentrations of $PGF_{2\alpha}$: Plasma concentrations of $PGF_{2\alpha}$ were significantly greater in the BWHE horses one hour prior the time when the peripheral leukocyte count was at its lowest point (Hurley et al., 2006) or had dropped by at least 30% (2-3 hours after administration of BWHE) when compared to the value for time 0 (Figure 4.6).

DISCUSSION

In the present study, we have determined that plasma concentrations of $PGF_{2\alpha}$ are increased during the prodromal stages of BWHE-induced laminitis. This increase in plasma $PGF_{2\alpha}$ was transient, with the peak in plasma $PGF_{2\alpha}$ concentrations coinciding with the nadir in white blood cell counts in these horses (Hurley et al., 2006). We also determined that laminar veins appear to be exquisitely sensitive to $PGF_{2\alpha}$, responding with robust and sustained constrictor responses whereas laminar arteries are virtually unresponsive to $PGF_{2\alpha}$. Taken together, these observations are consistent with the concept that the prodromal stages of laminitis involve a systemic inflammatory response that results in the production of inflammatory mediators, such as $PGF_{2\alpha}$, that may have profound effects upon the vasculature of the laminar dermis. Specifically, increases in circulating concentrations of eicosanoids may elicit venoconstriction within the digit thereby contributing to the pathogenesis of laminitis by increasing post-capillary resistance in the laminar dermis.

Laminar vessel contractile responses to $PGF_{2\alpha}$ or PE

$PGF_{2\alpha}$ elicited significantly greater contractile responses in laminar veins than in laminar arteries isolated from control horses or BWHE-administered horses. This veno-selective constrictor effect does not appear to be specific for $PGF_{2\alpha}$ as laminar veins have been reported to be generally more sensitive, and to constrict to a comparatively greater degree, than laminar arteries in response to a variety of physiologically-relevant agonists (Peroni et al., 2006). The inherent sensitivity of laminar veins to vasoactive agonists is consistent with the recently-proposed hypothesis that the equine digit may be pre-disposed to venoconstriction and, thereby, to the development of laminitis itself. The degree of constriction of laminar veins isolated from horses administered BWHE to $PGF_{2\alpha}$ and PE was significantly less than that measured in laminar veins isolated from control horses. This apparent reduction in efficacy mirrors that reported for serotonin and PE in laminar veins from BWHE-administered horses and is consistent with the latter report's finding of a dysfunction in laminar vein reactivity at Obel grade 1 laminitis. A similar reduction in maximal contractile responses to $PGF_{2\alpha}$ has also been reported in conduit digital arteries and veins isolated from horses in which Obel grade 1 laminitis was induced by carbohydrate overload (Baxter et al., 1989). However, the reduction in maximal contraction observed in digital arteries and veins was far greater (~80-90%) than that observed in laminar veins in the present study (~45%). Moreover, the EC_{50} values reported for conduit arteries and veins greatly exceeded those determined for laminar veins in the present study, indicating that laminar veins are far more sensitive to $PGF_{2\alpha}$ than are either digital arteries or veins. These observations, coupled with the fact that conduit digital arteries respond robustly to $PGF_{2\alpha}$ whereas laminar arteries do not (Baxter et al., 1989; Peroni et al., 2006) emphasize the importance of studying the physiologically relevant small laminar arteries and veins and the

potential problems that may arise from extrapolating data obtained with conduit vessels to the control of regional blood flow.

Effects of putative prostanoid receptor antagonists on laminar vessel responses to $PGF_{2\alpha}$ and PE

Purportedly, SQ 29,548 is a selective antagonist of the thromboxane (TP) receptor. In the present study, however, pre-incubation of laminar veins, isolated from either control horses or those with BWHE-induced Obel grade 1 laminitis, with SQ 29,548 ablated vasoconstrictor responses to $PGF_{2\alpha}$, while having no effect on responses to PE. These results suggest that $PGF_{2\alpha}$ may elicit its vasoconstrictor effects in laminar veins via activation of TP receptors. This finding is consistent with the results of another study in which $PGF_{2\alpha}$induced constriction of rat isolated aortic ring preparations via activation TP receptors (Dorn et al., 1992). However, it is also possible that SQ 29,548 may not be specific for TP receptors and part of its inhibitory action on $PGF_{2\alpha}$ may be due to non-specific blockade of other prostanoid receptors. The fact that SQ 29,548 did not reduce the contractile responses of laminar veins to PE in this study is, however, supportive of the antagonistic actions of this compound being confined to TP receptor activation.

In the presence of SQ 29,548, $PGF_{2\alpha}$ elicited a slight vasodilation in laminar veins isolated from control horses, whereas $PGF_{2\alpha}$ in the presence of the same antagonist induced a slight vasoconstriction in laminar veins isolated from BWHE-administered horses. These observations are consistent with the presence of a normal $PGF_{2\alpha}$-activatable vasodilator pathway in laminar veins that is compromised at Obel grade 1 laminitis and are similar to those we have recently reported for PGE_2, 11-deoxy-PGE_2 and for the isoprostane iso-$PGF_{2\alpha}$ (Noschka et al., 2007a, Noschka et al., 2007b). Because the primary aim of the current study was to

determine the vasoconstrictor effects of prostanoids, we have not addressed vasodilatory effects of these compounds. Future studies focusing in the vasodilator effects of eicosanoids may prove insightful with respect to the alterations in vascular function that occur in laminitis.

Pre-incubation of laminar veins from control horses with the putative prostaglandin EP_1 receptor antagonist, SC-19220, significantly reduced maximal contractile responses to $PGF_{2\alpha}$ by ~50%. In contrast, SC-19220 inhibited maximal contractile responses to $PGF_{2\alpha}$ in veins from BWHE horses to a much lesser extent. Together these results are consistent with the concept that contractile effects of $PGF_{2\alpha}$ are mediated, in part, via activation of EP1 receptors and that this pathway may be down-regulated in laminitis i.e., the lack of inhibition by SC-19220 in laminar veins isolated from BWHE horses may be due to down-regulation of these receptors. Ascribing eicosanoid actions to their respective classically-defined receptor subtypes should be done with caution as not only do PGE and $PGF_{2\alpha}$ activate their respective receptor counterparts (i.e., EP and FP receptors, respectively), but $PGF_{2\alpha}$ can also activate EP1, EP3 and TP receptors in some tissues (Okada et al., 2000). As with SQ 29,548, SC-19220 did not inhibit the contractile responses of laminar veins to PE, which is supportive of the effects of this compound being confined to eicosanoid receptors.

The putative EP_1, EP_2, EP_3 and DP_1 receptor antagonist, AH 6809, ablated contractile responses to $PGF_{2\alpha}$ in laminar veins isolated from both control and BWHE horses. However, this compound also markedly reduced the contractile responses of these vessels to PE, which raises concerns regarding the selectivity of this compound for eicosanoid receptors. The apparent lack of specificity of this compound renders interpretation of its effects on $PGF_{2\alpha}$ in laminar veins moot. Consequently, we feel that this compound should be used with caution. One obvious pharmacological intervention that we were not able to utilize in the current study was to block FP

receptors with specific receptor antagonists. Although putative FP-selective receptor antagonists have been described, they were not commercially available at the time of this study. However, our data would suggest that, similar to other vascular preparations, $PGF_{2\alpha}$ predominantly acts via TP receptors and that there may be minimal FP receptor population present in laminar veins and arteries.

In summary, the increase in plasma concentrations of $PGF_{2\alpha}$ during the prodromal stages of BWHE-induced laminitis is consistent with the occurrence of a systemic inflammatory response. The observation that equine laminar veins are exquisitely sensitive to $PGF_{2\alpha}$ is consistent with the concept that systemic inflammatory events in the horse may result in venoconstriction within the equine digit and that eicosanoids *per se* may act as conduits in these responses. The finding that a vasodilator pathway, which can be activated by $PGF_{2\alpha}$, is compromised at Obel grade laminitis is supportive of vascular perturbations within the laminar dermis being key events in the development of laminitis. It is hoped that the future development of more specific and chemically-distinct prostaglandin receptor antagonists will prove helpful in further delineating the roles of eicosanoids in the pathogenesis of this crippling condition.

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Table 4.1. Mean ± SEM effects of SC-19220, AH 6809 or SQ 29,548 on contractile responses to $PGF_{2\alpha}$ in equine laminar veins and arteries of Control and BWHE horses.

			Veins				Arteries		
Horses	**Treatment**	**No.**	**Max (%T_K)**	**EC_{50} (nM)**	**Max:EC_{50}**	**No.**	**Max (%T_K)**	**EC_{50} (nM)**	**Max:EC_{50}**
Control									
	Saline	8	112 ± 11	1585 ± 216	7.1 ± 0.8	10	27 ± 4	4444 ± 526	0.62 ± 0.12
	SQ 29,548	10	24 ± 6*	1175 ± 166	2.0 ± 0.5*	NA	ND	ND	ND
	SC-19220	9	96 ± 16	2377 ± 341*	4.1 ± 0.6*	NA	ND	ND	ND
	AH 6809	10	12 ± 4*	1096 ± 161	1.1 ± 0.3*	NA	ND	ND	ND
BWHE									
	Saline	9	194 ± 16†	1318 ± 266	14.7 ± 1.7†	11	50 ± 6*	6144 ± 529	0.81 ± 0.7
	SQ 29,548	10	-21 ± 6*	~0*	~0*	NA	ND	ND	ND
	SC-19220	10	104 ± 15*	1230 ± 204	8.5 ± 1.2*	NA	ND	ND	ND
	AH 6809	10	-8 ± 4*	~0*	~0*	NA	ND	ND	ND

Within group comparisons: *$P < 0.05$, treatments *versus* saline. Between group comparisons: $^{\dagger}P < 0.05$, Saline BWHE *versus* Control
Max = Maximal contraction. $\%T_K$ = Maximal response expressed as a percentage of the maximal contractile response to KCl-PSS. NA = Not applicable. ND = not determined.

Table 4.2. Mean ± SEM effects of SC-19220, AH 6809 or SQ 29,548 on contractile responses to PE in equine laminar veins and arteries of Control and BWHE horses.

			Veins				Arteries		
Horses	**Treatment**	**No.**	**Max (%T_K)**	**EC_{50} (nM)**	**Max:EC_{50}**	**No.**	**Max (%T_K)**	**EC_{50} (nM)**	**Max:EC_{50}**
Control									
	Saline	6	205 ± 18	186 ± 14	121 ± 15	NA	ND	ND	ND
	SQ 29,548	6	206 ± 14	196 ± 16	110 ± 9	NA	ND	ND	ND
	SC-19220	6	224 ± 12	173 ± 16	134 ± 16	NA	ND	ND	ND
	AH 6809	6	194 ± 14	468 ± 49*	41 ± 5*	NA	ND	ND	ND
BWHE									
	Saline	4	148 ± 18†	324 ± 46‡	46 ± 6†	NA	ND	ND	ND
	SQ 29,548	4	164 ± 15	355 ± 42	47 ± 5	NA	ND	ND	ND
	SC-19220	4	132 ± 14	407 ± 43	32 ± 5	NA	ND	ND	ND
	AH 6809	4	98 ± 13	479 ± 53	20 ± 6*	NA	ND	ND	ND

Within group comparisons: *$P < 0.05$, treatments *versus* saline. Between group comparisons: $^{\dagger}P < 0.05$, Saline BWHE *versus* Control
Max = Maximal contraction. %T_K = Maximal response expressed as a percentage of the maximal contractile response to KCl-PSS. NA = Not applicable. ND = not determined.

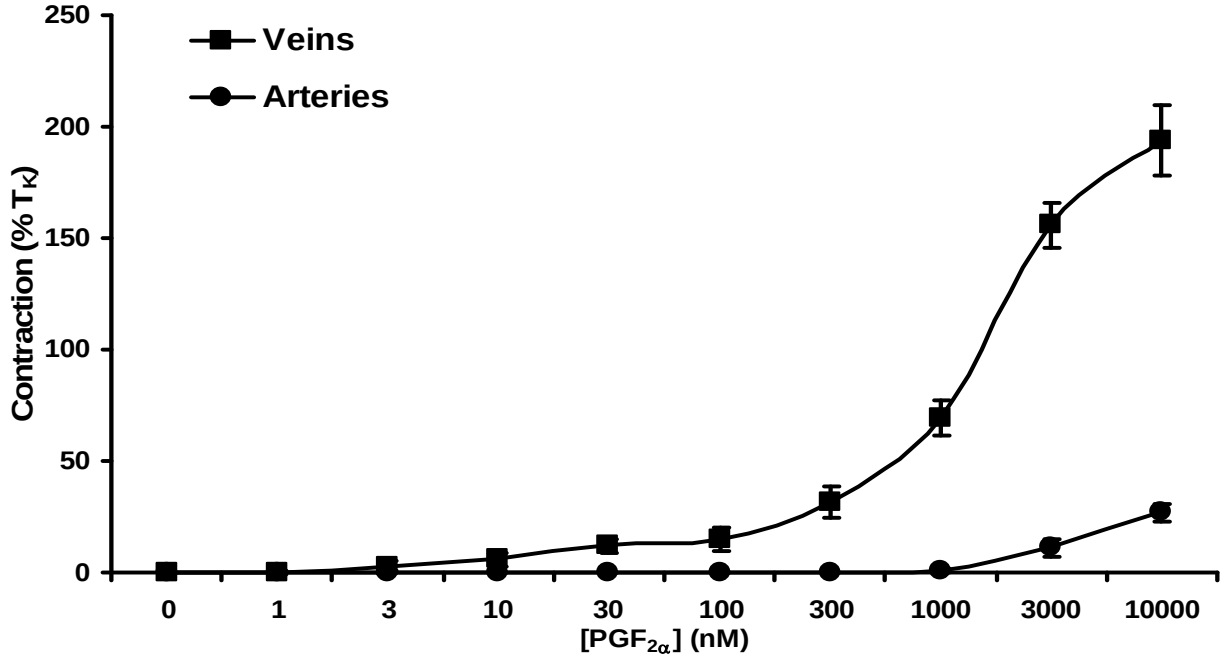

Figure 4.1. Mean ± SEM responses of laminar veins (■) and arteries (●) to increasing concentrations of $PGF_{2\alpha}$.

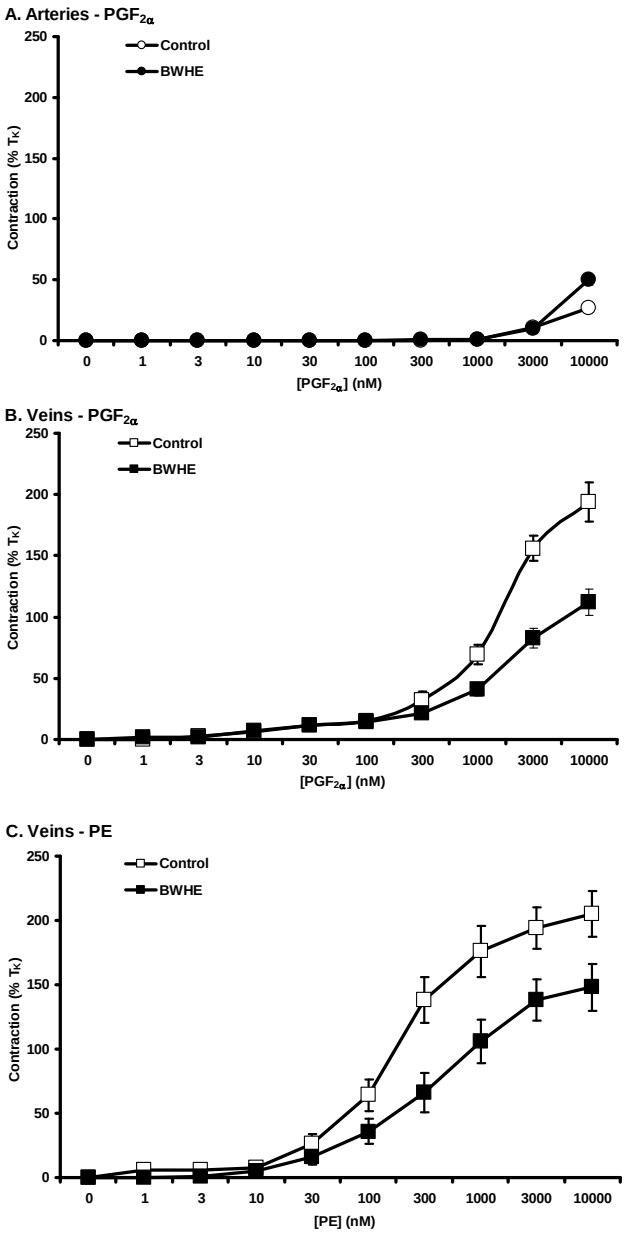

Figure 4.2. Mean ± SEM responses of: *Panel A*, control laminar arteries (○) and BWHE laminar arteries (●) to $PGF_{2\alpha}$; *Panel B*, control laminar veins (□) and BWHE laminar veins (■) to $PGF_{2\alpha}$, and *Panel C*, control laminar veins (□) and BWHE laminar veins (■) to PE.

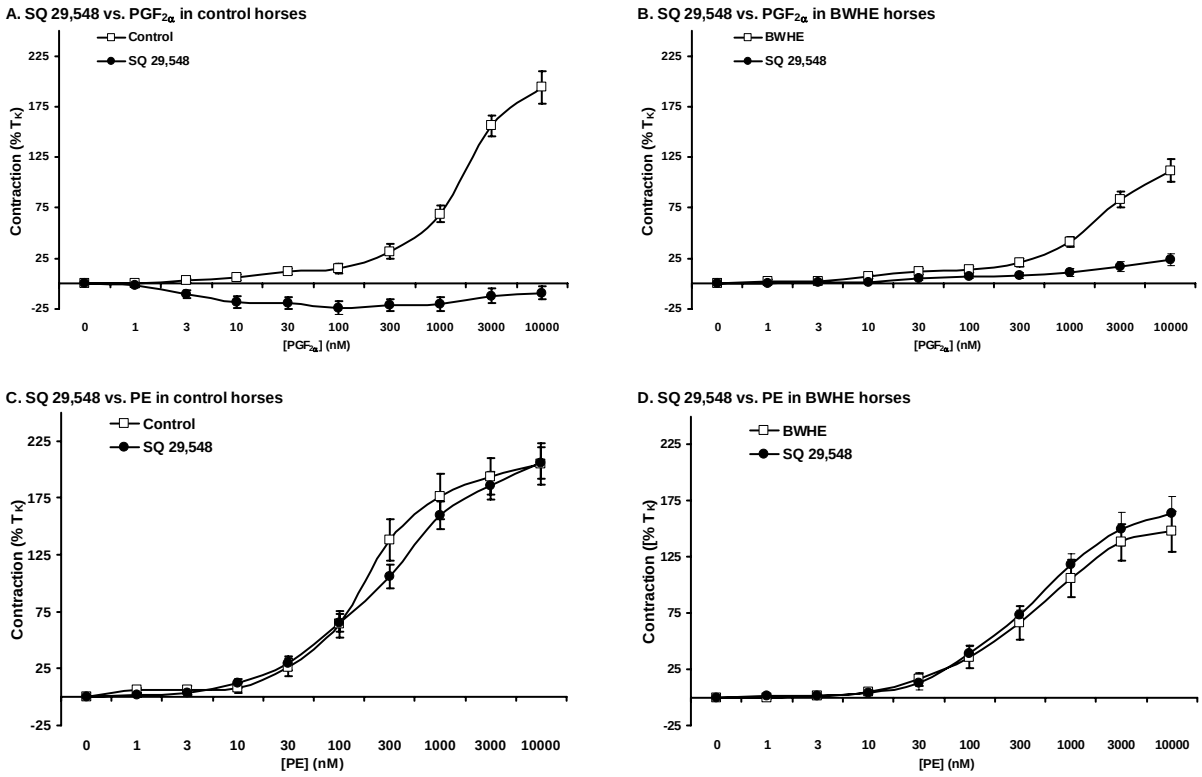

Figure 4.3. Mean ± SEM responses of laminar veins isolated from control (Panels A and C) or BWHE horses (Panels B and D) to increasing concentrations of PGF2□ (Panels A and B) or PE (Panels C and D) either in the absence (□) or presence (●) of SQ 29,548.

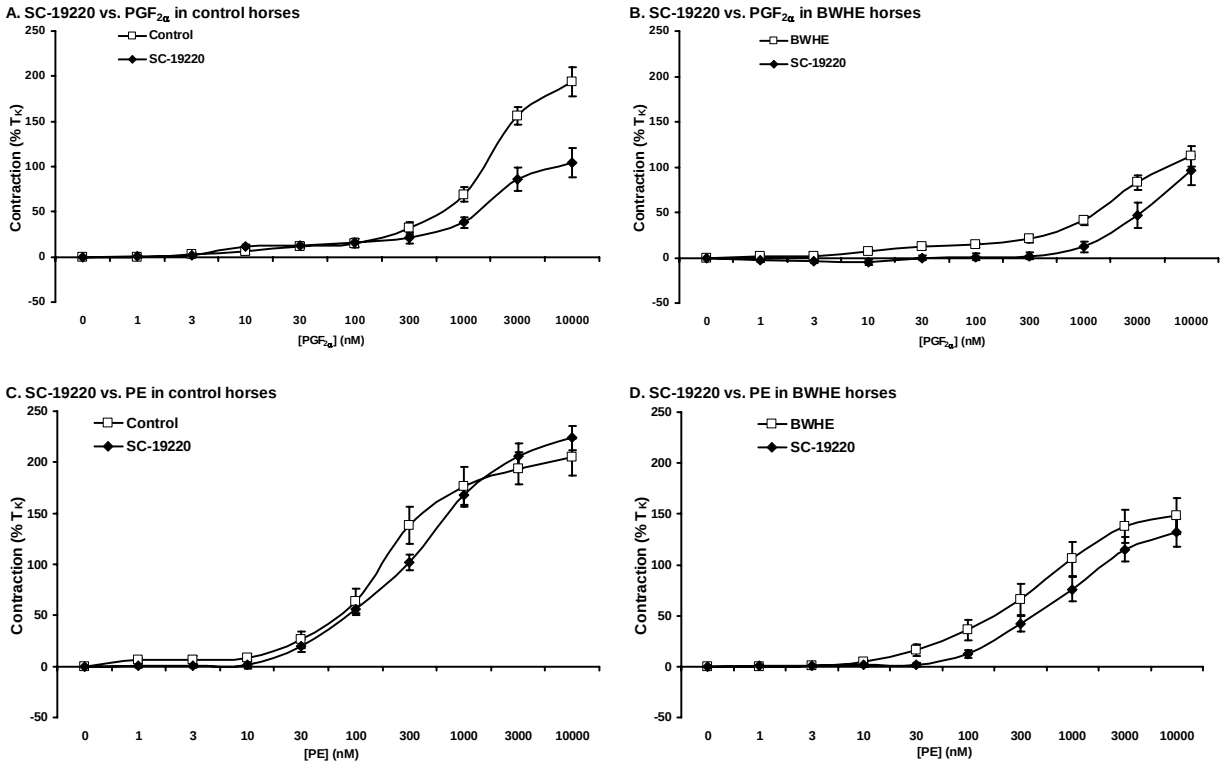

Figure 4.4. Mean ± SEM responses of laminar veins isolated from control (Panels A and C) or BWHE horses (Panels B and D) to increasing concentrations of $PGF_{2\alpha}$ (Panels A and B) or PE (Panels C and D) either in the absence (□) or presence (♦) of SC-19220.

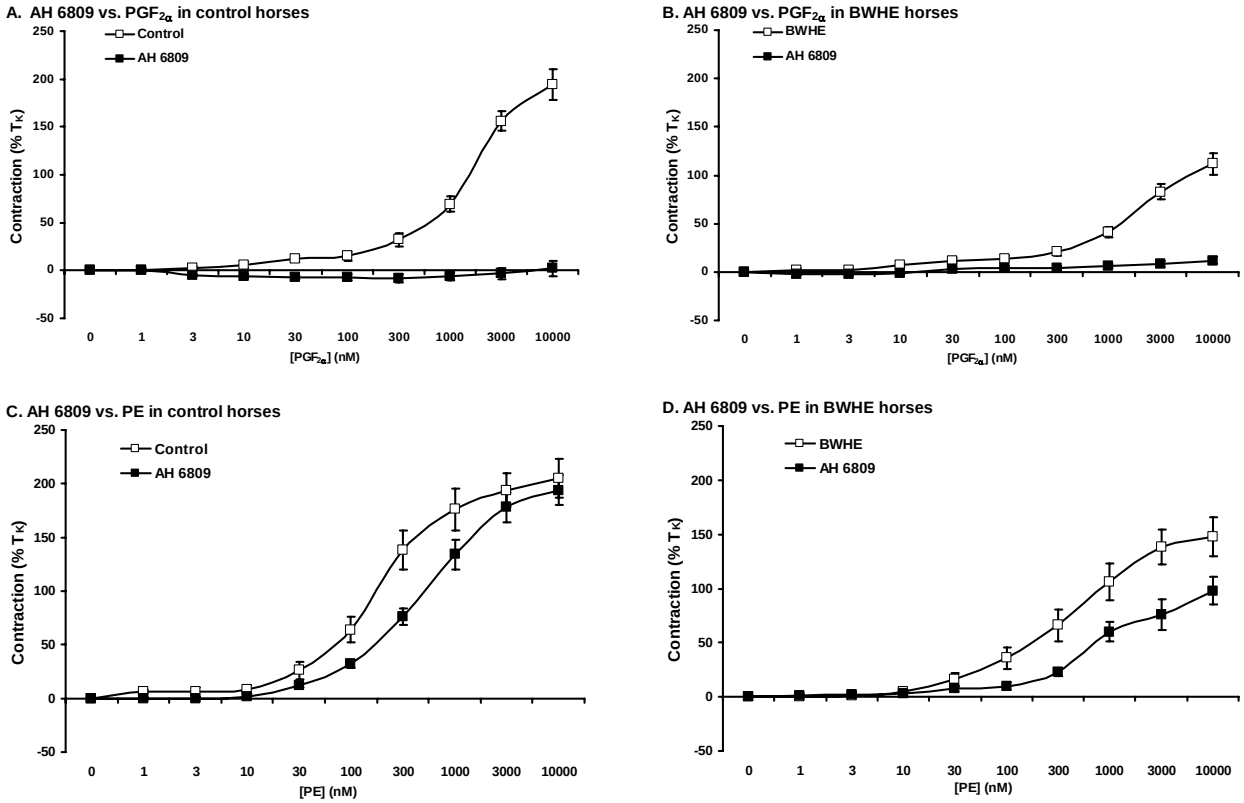

Figure 4.5. Mean ± SEM responses of laminar veins isolated from control (Panels A and C) or BWHE horses (Panels B and D) to increasing concentrations of $PGF_{2\alpha}$ (Panels A and B) or PE (Panels C and D) either in the absence (□) or presence (■) of AH 6809.

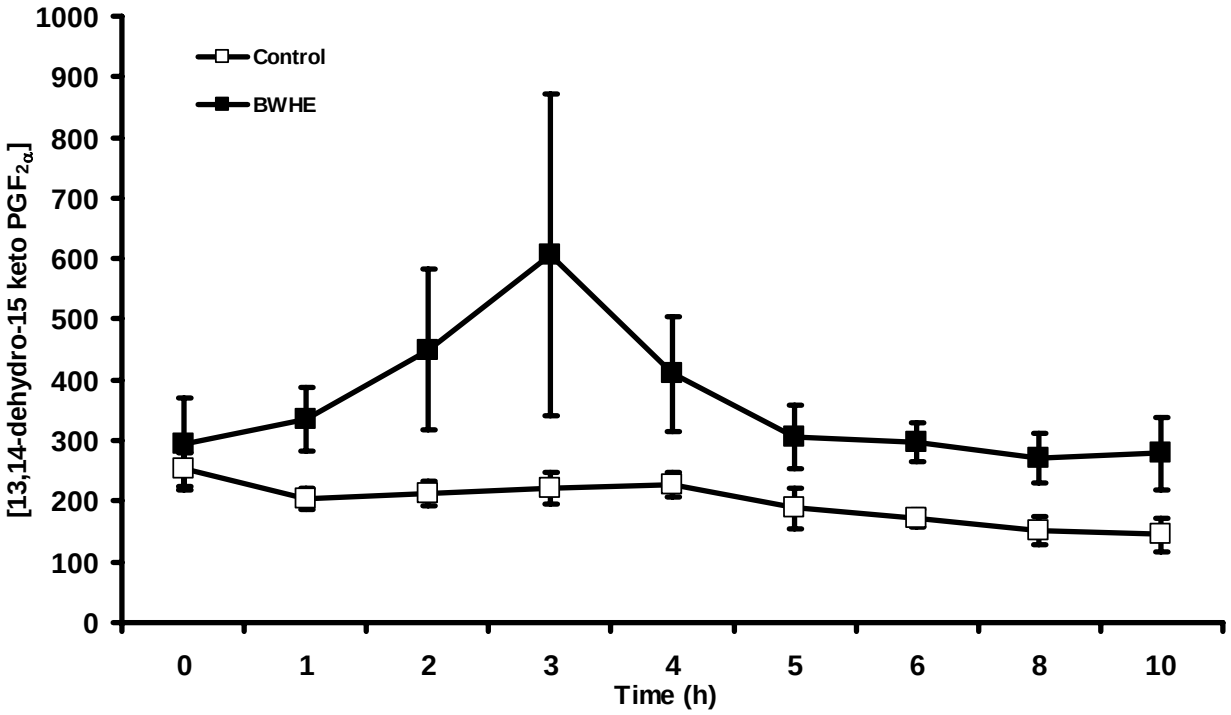

Figure 4.6. Mean ± SEM concentrations of plasma $PGF_{2\alpha}$ in control (□) or BWHE-administered (■) horses.

CHAPTER 5

EICOSANOIDS AND BLACK WALNUT HEARTWOOD EXTRACT INDUCED LAMINITIS: III. PROSTAGLANDIN E_2

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ABSTRACT

The possible links between the inflammatory and vascular events that occur during the prodromal stages of equine laminitis have yet to be determined. The aim of this study was to provide initial insights into the role that prostaglandin E_2 (PGE_2) may play in the development of equine laminitis induced by black walnut heartwood extract (BWHE). Horses were divided into two groups, either control or BWHE-administered horses. Blood samples were collected at scheduled times after administration of either water (control horses) or BWHE for determination of white blood cell counts and plasma concentrations of PGE_2.

At 12 hours (for the control group) or Obel grade 1 laminitis (for BWHE group) the horses were euthanized and laminar tissue collected for the isolation of laminar arteries and veins (200-800 micron internal diameter). Vasoconstrictor responses of laminar vessels to either PGE_2 or the PGE_2 analogues 11-d-PGE_2 or 17-pt-PGE_2 were determined using small vessel myographs. In some vessels, the effects of putative prostanoid and thromboxane receptor antagonists, SQ 29,548, SC-19220 and AH 6809, upon these contractile responses were determined.

Plasma concentrations of PGE_2 increased transiently and coincided with the nadir in white blood cell counts in horses administered BWHE. PGE_2 elicited small dilator responses in laminar veins isolated from control horses, whereas a small constrictor response was observed in response to PGE_2 in laminar veins from BWHE-administered horses. 11-d-PGE_2 elicited similarly robust contractile responses in laminar veins isolated from either control or BWHE horses. The responses of laminar veins to 11-d-PGE_2 were greater than those observed in laminar arteries and were abolished by pre-incubation with the thromboxane receptor antagonist, SQ 29,548. 17-pt-PGE_2 did not affect tone in either laminar veins or arteries.

In summary, the results of the present study are consistent with systemic inflammatory events occurring at an early stage in the pathogenesis of laminitis. The relative selectivity of eicosanoids *per se* for laminar veins, in terms of eliciting constrictor responses, supports the concept that these factors may be important conduits in the inflammatory and vascular perturbations evident in laminitis.

INTRODUCTION

Prostaglandin E_2 (PGE_2), which is formed by PGE synthase-dependent isomerization of PGH_2, plays key roles in numerous physiological processes such as inflammation and the regulation of vascular tone (Breyer and Breyer, 2000; Reiss and Edelman, 2006). To date, no studies exist that have examined the possible role of this key eicosanoid in the development of equine laminitis.

PGE_2 exerts its autocrine/paracrine effects on target cells by coupling to four subtypes of G-protein-coupled receptors, which have been classified as EP_1, EP_2, EP_3, and EP_4 (Coleman et al., 1994). These receptors are often co-expressed in the same cell and utilize discrete and, in some cases, opposing intracellular signaling pathways (Ashby, 1998). *In vitro* and *in vivo* data suggest that vasoconstrictor responses to PGE_2 are mediated via activation of EP_1 and EP_3 receptors and also via the thromboxane receptor (TP) activation (Coleman and Kennedy, 1980; Dorn et al., 1992; Jadhav et al., 2004; Qian et al., 1994; Walch et al., 2001). In mammals, activation of EP_3-receptors induces smooth muscle contraction in ileum (Botella et al., 1993), colon (Crankshaw and Gaspar, 1995), myometrium and corpus luteum (Sharif et al., 1998). PGE_2

has also been reported to induce contractile responses via EP_1 receptor activation in guinea-pig trachea (Coleman and Kennedy, 1985) or gastrointestinal tract (Kennedy et al., 1982).

In terms of its vascular actions, PGE_2 is generally regarded as a vasodilator eicosanoid in peripheral (Coleman et al., 1994; Lawrence and Jones, 1992; Lydford et al., 1996) and cerebral vascular beds (Li et al., 1994) in humans and laboratory animals, where it directly relaxes vascular smooth muscle cells (Lawrence and Jones, 1992). This effect is purported to be mediated via activation of either EP_2 or EP_4 receptors and subsequent increases in cAMP generation within vascular smooth muscle (Lawrence and Jones, 1992; Lydford et al., 1996). However, in the veins of the human hand, PGE_2 has been reported to elicit a contractile response that is purportedly due to the activation of EP_1 receptors (Arner and Hogestatt, 1991).

The aim of the present study was to determine whether plasma concentrations of PGE_2 changed during the prodromal stages of experimentally-induced laminitis and to determine the vasoconstrictor effects of PGE_2 and its synthetic analogs, 11-deoxy-PGE_2 (11-d-PGE_2) and 17-phenyl-trinor-PGE_2 (17-pt-PGE_2), in laminar arteries and veins isolated from control horses or those with Obel grade 1 laminitis. We also report, for the first time, the effects of a thromboxane receptor antagonist, (SQ 29,548), prostaglandin EP_1 receptor antagonist (SC-19220) and prostaglandin EP_1, EP_2, EP_3 and DP_1 receptor antagonist (AH 6809), on the responses of equine laminar vessels to PGE_2 and its synthetic analogs 11-d-PGE_2 and 17-pt-PGE_2.

MATERIALS AND METHODS

Ten mixed breed horses ranging in age from 4 to 12 years old (mean 9 years) were used in this study. Each horse lacked clinical evidence of lameness and survey radiographs of the

forelimb digits were within normal limits. Horses were randomly assigned to control or black walnut heartwood extract (BWHE) groups. Horses in the control group (5 horses) received 6 L of water via nasogastric tube, and were euthanatized after the collection of the final (12 hour) blood sample. Horses in the BWHE group (5 horses) received BWHE via nasogastric tube and were euthanatized at the onset of Obel grade 1 laminitis (clinical signs consisting of weight shifting and bounding digital pulses without evidence of lameness at a walk) or at 12 hours after intubation, if signs of Obel grade 1 laminitis had not developed by that time. Each horse was evaluated prior to intubation and every hour thereafter for attitude, heart rate, respiratory rate, capillary refill time, hoof temperature, digital pulses, and evidence of lameness consistent with Obel grade 1 laminitis. Blood samples were obtained via a jugular catheter at 0, 1, 2, 3, 4, 6, 8, 10, and 12 hours. Catheter patency was maintained by flushing the catheter with 20 ml heparinized saline (5 IU heparin/ml) after each blood collection. All protocols were approved by the University of Georgia Institutional Animal Care and Use Committee. The horses were euthanatized using a penetrating captive bolt, as approved by the Report of the American Veterinary Medical Association's Panel on Euthanasia (AVMA, 2001).

The blood samples were aliquoted into three ice chilled vacuum-evacuated tubes containing EDTA, one of which was used for determination of the peripheral WBC count. The other samples were placed on ice for 10 minutes and then immediately centrifuged at 400 x g for 10 min at 4 ºC. Plasma from the latter tubes was frozen at -80ºC until assayed for Prostaglandin E_2 (PGE_2) concentrations. Plasma concentrations of PGE_2 were determined using a commercially available enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI).

Isolation of laminar vessels

Laminar arteries and veins were isolated as described previously (Peroni et al., 2005; Peroni et al., 2006; Robertson et al., 2005). Briefly, two full thickness segments of the dorsal hoof were placed in ice-cold physiological salt solution (PSS) containing (in mM): NaCl 118; $NaHCO_3$ 24; $MgSO_4$ 1; NaH_2PO_4 0.435; glucose 5.56; $CaCl_2$ 1.8, and KCl 4; gassed with 21% O_2 and 5% CO_2 (pH = 7.40 ± 0.01). On the stage of a high-powered microscope, the lamellar portion of the dermis was shaved until only a thin layer covered the laminar vascular bed. Laminar arteries and veins (2 - 3 cm distal to the coronary band, 200 - 800 μm internal diameter, 1-2 mm in length) were isolated using micro-fine surgical instruments and mounted on small vessel myographs (Model 500A, Danish Myo Technology, Denmark). The vessels were bathed in PSS and the bath temperature was raised to, and maintained at, 37^{o}C for one hour while the vessels equilibrated. Laminar arteries and veins were then stretched to produce equivalent transmural pressures of 3.1kPa and 1.9kPA, respectively (Peroni et al., 2005). Data were collected for each agonist and, where appropriate, in the presence of specific antagonists, from at least 7 arteries and veins. The numbers of vessels and horses used to obtain the data in this study were based on our previous experience with isolated laminar arteries and veins (Peroni et al., 2005; Peroni et al., 2006; Robertson et al., 2005).

Experimental vessel protocols

All vessels were given three 2-minute exposures to 80 mM KCl-PSS (isotonic replacement of NaCl with KCl, KPSS), 15 minutes apart to establish the maximal contractile response to a depolarizing stimulus. Concentration response curves were then obtained for either

PGE_2 (PGE_2, 1 nM – 10 μM), 11-deoxy-PGE_2 (11-d-PGE_2, 1 nM – 10 μM) or 17-phenyl-trinor-PGE_2 (17-pt-PGE_2, 1 nM – 100 μM) by cumulative addition of each agonist.

Similar experiments were performed in which vessels were pre-incubated with each of the following antagonists: SQ 29,548 (thromboxane receptor antagonist, 50 μM), SC-19220 (prostaglandin EP_1 receptor antagonist, 50 μM), or AH 6809 (prostaglandin EP_1, EP_2, EP_3 / DP_1 receptor antagonist, 50 μM) 10 min prior to commencement of the appropriate concentration response curve. The concentration of each antagonist was based upon published reports of their efficacy in isolated blood vessels (Daray et al., 2004; Sametz et al., 2000; Walch et al., 2001).

Sample preparation for enzyme immunoassay (EIA)

Prior to determining concentrations of PGE_2, plasma was subjected to methanol extraction with one ml of plasma added to 9 ml methanol. The samples were then centrifuged for 10 minutes at 400 x g, and the supernatant was decanted and concentrated by vacuum centrifugation (Hetovac Vacuum Rotator model VR-1, Heto-Holten, Denmark). The concentrated supernatants were stored at −80ºC until reconstituted to a 2 x concentration in the EIA buffer immediately before performing the assay according to the manufacturer's instructions.

EIA for PGE_2

Plasma PGE_2 concentrations were determined using a commercially available EIA kit (Cayman Chemical, Ann Arbor, MI). Briefly, 50 μl of standard or extracted plasma samples were placed in a 96-well plate pre-coated with mouse monoclonal antibody. Thereafter, 50 μl of PGE_2 tracer and PGE_2 antiserum were added into each well and incubated for 18 hours at room

temperature. After washing with wash buffer, 200 µl of Ellman's reagent containing acetylcholinesterase was added. The plates were read at 412 nm and concentrations of PGE_2 were calculated from a standard curve generated with known concentrations of PGE_2. The detection limit of the assay was 11 pg/ml.

Data and statistical analyses

Data are presented as mean ± SEM. Contractile responses were calculated as a percentage of the maximal response to KPSS (% T_K) for each vessel. The data were analyzed by repeated measures analysis of variance (ANOVA). Differences between individual means were identified by Student's modified *t*-test using the Bonferroni correction for multiple comparisons between means using the error mean square term from the ANOVA (Wallenstein et al., 1980; Winer, 1971). PGE_2 plasma concentration data were analyzed using a unpaired Student's t test to compare mean values for the control and BWHE horses.

RESULTS

Responses of laminar arteries and veins to PGE_2, 11-d-PGE_2 and 11-pt-PGE_2

PGE_2 elicited a slight vasodilation in laminar veins isolated from control horses, but not in laminar veins from horses with BWHE-induced Obel grade 1 laminitis. In contrast, a small, but consistent, vasoconstrictor effect was observed at the highest concentration of PGE_2 in vessels from BWHE horses. PGE_2 had no effect on tone in similarly sized laminar arteries isolated from either control horses or those administered BWHE. The synthetic analogue of PGE_2, 11-d-PGE_2, elicited robust contractile responses in laminar veins from control horses, but

substantially smaller contractile responses in laminar arteries from control horses. The vasoconstrictor effects of 11-d-PGE_2 were similar in veins from control and BWHE-administered horses; 11-d-PGE_2 elicited larger contractile responses in laminar arteries isolated from BWHE-administered horses than in laminar arteries from control horses. The PGE_2 analogue, 17-pt-PGE_2, did not induce vasoconstriction of laminar arteries or veins from either control horses or those administered BWHE (Figures 5.1-5.2 and Tables 5.1-5.3).

Modulation of contractile responses of laminar veins to PGE_2, 11-d-PGE_2 and 11-pt-PGE_2 by eicosanoid receptor antagonists

Effects of SQ 29,548 on laminar veins: Pre-incubation of laminar veins from control and BWHE horses with the thromboxane receptor antagonist SQ 29,548 (50 μM) reduced the contractile responses to 11-d-PGE_2. Indeed, in the presence of SQ 29,548, 11-d-PGE_2 induced a small vasodilation in laminar veins isolated from control horses. While the contractile effects of 11-d-PGE_2 were similarly ablated by SQ 29,548 in laminar veins from BWHE-administered horses, no vasodilation was observed in the presence of this antagonist. SQ 29,548 did not affect the responses to either PGE_2 or 17-pt-PGE_2 in laminar veins from control horses or those with BWHE-induced laminitis (Figure 5.3 and Tables 5.1-5.3).

Effects of SC-19220 on laminar veins: Pre-incubation of laminar veins from control and BWHE horses with the prostaglandin EP_1 receptor antagonist, SC-19220, reduced the contractile responses of laminar veins from control or BWHE-administered horses to low concentrations of 11-d-PGE2, although the maximal contractile responses were not affected. In the presence of SC-19220, laminar veins from control horses dilated slightly upon exposure to 17-pt-PGE_2. In

contrast, 17-pt-PGE_2 in the presence of SC-19220 did not elicit vasodilation of laminar veins from BWHE-administered horses. Responses of laminar veins isolated from control or BWHE horses to PGE_2 were unaffected by pre-incubation with SC-19220 (Figure 5.4 and Tables 5.1-5.3).

Effects of AH 6809 on laminar veins: Pre-incubation of laminar veins from control or BWHE horses with the prostaglandin EP_1, EP_2, EP_3 and DP_1 receptor antagonist AH 6809 resulted in a rightward shift in the concentration-response curve to 11-d-PGE_2 and a reduction in the maximal contractile effect of 11-d-PGE_2 in these vessels. AH 6809 had no effect on the responses of laminar veins isolated from control horses, but did inhibit the small contractile response observed in laminar veins isolated from BWHE-administered horses observed at the highest concentration of PGE_2. There was no difference in the responses of laminar veins from either control or BWHE horses to 17-pt-PGE_2 either in the presence or absence of AH 6809 (Figure 5.5 and Tables 5.1-5.3).

Plasma concentrations of PGE_2

Plasma concentrations of PGE_2 were significantly greater in the BWHE horses one hour prior the time when the peripheral leukocyte count was at its lowest point (Hurley et al., 2006) or has dropped by at least 30%, 2-3 hours after administration of BWHE, when compared to the value for time 0.

DISCUSSION

This is the first study to determine that PGE_2 levels are increased during the developmental stages of BWHE-induced laminitis and to compare the vasoconstrictor responses of equine laminar arteries and veins to PGE_2 and to the synthetic PGE_2 analogs 11-d-PGE_2 and 17-pt-PGE_2. PGE_2 is an important eicosanoid involved in the regulation of many physiological processes, including inflammation and minute-to-minute regulation of local blood flow. PGE_2 also modulates immune responses by regulating the function of cells such as macrophages, T and B lymphocytes leading to pro- and anti-inflammatory effects (Rolin et al., 2006). For example, PGE_2 can stimulate the release of vascular endothelial growth factor (Fukuda et al., 2003), induce cell migration (Sheng et al., 2001), and increase matrix metalloproteinase-2 expression and activation (Dohadwala et al., 2002). Although PGE_2 is principally regarded as a vasodilator, this is not exclusively the case as PGE_2 can elicit venoconstriction in certain vascular beds (Arner and Hogestatt, 1991; Muller-Schweinitzer, 1979). Purportedly, PGE_2 acts via activation of specific receptors, namely EP receptors, which have been subclassified as EP_1, EP_2, EP_3 and EP_4 receptors. Of these receptors, EP_1 and EP_3 mediate the constrictor effects of PGE_2 in smooth muscle, whereas EP_2 and EP_4 mediate the vasodilatory effects of PGE_2 (Coleman et al., 1994; Narumiya et al., 1999).

The identification of the specific EP receptors that are responsible for the actions of PGE_2 in mammalian tissues has been aided by the development of synthetic analogues of PGE_2 that are specific for certain EP receptor subtypes. 11-d-PGE_2 is almost identical to PGE_2, except that 11-d-PGE_2 lacks hydroxyl group at position 11. 11-d-PGE_2 was selected for this study as it has been purported to be a potent analogue of PGE_2 (Crider et al., 2000; Noguchi et al., 2000), and has

been utilized as a substitute for PGE_2 in studies where the physiological role of PGE_2 has been examined (Qin et al., 2003). However, the simple substitution of 11-d-PGE_2 for PGE_2 may be imprudent, as it is apparent that 11-d-PGE_2 has biological activities distinct from those of PGE_2, at least with respect to its action on smooth muscle. Karim and co-workers (1980) were the first group to synthesize 11-d-PGE_2 and provide data as to this compound's biological activity. These investigators reported that whereas PGE_2 possessed both dilatory and constrictor activity in human airways (trachea, bronchus and small bronchioles), 11-d-PGE_2 was a potent constrictor of airway smooth muscle (Karim et al., 1980). In the current study we have determined that PGE_2 is devoid of constrictor activity in laminar arteries and veins from control horses. In contrast, 11-d-PGE_2 is a potent constrictor agonist in laminar veins and elicit contractions, albeit much smaller, in laminar arteries. The constrictor effects of 11-d-PGE2 were similar in laminar veins from horses with BWHE-induced laminitis when compared to those observed in laminar veins from control horses. This is in contrast to our observations that the constrictor effects of $PGF_{2\alpha}$, 5-HT and phenylephrine in laminar veins from BWHE-administered horses were smaller than responses in laminar veins from control horses. Maintenance of the contractile responses to 11-d-PGE_2 as well as to the isoprostanes, iso-$PGF_{2\alpha}$ and iso-PGE_2 (Noschka et al., 2007a), demonstrates that laminitis is not associated with a generalized depression of agonist-induced venoconstrictor responses, but that perturbations in contractile responses appear to be agonist specific.

In the current study, PGE_2 elicited a slight dilation in laminar veins from control horses that was not evident in laminar veins from BWHE-administered horses. Similarly, the vasodilation observed in laminar veins from control horses to 11-d-PGE_2 in the presence of the thromboxane receptor antagonist, SQ 29,548, was absent in laminar veins from BWHE-

administered horses. These results are consistent with normal PGE_2 and 11-d-PGE_2–activatable venodilator pathways being compromised at Obel grade 1 laminitis and are similar to our recent findings with $PGF_{2\alpha}$ and the isoprostane iso-$PGF_{2\alpha}$ in these vessels (Noschka et al., 2007a; Noschka et al., 2007b). The fact that the vasodilatory effects of 11-d-PGE_2 were not present when laminar veins were pre-incubated with AH 6809 is consistent with the possibility that 11-d-PGE_2 exerts its vasodilatory effects on laminar veins via activation of EP_2 receptors (AH 6809 has been reported to block EP_2 receptors but not EP_4 receptors) (Jadhav et al., 2004; Kiriyama et al., 1997). In contrast, AH 6809 did not affect the slight PGE_2-mediated dilation in laminar veins isolated from control horses, which is consistent with PGE_2 activating EP_4, rather than EP_2, receptors in laminar veins. Together, these results provide evidence that both EP_2 and EP_4 receptor mediated vasodilatory pathways may be compromised at Obel grade 1. However, this assertion must be tempered by the possibility that AH 6809 may possess non-prostanoid receptor specific effects (Noschka et al., 2007b).

The fact that the vasoconstrictor responses to 11-d-PGE_2 were abolished in the presence of SQ 29,548 is consistent with these effects of 11-d-PGE_2 being mediated via activation of TP receptors. However, the finding that AH 6809 also abolished the vasoconstrictor effects of 11-d-PGE_2, argues against this possibility as AH 6809 has not been reported to inhibit TP receptors in any species. This apparent conflict may be reconciled by our determination that AH 6809 inhibits contractile responses in laminar veins independently of prostanoid receptor antagonism (Noschka et al., 2007b). In light of these observations, attempting to interpretation of the effects of AH 6809 upon vascular contractility may be futile.

17-pt-PGE_2 is a synthetic analog of PGE_2 that is an agonist for EP_1 and EP_3 receptors in several tissues (Kiriyama et al., 1997; Lawrence and Jones, 1992; Okada et al., 2000; Walch et

al., 2003). Because EP_1 and EP_3 receptors are thought to mediate the vasoconstrictor actions of PGE_2, 17-pt-PGE_2 was selected for use in this study to aid in the delineation of the roles of specific prostanoid receptor subtypes in the contractile responses of laminar veins to eicosanoids. Unexpectedly, we found that 17-pt-PGE_2 was virtually inert with respect to altering tone in laminar vessels. The lack of effect of 17-pt-PGE_2 on tone in laminar veins may be explained by a lack of EP_1 and EP_3 receptors in these vessels and if the constrictor effects of PGE_2 are mediated via activation of the thromboxane receptor. Our observations with the thromboxane receptor antagonist, SQ 29,548, would appear to support this concept. Indeed, contractile responses elicited by PGE_2 in other vascular preparations also appear to be mediated via thromboxane-receptor activation (Baxter et al., 1995; Jones et al., 1982; Sametz et al., 2000). Alternatively, it is possible that 17-pt-PGE_2 lacks affinity for equine EP_1 and EP_3 receptors and, as such, may not act as an agonist for these receptors in isolated laminar blood vessels.

In summary, the present study represents our initial steps in determining whether eicosanoids, and more specifically PGE_2, act as conduits between the inflammatory and vascular alterations that contribute to the development of equine laminitis. The increases in plasma concentrations of PGE_2 are consistent with systemic inflammatory events occurring at an early stage in the pathogenesis of laminitis. The relative selectivity of eicosanoids *per se* for laminar veins, in terms of eliciting constrictor responses (Noschka et al., 2007a; Noschka et al., 2007b), supports the concept that these factors may be important elements in the inflammatory and vascular perturbations evident in laminitis.

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Table 5.1. Mean ± SEM effects of SC-19220, AH 6809 or SQ 29,548 on contractile responses to PGE_2 in equine laminar veins and arteries of Control and BWHE horses.

			Veins				Arteries		
Horses	**Treatment**	**No.**	**Max (%T_K)**	**EC_{50} (nM)**	**Max:EC_{50}**	**No.**	**Max (%T_K)**	**EC_{50} (nM)**	**Max:EC_{50}**
Control									
	Saline	8	-14 ± 4	2.6 ± 0.42	-540 ± 120	8	-0.3 ± 0.1	~0	~0
	SQ 29,548	8	-25 ± 5	1.6 ± 0.14	-1530 ± 260*	NA	ND	ND	ND
	SC-19220	7	-18 ± 7	~251	~-7	NA	ND	ND	ND
	AH 6809	8	-14 ± 4	9 ± 2	-152 ± 22*	NA	ND	ND	ND
BWHE									
	Saline	10	14 ± 2†	3631 ± 468†	0.37 ± 0.06†	10	1.3 ± 0.5	~0	~0
	SQ 29,548	10	8 ± 2	~30	~26	NA	ND	ND	ND
	SC-19220	10	15 ± 5	2692 ± 344	0.54 ± 0.07	NA	ND	ND	ND
	AH 6809	10	2 ± 2*	None*	None*	NA	ND	ND	ND

Within group comparisons: *$P < 0.05$, treatments *versus* saline. Between group comparisons: †$P < 0.05$, Saline BWHE *versus* Control
Max = Maximal contraction. $\%T_K$ = Maximal response expressed as a percentage of the maximal contractile response to KCl-PSS. NA = Not applicable. ND = not determined.

Table 5.2. Mean ± SEM effects of SC-19220, AH 6809 or SQ 29,548 on contractile responses to 11-d-PGE_2 in equine laminar veins and arteries of Control and BWHE horses.

			Veins				Arteries		
Horses	**Treatment**	**No.**	**Max (%T_K)**	**EC_{50} (nM)**	**Max:EC_{50}**	**No.**	**Max (%T_K)**	**EC_{50} (nM)**	**Max:EC_{50}**
Control									
	Saline	10	148 ± 12	203 ± 26	74 ± 8	10	24 ± 5	128 ± 32	21 ± 6
	SQ 29,548	10	-32 ± 9*†,‡	9.2 ± 2.6*	-331 ± 56*	NA	ND	ND	ND
	SC-19220	10	149 ± 12	721 ± 53*	21 ± 3*	NA	ND	ND	ND
	AH 6809	10	79 ± 9*†	3801 ± 396*	2.1 ± 0.4*	NA	ND	ND	ND
BWHE									
	Saline	10	133 ± 13	156 ± 12	83 ± 9	10	60 ± 11	131 ± 28	46 ± 6*
	SQ 29,548	10	-3.8 ± 1.7*	12.4 ± 6.9*	-31 ± 16*	NA	ND	ND	ND
	SC-19220	10	147 ± 12	263 ± 21*	57 ± 6*	NA	ND	ND	ND
	AH 6809	10	65 ± 12*	3014 ± 466*	2.2 ± 0.6*	NA	ND	ND	ND

Within group comparisons: *$P < 0.05$, treatments *versus* saline. Between group comparisons: $^{\dagger}P < 0.05$, Saline BWHE *versus* Control
Max = Maximal contraction. $\%T_K$ = Maximal response expressed as a percentage of the maximal contractile response to KCl-PSS. NA = Not applicable. ND = not determined.

Table 5.3. Mean ± SEM effects of SC-19220, AH 6809 or SQ 29,548 on contractile responses to 17-pt-PGE_2 in equine laminar veins and arteries of Control and BWHE horses.

			Veins				Arteries		
Horses	**Treatment**	**No.**	**Max (%T_K)**	**EC_{50} (nM)**	**Max:EC_{50}**	**No.**	**Max (%T_K)**	**EC_{50} (nM)**	**Max:EC_{50}**
Control									
	Saline	8	7 ± 6	~0	~0	10	1 ± 1	~0	~0
	SQ 29,548	10	7 ± 4	~0	~0	NA	ND	ND	ND
	SC-19220	9	-11 ± 3	~0	~0	NA	ND	ND	ND
	AH 6809	10	3 ± 3	~0	~0	NA	ND	ND	ND
BWHE									
	Saline	11	20 ± 9	~0	~0	13	0.8 ± 0.2	~0	~0
	SQ 29,548	12	11 ± 3	~0	~0	NA	ND	ND	ND
	SC-19220	12	10 ± 4	~0	~0	NA	ND	ND	ND
	AH 6809	12	9 ± 3	~0	~0	NA	ND	ND	ND

Within group comparisons: $^{*}P < 0.05$, treatments *versus* saline. Between group comparisons: $^{\dagger}P < 0.05$, Saline BWHE *versus* Control
Max = Maximal contraction. $\%T_K$ = Maximal response expressed as a percentage of the maximal contractile response to KCl-PSS. NA = Not applicable. ND = not determined.

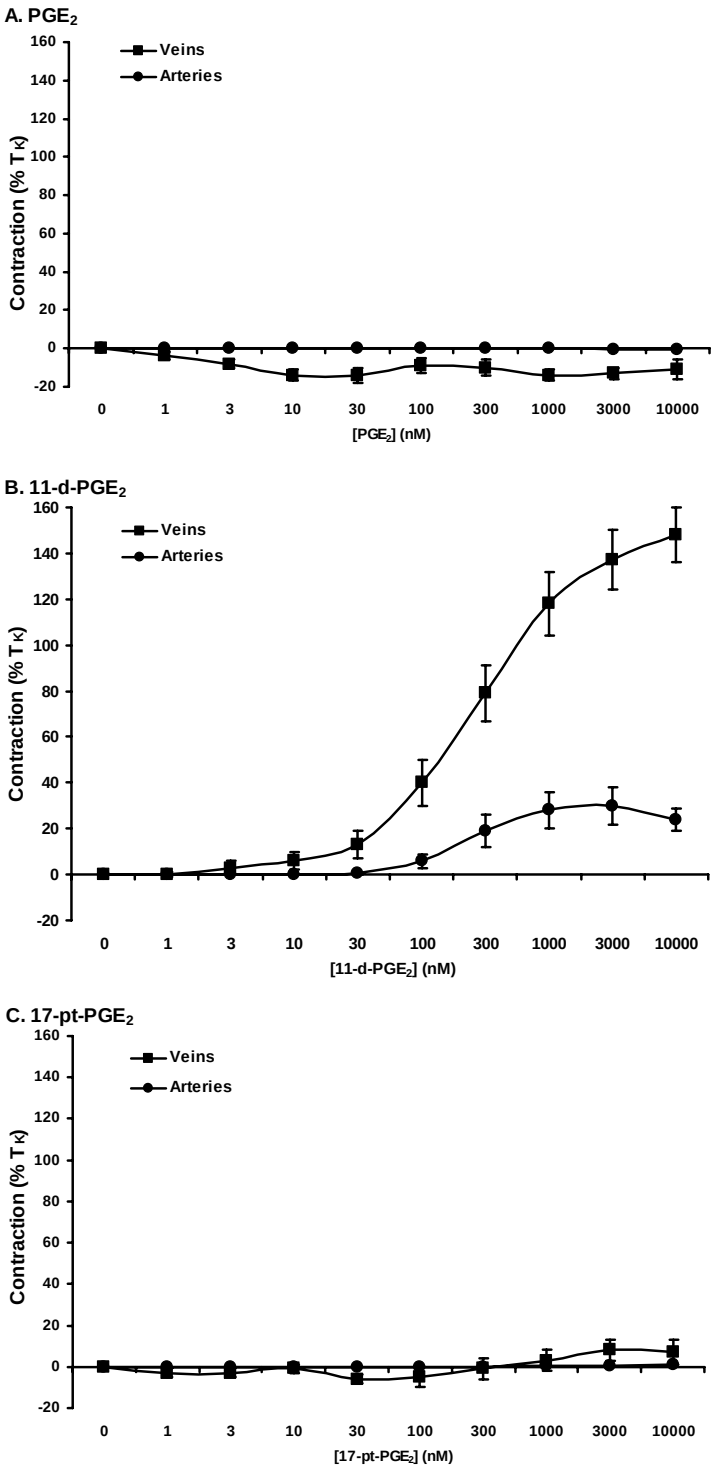

Figure 5.1. Mean ± SEM responses of laminar veins (■) and arteries (●) to increasing concentrations of PGE_2 (Panel A), 11-d-PGE_2 (Panel B) or 17-pt-PGE_2 (Panel C).

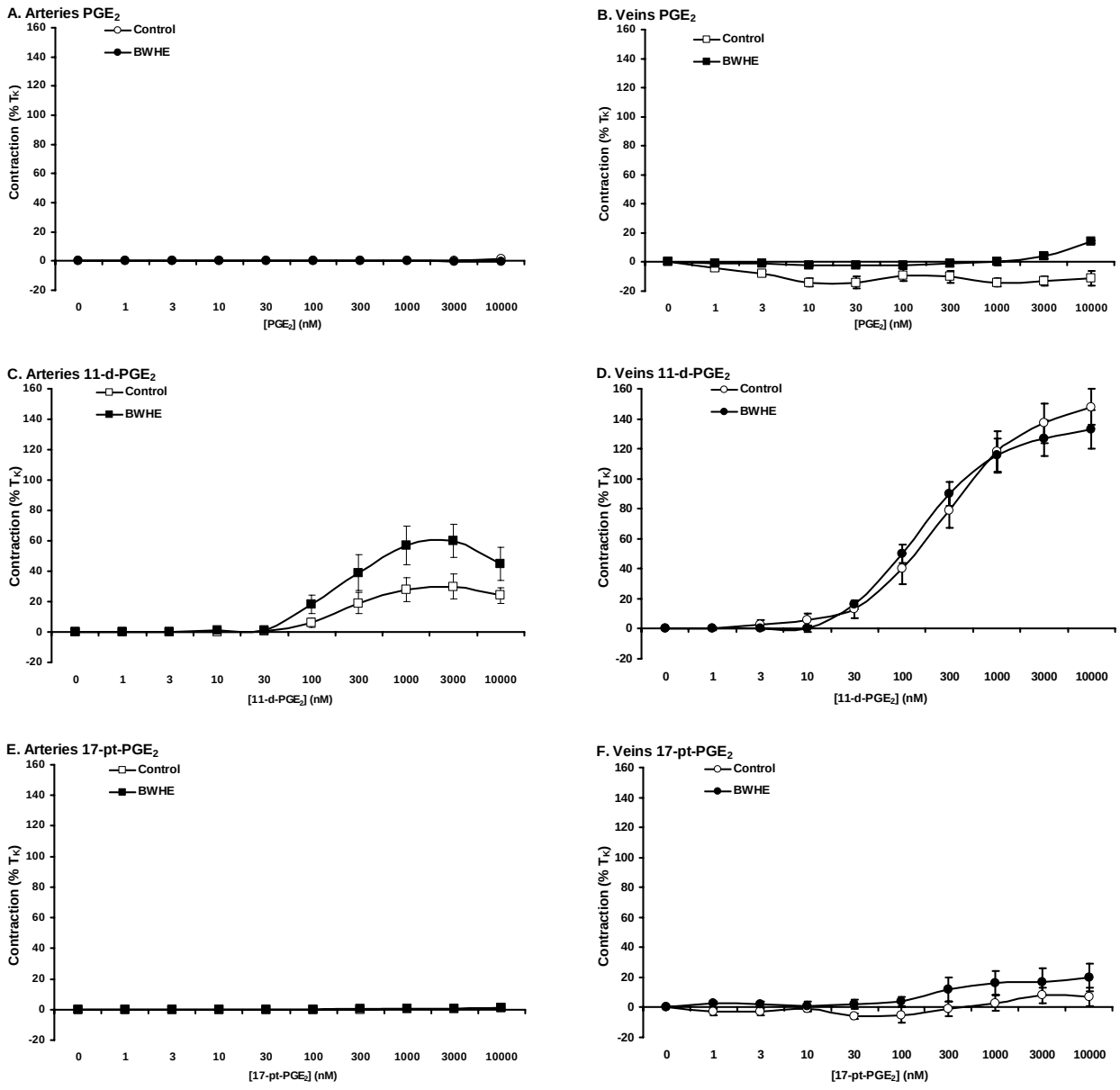

Figure 5.2. Mean ± SEM responses of: *Panel A*, control laminar arteries (○) and BWHE laminar arteries (●) to PGE_2; *Panel B*, control laminar veins (□) and BWHE laminar veins (■) to PGE_2; *Panel C*, control laminar arteries (□) and BWHE laminar arteries (■) to 11-d-PGE_2; and *Panel D*, control laminar veins (○) and BWHE laminar veins (●) to 17-pt-PGE_2.

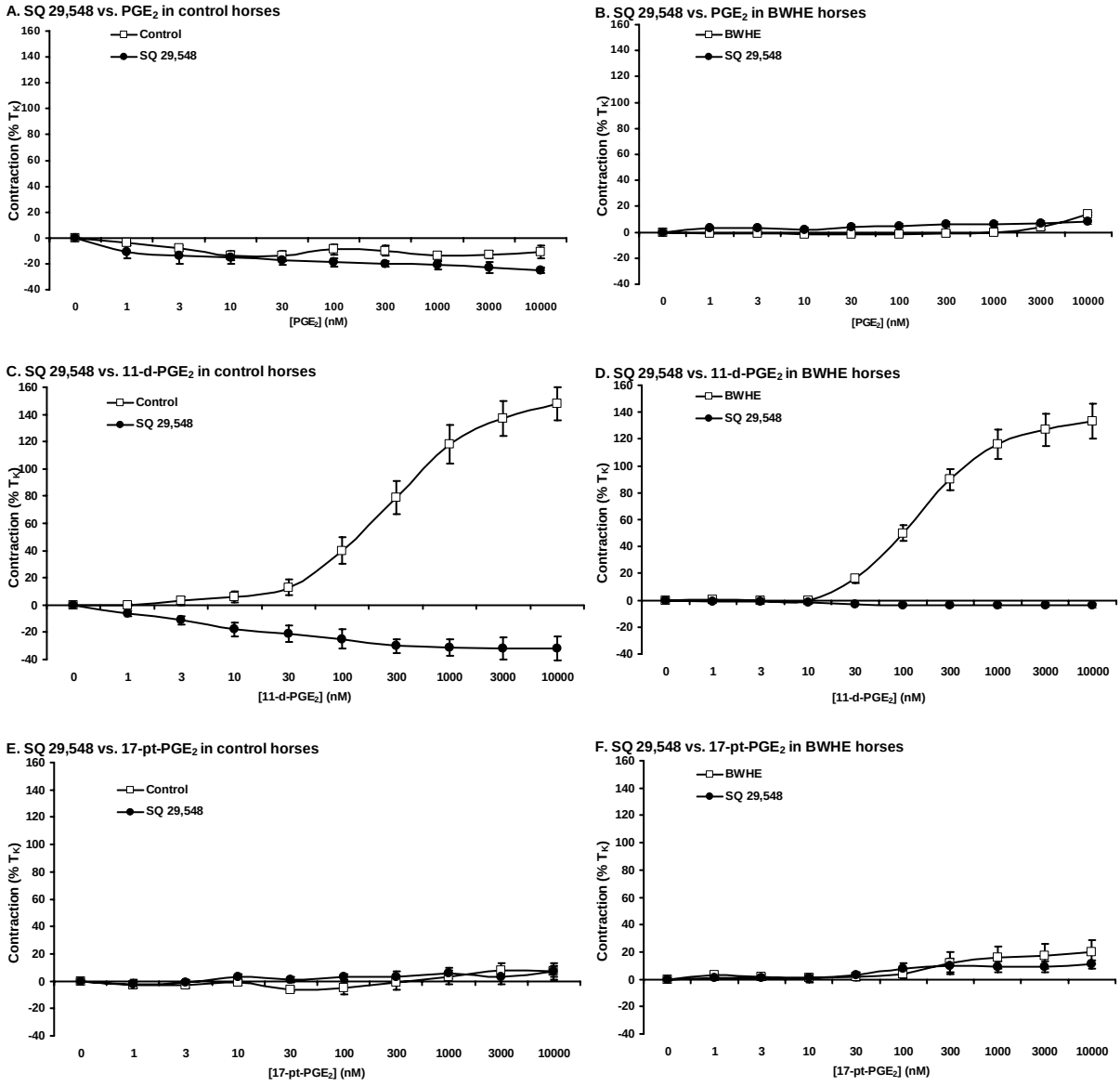

Figure 5.3. Mean ± SEM responses of laminar veins isolated from control (Panels A, C and E) or BWHE horses (Panels B, D and F) to increasing concentrations of PGE_2 (Panels A and B), 11-d-PGE_2 (Panels C and D) or 17-pt-PGE_2 (Panel E and F) either in the absence (□) or presence (●) of SQ 29,548.

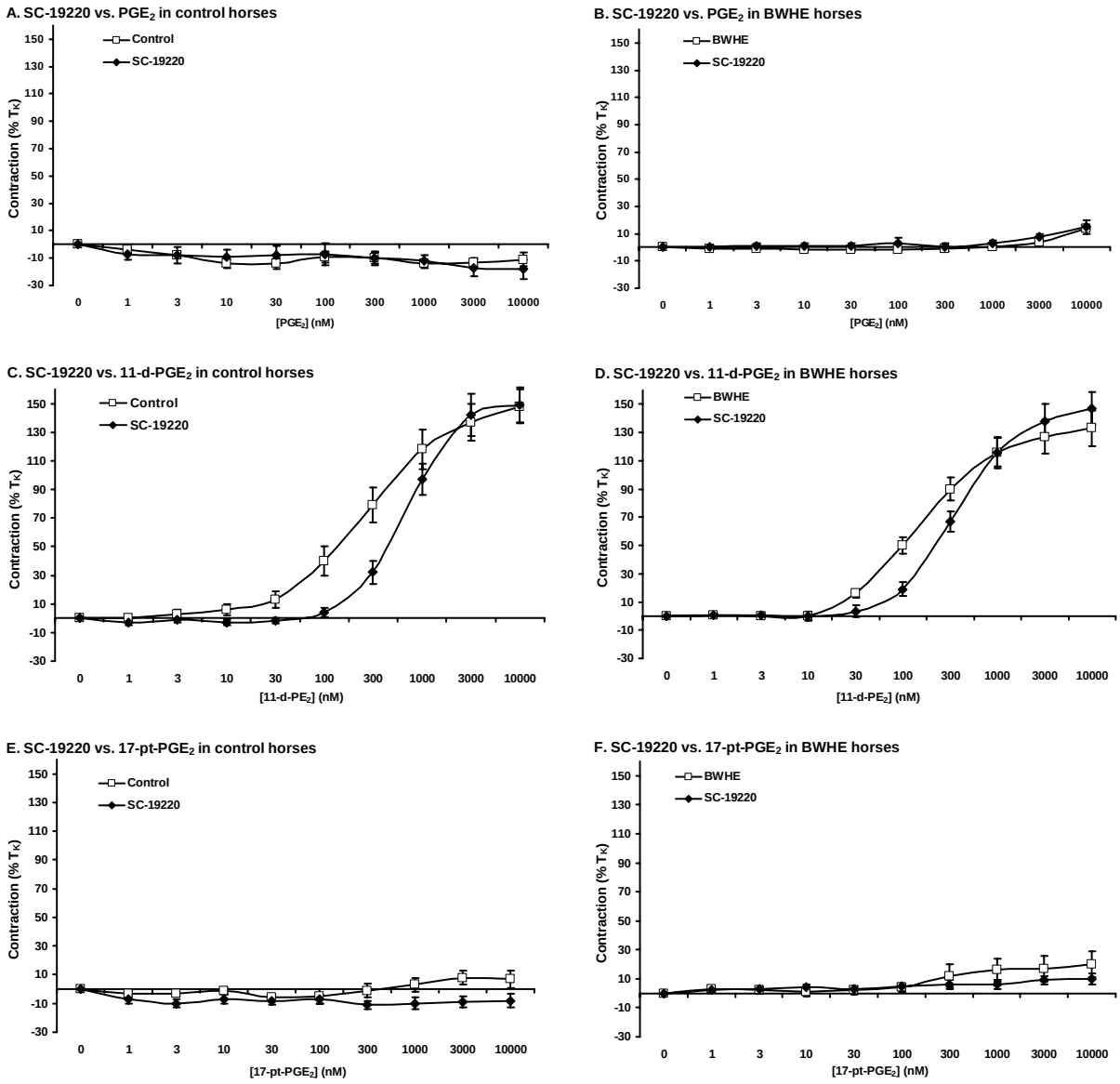

Figure 5.4. Mean ± SEM responses of laminar veins isolated from control (Panels A, C and E) or BWHE horses (Panels B, D and F) to increasing concentrations of PGE_2 (Panels A and B), 11-d-PGE_2 (Panels C and D) or 17-pt-PGE_2 (Panel E and F) either in the absence (□) or presence (♦) of SC-19220.

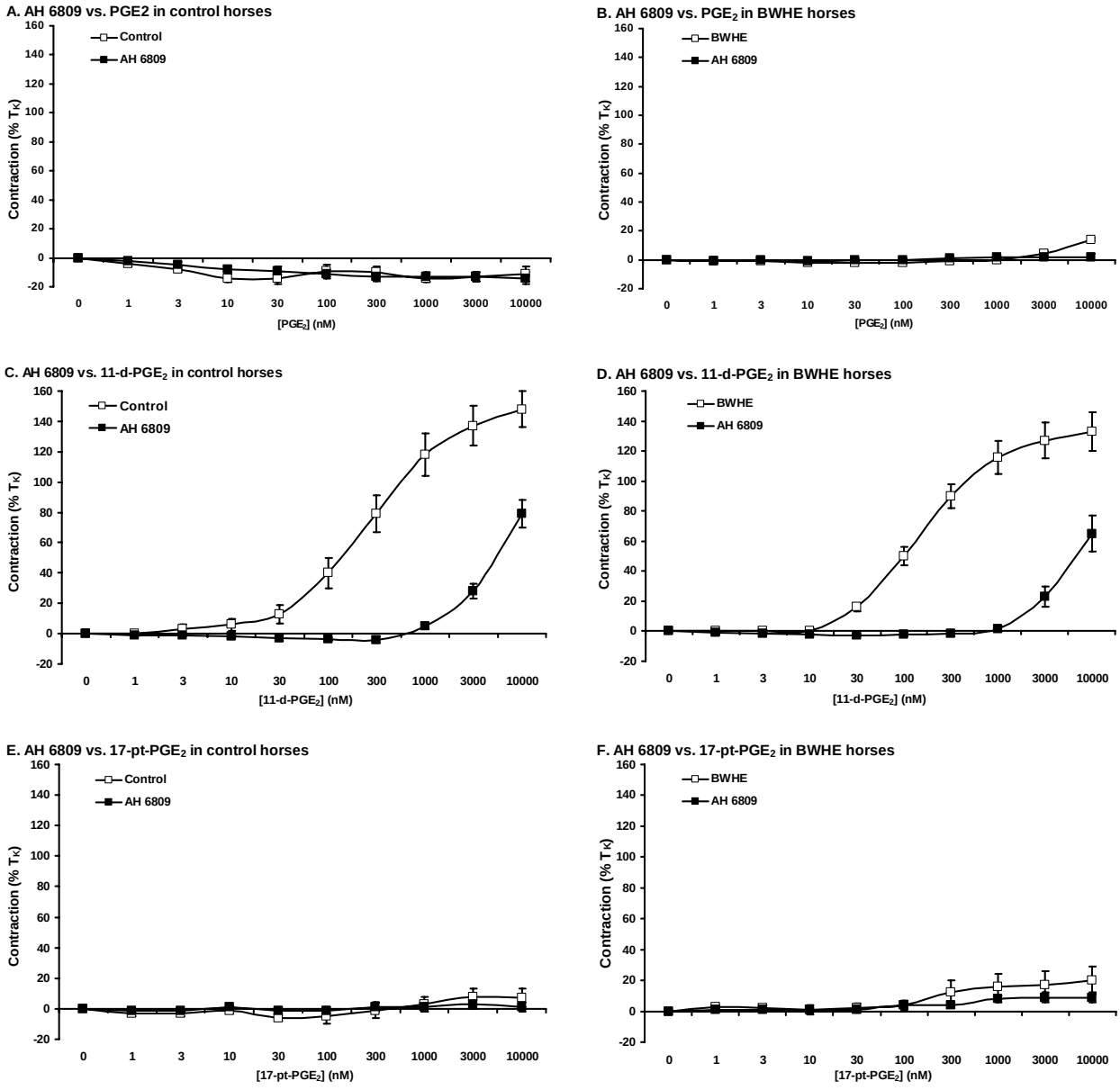

Figure 5.5. Mean ± SEM responses of laminar veins isolated from control (Panels A, C and E) or BWHE horses (Panels B, D and F) to increasing concentrations of PGE_2 (Panels A and B), 11-d-PGE_2 (Panels C and D) or 17-pt-PGE_2 (Panel E and F) either in the absence (□) or presence (■) of AH 6809.

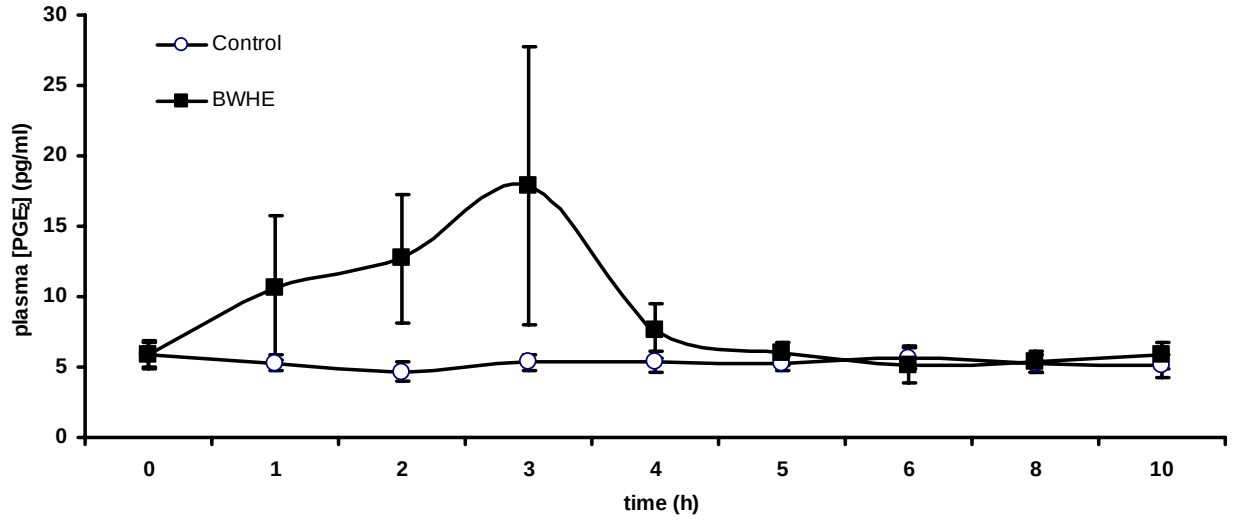

Figure 6.6. Mean ± SEM concentrations of plasma TxB_2 (Panel A) and iso-$PGF_{2\alpha}$ (Panel B) and white blood cell counts (Panel C) in control (○) or BWHE-administered (■) horses.

CHAPTER 6

TEMPORAL ASPECTS OF LAMINAR GENE EXPRESSION DURING THE DEVELOPMENTAL STAGES OF EQUINE LAMINITIS[1]

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ABSTRACT

The results of recent studies indicate that inflammatory responses occurring in the early stages of equine laminitis lead to downstream events that eventually result in failure of the bond between the hoof wall and the distal phalanx. In order to gain further insights into the molecular mechanisms involved in the development of laminitis, an equine-specific cDNA microarray consisting of transcripts for more that 3,000 genes was used to assess temporal changes in gene expression in laminar tissues at 1.5, 3 and 12 hr after administration of either a laminitis-inducing agent (black walnut heartwood extract; BWHE) or an equal volume of water (control). As early as 1.5 hr after BWHE administration, proinflammatory genes associated with leukocyte activation and emigration, including MCP-3/CCL7, MCP-1/CCL2, IP-10/CXCL10 and ICAM-1 were up-regulated. At both 1.5 and 3 hr after administration of BWHE, expression of B-cell specific transcripts (e.g. Ig-γ 3, Ig-γ 1 and λ-light chain) were decreased in the laminar tissues. At the onset of Obel grade 1 lameness in horses administered BWHE, other genes involved in inflammatory processes (*e.g.,* serum amyloid A, calgranulin C and NFAT-activation molecule 1), regulation of inflammation (e.g., inter-alpha-trypsin inhibitor, BiP/GRP78 [Ig binding protein], L-plastin, serpin and nexin-1), antioxidant responses *(e.g.,* superoxide dismutase), matrix turnover (*e.g.,* MMP-9 and TIMP-1), and anti-microbial responses (e.g. serotransferrin, β-defensin-1 and elafin) were up-regulated. These results provide convincing evidence that genes associated with inflammation, activation and extravasation of leukocytes, antimicrobial activities, and destruction of the lamellar basement membrane are induced during the early stages of development of laminitis in response to administration of BWHE.

INTRODUCTION

Laminitis is a debilitating musculoskeletal disease that affects the sensitive and insensitive laminae in the digits of adult horses and ponies. Laminitis causes structural changes in the digit that lead to the breakdown of the lamellar structure of the inner hoof wall, pain, and a characteristic lameness. Laminitis is often associated with conditions such as strangulating intestinal obstruction, colitis, enteritis, grain overload, retained fetal membranes, and pleuro-pneumonia (Cohen et al., 1994; Hunt et al., 1986). Although there are several hypotheses concerning the pathophysiology of laminitis, in general the disease is considered to be due to either failure of digital vascular function (Hood, 1999) or excessive local production of inflammatory mediators (Belknap et al., 2007; Blikslager et al., 2006; Waguespack et al., 2004b). In support of the latter hypothesis, the results of recent studies of experimentally induced laminitis have revealed marked increases in the number of leukocytes, concentration of myeloperoxidase, and expression of pro-inflammatory cytokines, mediators and COX-2 enzymes in laminar tissue during the development of the disease (Blikslager et al., 2006; Fontaine et al., 2001; Riggs et al., 2007; Waguespack et al., 2004b). Notable examples of cytokines that increase in laminar tissues include interleukin-1β, (Fontaine et al., 2001; Waguespack et al., 2004b) IL-6, (Waguespack et al., 2004b) and the molecule possessing ankyrin-repeats induced by lipopolysaccharide (MAIL;(Waguespack et al., 2004b)) that plays a key role in regulation of cytokine expression. To date, no studies have been performed in which a more global picture of changes in gene expression in the laminar tissues has been presented.

The aim of the present study was to gain further insights into the molecular mechanisms involved in the development of equine laminitis using an equine cDNA microarray. This

microarray was used to assess expression of more than 3,000 genes in laminar tissues harvested at 3 times during the development of acute laminitis, and compare their level of expression with the same genes in laminar tissues of healthy control horses.

MATERIALS AND METHODS

Mixed breed horses ranging in age from 4 to 12 years (mean = 9 years) were used in this study as approved by the IACUC at the University of Georgia and The Ohio State University. Each horse was free of clinical evidence of lameness and survey radiograph of the forelimb digits revealed no abnormalities. The horses were randomly assigned to control (3 hr and 12 hr) or black walnut heartwood extract (BWHE; 1.5 hr, 3 hr and Obel grade 1) groups. The times selected for evaluation of the effects of BWHE were based on the fact that there are no outward clinical effects attributable to BWHE at 1.5 hr, the earliest readily monitored response to BWHE (leucopenia) occurs at about 3 hr, and horses with Obel grade 1 laminitis by definition exhibit the earliest clinical signs indicative of laminitis. Obel grade 1 was defined by observation of clinical signs consisting of weight shifting and bounding digital pulses without evidence of lameness at a walk.

Horses in the control groups (5 horses/group) received 6 L of water via nasogastric tube, and were euthanatized either 3 or 12 hr later. Horses in the three BWHE groups received BWHE via nasogastric tube and were euthanatized either at 1.5 hr (n=5), 3 hr (n=5) or onset of Obel grade 1 laminitis (n=12). Horses in the latter group that did not develop Obel grade 1 laminitis by 12 hr after administration of BWHE were euthanatized at that time. For simplicity, we refer to this group as Obel grade 1 laminitis. We assessed the gene expression profiles at 1.5, 3 and 12 hr

as representative of immediate early, early, and late response genes, respectively. Each horse was evaluated prior to intubation and every hr thereafter for attitude, heart rate, respiratory rate, capillary refill time, hoof temperature, digital pulses, and evidence of lameness consistent with Obel grade 1 laminitis. Blood samples were obtained via a jugular catheter at 0, 1, 2, 3, 4, 6, 8, 10, and 12 hr. Catheter patency was maintained by flushing the catheter with 20 ml heparinized saline (5 IU heparin/ml) after each blood collection. The horses in the Obel grade 1 and 12 hr control groups were used under protocols approved by the University of Georgia Institutional Animal Care and Use Committee. Horses in this group were euthanatized using a penetrating captive bolt, as approved by the Report of the American Veterinary Medical Association's Panel on Euthanasia (AVMA, 2001). Horses in the 1.5 and 3 hr groups were studied at The Ohio State University as previously described (Loftus et al., 2007).

Collection of laminar tissue

Both forelimbs were disarticulated immediately after euthanasia at the level of the metacarpophalangeal joint, and hooves were cut into sections with a band saw. During the procedure, thermal damage was minimized by constant irrigation of the tissue with ice-cold physiologic salt solution containing the following: 118 mM NaCl, 24 mM $NaHCO_3$, 1 mM $MgSO_4$, 0.43 5mM NaH_2PO_4, 5.56 mM glucose, 1.8 mM $CaCl_2$, and 4 mM KCl. Full-thickness segments from each forelimb foot were placed in the ice-cold physiologic salt solution, and the keratinized portion of the hoof and the distal phalanx were removed. Specimens of laminar tissue were removed by dissection from the underlying coffin bone. Laminar specimens were rapidly frozen in liquid nitrogen and stored at −80ºC until RNA was isolated for gene expression studies.

RNA isolation

Total **e**quine laminar tissue RNA for microarray analysis was isolated using the Versagene^{TM} RNA Tissue Kit (Gentra Systems**,** Minneapolis, MN). Briefly, 80-100 mg of frozen laminar tissue was finely cut with a blade and homogenized in reducing lysis buffer using a motorized micro-dounce (homogenizer) according to the manufacturer's protocol for isolation of RNA. RNA quality and quantity were determined using a micro-photospectrometer (ND-1000, NanoDrop Technologies, USA). An OD 260/280 ratio greater than 2.0 was considered to be indicative of high quality RNA.

Equine 3,076 gene cDNA microarray

Our laboratory developed an equine-specific 3,076-gene cDNA microarray using cDNA inserts from an equine expressed sequence tag (EST) unigene set. The inserts were used as templates to generate PCR-amplified, immobilized gene probes printed on mirrored glass slides. The ESTs were obtained as part of a project that generated ~14,000 equine-specific ESTs from several equine cDNA libraries, mostly of leukocyte origin. Full information on these ESTs, including sequence quality, BLAST annotation and E-value, can be obtained at http://fungen.org. The 3,043 PCR amplified cDNA probes encode for a wide range of inducible and constitutive proteins. Because the majority of the ESTs used as PCR templates were obtained from leukocyte cDNA libraries, the genes encode for many proteins involved in inflammatory responses, including genes associated with leukocyte activation, cellular receptors, secreted proteins, housekeeping genes, nuclear proteins, and intracellular signal transducers. Collectively, these probes represent ~10% of the equine transcriptome. Examples of the probes included on the microarray are presented in the tables and figures in the results section.

After EST contig assembly and BLAST annotation of the sequences against both the Protein Information Resource (http://pir.georgetown.edu) and Uniprot databases (http://www.pir.uniprot.org), a unigene set of 3,043 individual genes was identified. Clones representing this unigene set were re-arrayed into new plates, and the plasmids in these clones were isolated by alkaline lysis.

Using the plasmids as template, an equine-specific 3,076-gene cDNA microarray was printed at the Vanderbilt University Microarray Shared Resource facility (Nashville, TN). Briefly, the cDNA inserts were amplified by PCR using primers flanking the cDNA insertion sites and PCR products were spotted onto mirrored glass slides (Exprexions, New Jersey) using a robotic printer (BioRobotics MicroGrid, Genomic Solutions, Ann Arbor, MI) with a spot diameter of ~120 μm. The arrays were then tested for spot morphology and uniformity of spot deposition before being hybridized with equine RNA provided by our laboratory to confirm the overall array quality.

The resulting microarrays consist of 2 printed subarrays, one on the upper half and one on the lower of the slide. Each subarray is comprised of 4 x 4 blocks of gene spots that are contact-printed in duplicate adjacent to each other. Each block has 16 x 16 rows and columns of spots; the top of the block contains empty spots as required to permit the entire gene and control sets to be included. Consequently, each PCR product representing a unique equine gene was printed 4 times on each slide. In addition to the aforementioned equine EST gene sequences, 26 cloned equine genes (TIMP-2, Bax, GLUT-1, E-selectin, Bcl-2, ICAM, tissue factor, IL-10, MIP-1α, MMP-2, cytochrome oxidase, MAIL, RANTES, COX-1, HIF-1α, IL-12, IL-6, XANDH, COX-2, IL-4, VCAM, TNFα, TIMP-1, GLUT-4, VEGF and IL-8) obtained from Dr. Jim Belknap (College of Veterinary Medicine, Ohio State University, Columbus, OH) were added to the

printed gene set. Controls printed onto the microarrays included print buffer only and non-mammalian array control spots (Ambion Array Control, Applied Biosystems, Foster City, CA). Inclusion of these spots allowed determination of signal dynamic range and specificity of hybridization.

cDNA target preparation

Equivalent amounts of RNA (0.5 μg) from the laminar tissue of each of the control horses in the 3-hr water treatment group were pooled to generate control targets for hybridizations against targets prepared from laminar tissue RNA from the individual horses in the 1.5-hr and 3-hr BWHE treatment groups. Similarly, pooled laminar tissue RNA (0.5 μg/horse) isolated from the horses in the 12-hr control group was used to prepare control targets for hybridizations against targets prepared from laminar tissue RNA from the individual horses in the Obel grade 1 laminitis group. Control target cDNA synthesis reactions contained 2.5 μg of pooled RNA hybridized against targets prepared from 2.5 μg of RNA of the individual BWHE treated horses. All RNA samples isolated from the laminar tissue from control and BWHE-treated horses were concentrated to < 9 μl using Microcon YM-30 spin filters (Millipore, Billerica, MA) when needed, and supplemented with 1 μl (0.5 μg) non-mammalian RNA control spikes (Ambion Array Control™, Applied Biosystems, Foster City, CA) before carrying out the cDNA synthesis reactions. The control spikes were used to determine the dynamic range of hybridizations including detection threshold and feature saturation.

cDNA targets were prepared using Cy3 and Cy5 indirect dendrimer labeling kits (Dendrimer Labeling Technique 3DNA® Array 900™ Expression Array Detection Kit, Genisphere Inc., Hatfield PA) with SuperScript™ II reverse transcriptase (Invitrogen

Corporation, Carlsbad, California) according to the manufacturers' protocol. Product prepared using these kits allowed for a 2-step hybridization procedure that avoided potential for dye bias during hybridization. After performing dye swap validation experiments, the Cy3 labeling kit was used to prepare pooled control cDNA targets; the Cy5 labeling kit was used to prepare experimental target cDNA from the individual BWHE-treated horses. Because this is a cDNA microarray, genetic variation between animals (e.g., SNPs and Indels) do not affect overall hybridization kinetics.

Microarray hybridization and analysis

For prehybridization, slides were washed in 0.2% SDS and then transferred into a prehybridization solution containing 5x SSC/1% BSA/1% SDS for 45 min at 55^{o}C. The slides were then washed in ultrapure water (>18 MΩ/cm), rinsed with isopropanol and allowed to air dry. The prehybridized slides were placed into a GeneMachines HybChamber (Genomic Solutions, Ann Arbor, MI), overlaid with a clean LifterSlip (Erie Scientific Company, Portsmouth, NH) and placed into a hybridization oven at 65^{o}C for at least 10 min before use.

Equivalent volumes (14 µl) of control and BWHE cDNA target were placed in a microfuge tube containing 2 µl prewarmed locked nucleic acid (LNA) dT blocker and 30 µl 2X SDS hybridization buffer (Genisphere, Inc.). The mixed target DNA was linearized by heating the solution for 10 min at 75°C, cooled to 65°C and then placed under the lifter slip onto the slide using capillary action. Hybridization of target to the immobilized probe was carried out at 65°C for at least 16 hr. Post hybridization washes of 10 min each included washes of increasing stringency in 2X SSC/0.2% SDS at 65°C, 2X SSC/0.2% SDS at room temperature, 2X SSC at room temperature and 0.2X SSC at room temperature.

The slides were washed briefly in 95% ethanol and dried by centrifugation at 600 *xg* for 2 min. For the secondary hybridization, 2.5 µl of heat denatured Cy3 and Cy5 dendrimer were mixed with 24.7 µl water, 30 µl 2X SDS hybridization buffer and 0.3 µl antifade reagent from the Genisphere dendrimer labeling kits. Hybridization for 4 hr at 65^{o}C and postwashes were similar to those described above, except that the post hybridization wash buffers contained 1 mM dithiotreitol (DTT) and all steps were performed in the dark. After the slides were dried, they were kept in dark tubes containing 20 µl of 100 mM DTT to create a reducing atmosphere.

Fluorescent images from the arrays were acquired using a ProScanArray Microarray Scanner and its accompanying software (PerkinElmer Life And Analytical Sciences, Inc, Waltham, MA) set at a resolution of 10 µm. Saved image files were normalized using ImaGene software and statistically analyzed for change in expression using GeneSight software (both from BioDiscovery Inc., El Segundo, CA). Mean replicate signal intensity for each slide within each of the 3 time periods (1.5 hr, 3 hr and Obel grade 1 laminitis) were combined after local background signal had been subtracted and poor quality spots were excluded. The data were $\log_2$ transformed, normalized by subtraction of mean, and expressed as a ratio of BWHE-treated/control. Data were calculated as fold change in expression (up- or down-regulated) with a greater than 2-fold change in expression considered to be significant at a 95% confidence interval. Pearson correction was used as distance metric in the heat maps and statistical significance set at $p > 0.001$ where necessary.

Microarray validation experiments

To confirm reproducibility of data among microarray hybridizations, the following validation experiments were performed: 1) dye swap experiments, 2) experimental (*i.e.,* within

an animal) and biological (*i.e.,* among animals) replicates, and 3) determination of variation among individual horses in the pooled control groups. Self:self hybridizations (control versus control) with differentially Cy3 and Cy5 labeled probes in scatter plots revealed even distribution of spots for the 2 fluors around the identity axis (x = y line) with an overall <2-fold change in gene expression profiles. Experimental and biological replicate experiments revealed minor differences in the magnitude of induction/suppression of gene expression profiles among tissues isolated from horses at the same time after administration of BWHE. These differences were averaged for the final analysis. When gene expression of the pooled control animal probe was compared against probe prepared from the individual animals within the control group, < 2-fold change in gene expression was observed. As our criterion for a significant change in gene expression as a consequence of BWHE treatment was defined as a > 2-fold change in expression relative to the pooled controls, variation contributed by individual horses in the pooled control groups was controlled. Furthermore, pooling of the control animal RNA templates yielded a consistent control against which the effects of BWHE on gene expression were examined.

RESULTS

Clinical signs and gene expression profiles at 1.5 hr

There were no significant alterations in clinical or hematologic parameters in the 1.5-hr BWHE treatment group or in the horses in the control group. Gene expression profiles in the latter animals are considered to reflect those of normal laminar tissue, with minor alterations potentially caused by the intragastric administration of water.

Gene expression data at 1.5 hr are summarized graphically as a scatter plot comparing gene expression in laminar tissues of BWHE and control horses (Figure 6.1). In the scatter plot, unaltered transcripts are depicted by spots that are evenly distributed about the identity axis. By our analysis criteria, expression of these genes is changed by < 2-fold when compared to their expression in the control group. The distance from the graph origin, a measure of signal intensity, reflects transcript intensity, with those closest to the origin being present in low abundance, and those furthest from the origin having high intensity, regardless of the effects of BWHE on transcription. The even distribution of the transcripts is evidence of the quality of the microarray, as poor hybridization would have been reflected by concentration of the spots towards the graph origin, and tailing of the spots would be indicative of dye-bias or other hybridization artifacts. No such artifacts are evident, and only a few spots have weak signal intensity.

As is evident in Figure 1, 8 transcripts were significantly up-regulated and 6 transcripts were significantly down-regulated in laminar tissues of the BWHE horses at 1.5 hr when compared to expression in the control group.

Transcripts with a >2-fold change in expression 1.5 hr after administration of BWHE are listed in Table 6.1, along with the E-value of the EST gene annotation, the condensed gene annotation, and the EST gene ID. Changes in expression are presented using a $\log_2$ scale and are ranked from high to low relative to their appropriate controls. The E-values provide an index of annotation accuracy obtained from EST BLAST annotations, with low E-values (< 1.00E-9) reflecting high quality annotations ("strength" of the match); all but 3 genes whose level of expression changed by >2-fold had E-values < 1.00E-9 (EST gene ID's: MONO1_20-G07, LeukoN6_3-D05 and LeukoN3_8-B12). Annotations with higher E-values values may change as

the sequence databases against which the BLAST annotation was performed receives more accurately curated sequences. This latter group has a high likelihood of including genes unique to horses. The information in the EST gene ID column in Table 6.1 provides the sequence names for the ESTs from which PCR gene targets were prepared (full information about these ESTs is accessible via http://fungen.org.).

The transcripts in Table 6.1 include proinflammatory cytokines, chemokines, cell surface molecules and signaling molecules, suggesting that products originating from different cellular compartments play critical roles in mediating the early responses to BWHE. The 8 immediate-early response genes whose expression increased > 2-fold include transcripts for secreted mediators such as CCL7/MCP-3, MCP-1, CXCL10/IP-10 and SAA, as well as intracellular signal modulators such TNFAIP3; all of these genes play critical roles in inflammatory processes. Six gene transcripts were down-regulated in laminar tissue 1.5 hr after administration of BWHE (Table 6.1); all but one of these genes are associated with B-cell immunoglobulin production. At this time, it is unclear whether this decrease in expression reflects changes in gene expression or remodeling of leukocyte populations (e.g. B-cell death or trafficking from the tissue) during the progression of laminitis.

Clinical signs and gene expression profiles at 3 hr

There were no significant alterations in clinical or hematologic parameters in the 3 hr control group. The number of circulating leukocytes decreased by at least 30% in the horses administered BWHE; this change has been reported previously with this model of laminitis induction (Galey et al., 1991). The gene expression data at 3 hr are summarized graphically as a scatter plot comparing gene expression in horses administered BWHE with their respective

control group (Figure 6.2).

As is evident in Figure 6.2, 17 transcripts were significantly up-regulated and 5 transcripts were significantly down-regulated in laminar tissues from the horses in the BWHE treatment group when compared to expression in the control group.

Increased expression of transcripts for CCL7/MCP-3, MCP-1, CXCL10/IP-10, SAA and TNFAIP3 persisted at 3 hr after administration of BWHE, and 12 additional gene transcripts were up-regulated at this time (Table 6.2). The latter include transcripts such as IFN regulatory factor 1, IER5 and granulocyte colony-stimulating factor (G-CSF). All but 1 of the 5 down-regulated genes were associated with B-cell immunoglobulin production; the remaining down-regulated gene encodes for a protein of unclear function. Four of the genes that were down-regulated at 1.5 hr and 1 gene (clone CH241-121C14) encoding for a protein of unclear function were down-regulated at 3 hr relative to control. Two of the transcripts that were down-regulated at 1.5 hr (Acidic 82 kDa protein and Ig variable region VDJ) returned to expression equivalent to that of control group by 3 hr.

Clinical signs and gene expression profiles at Obel grade 1 laminitis

There were no significant alterations in clinical or hematologic parameters in the 12 hr control group. All horses in the BWHE treatment group became leukopenic between 3-4 hr and clinical signs indicative of Obel grade 1 laminitis developed in 8 (of 12) horses.

As evident in Figure 6.3, 64 transcripts were significantly up-regulated and 8 transcripts were significantly down-regulated in laminar tissues from the BWHE treatment group when compared to expression in the control group.

At 12 hr after administration of BWHE, not only did expression of chemokines CCL7/MCP-3 and MCP-1 remain elevated, but several additional gene transcripts were up-regulated (Table 6.3). The latter included transcripts for antimicrobial compounds (e.g., calgranulin C , β-defensin 1) and for genes that regulate proteolytic activity (e.g., MMP-9, nexin-1 and metalloproteinase inhibitor 1 [TIMP-1]). Other newly up-regulated transcripts included MyD-1, which is involved in the negative regulation of receptor tyrosine kinase signaling pathways, superoxide dismutase (SOD), which is an important component of the endogenous cellular defense system that converts superoxide into oxygen and hydrogen peroxide, serum amyloid A (SAA), which is regarded as a classic acute phase serum protein, and the transcription factor, Egr-1, which is involved in the enhanced expression of cytokines such as TNFα. Egr-1 may contribute to the induction of cytokine expression in laminar tissue in response to BWHE treatment. Eight genes were down-regulated including B-cell associated genes such as those for λ-immunoglobulin and MHC class I antigen, lymphocyte antigen Ly-6H, X2 box repressor (zinc finger transcription factor) homeodomain-only protein (HOP, a small protein that modulates target gene transcription without direct binding to DNA), odd homeobox 1 protein (mitotic checkpoint gene MOB1), and 1 gene for a protein of unclear function.

General features of BWHE-induced gene expression in equine laminar tissues

The number of genes whose expression was significantly altered after administration of BWHE increased over time; 4 genes (CCL7/MCP-3, MCP-1, CXCL10/IP-10 and SAA) were consistently up-regulated and 1 gene transcript (λ-light chain) was consistently down-regulated at all 3 time points. Three genes were up-regulated and 3 genes were down-regulated at both the

1.5 and 3-hr time-points. Fifty-five genes were up-regulated and 7 genes were down-regulated at the Obel grade 1 laminitis group only.

Figures 6.1-6.3 provide visual evidence of the increase in differentially expressed genes over the 3 time periods. While the number of genes that were significantly down-regulated remained stable, there was an obvious increase in the number of genes that were up-regulated at the 3 and Obel grade 1 time points. The scatter plots also demonstrate that the vast majority of genes in the laminar tissue are altered little by administration of BWHE. Certain of these unchanged transcripts encode for known housekeeping genes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which is involved in cellular energetics, cyclophylin, which is involved in protein binding and folding, acidic ribosomal protein, a ribosomal protein, and β_2-microglobulin, a structural protein. Other genes that are minimally altered by administration of BWHE encode for inducible transcripts such as Toll-like receptor 8, GLUT 4, and IL-10.

The transcripts listed in Tables 6.1-6.3 include proinflammatory cytokines, chemokines, cell surface molecules, signaling molecules, and enzymes, suggesting that products originating from different cellular compartments play critical roles in mediating the responses to BWHE.

Several of the transcripts that were up-regulated after administration of BWHE encode for proteins of unclear function, as the annotation process yielded hypothetical or predicted proteins, clone numbers or only DNA sequence numbers. Additional work will be required to determine the functional importance of these transcripts in laminitis.

Of the genes up-regulated in response to administration of BWHE were a group of IFN-inducible genes, including CCL7/MCP-3, Cxcl10/IP-10, transcription factor/ signaling molecules IRF1, and genes encoding cell surface receptors including inhibitory receptor PILRα, RGS2, IgE

receptor γ, INF-γ receptor 2 and ICAM-1. Up-regulation of these genes as early as 1.5 hr after administration of BWHE is consistent with induction of inflammatory responses in the laminar tissue.

The temporal changes in gene expression are presented as heat maps (Figure 6.4) in which changes in gene expression across the 3 time points are represented by changes in colors in a 2-dimensional map, with each row representing expression of a specific transcript.

DISCUSSION

Microarrays provide an effective way to evaluate changes in expression of large numbers of genes simultaneously, and has become a sophisticated and affordable way to study processes as complex as inflammation (Fulop and Falus, 2004). For example, in studies of equine articular disease, oligonucleotide microarrays have proved useful in identifying specific genes that may contribute to the pathophysiologic state (Gu and Bertone, 2004; Santangelo et al., 2007). In the current study, our goal was to identify temporal changes in expression of more than 3,000 genes in laminar tissues during the onset of acute laminitis. In an effort to minimize the generation of artifacts and to focus on the most pronounced changes in gene expression in the laminar tissue during the onset of acute laminitis, we applied quite stringent criteria to the analysis of the gene expression data generated in this study. In doing so, we determined that expression of less than 100 of the 3,069 genes monitored changed significantly during the first 12 hr after administration of BWHE.

Of the genes induced by administration of BWHE in the present study, a few had previously been determined to be increased during the development of acute laminitis (e.g.

MMP-9 (Loftus et al., 2006; Mungall et al., 2001; Mungall and Pollitt, 1999; Mungall et al., 1998). Our finding that a relatively small complement of genes were induced or suppressed in response to administration of BWHE are consistent with those of other studies in which microarrays were used to monitor the effects of potent and pleuotropic agents (e.g. lipopolysaccharide) on gene expression (Bjorkbacka et al., 2004; Hirotani et al., 2005). When less stringent analysis criteria were applied to our microarray data, expression of genes encoding for IL-1β, TNF-α, MMP-2 and Cox-2 (*data not shown*) were increased, as has been reported previously using real-time quantitative PCR methods as an accurate means of assessing gene expression (Kyaw-Tanner and Pollitt, 2004; Rodgerson et al., 2001; Waguespack et al., 2004a; Waguespack et al., 2004b).

At present, there are several principal theories for the development of equine laminitis, namely the ischemic/vascular, mechanical/traumatic, inflammatory, and toxic/enzymatic theories (Hood, 1999). Rather than involving a single pathway, the mechanisms involved in the pathogenesis of laminitis probably are interrelated with several factors contributing to the development of the disease. In the current study, we identified changes in expression of genes that could be linked with more than 1 of the aforementioned theories. For example, at the early (1.5 and 3 hr) sample times, we identified up-regulation of several pro-inflammatory genes (MCP-3 MCP-1, IP-10 and ICAM-1) and concomitant suppression of genes encoding for products associated with B-cell related immune responses. Of particular relevance to the development of acute laminitis is the fact that the chemokines MCP-1 and MCP-3 (Proost et al., 1996) attract leukocytes, activate platelets, and induce inflammatory responses. MCP-1 also triggers recruitment, diapedesis and homing of T-lymphocytes and monocytes, as well as activation of macrophages (Simionescu, 2007). Additionally, during the first 3 hr after

administration of BWHE, IP-10 is also up-regulated. IP-10 stimulates monocytes, promotes migration of natural killer and T-cells, regulates T-cell and bone marrow progenitor maturation, and promotes expression of adhesion molecules (Neville et al., 1997). Increased accumulation of IP-10 transcripts could in part be due to increased mRNA stability, as transcripts for S-100 protein isoforms, which extend the half-life of IP-10 mRNA, are expressed to a greater extent starting at 3 hr after administration of BWHE. At the same time, the increased expression of ICAM-1, a cell surface adhesion molecule involved in neutrophil and monocyte extravasation (Carman and Springer, 2004; Konstantopoulos et al., 1998), would promote movement of these cells into the laminar tissue. Collectively, the increased expression of MCP-3, MCP-1, IP-10, and ICAM-1 may be responsible for the early activation and migration of leukocytes from the circulation into the laminar tissues.

Granulocyte colony stimulating factor (G-CSF), a potent chemokine that triggers the production and release of neutrophils from bone marrow stores (Christopher and Link, 2007) and their activation for enhanced antimicrobial activity (Marshall, 2005), appears to be selectively up-regulated at the 3 hr time point. Thus, although expression of the chemokines is increased from 1.5 hr after administration of BWHE through the onset of Obel grade 1 lameness, expression of ICAM-1 returns to basal levels by this time. These findings may account for the relatively short period of leucopenia, as recruitment of cells is curtailed at the same time genes are being expressed whose function is to promote the production and release of new leukocytes into the circulation.

At the time of Obel grade 1 laminitis, several additional genes are up-regulated that have roles in regulation of inflammation; these genes include inter-alpha-trypsin inhibitor, BiP, L-plastin, serpin and nexin-1. The product of the inter-alpha-trypsin inhibitor gene regulates

adhesion of infiltrating leukocytes (de la Motte et al., 2003; Zhuo et al., 2006), whereas BIP, an endoplasmic reticulum chaperone with anti-inflammatory and immunomodulatory properties, stimulates expression of anti-inflammatory genes in monocytes and leads to the development of T-cells that secrete regulatory cytokines such as interleukin-10 and interleukin-4 (Panayi and Corrigall, 2006). Thus, by the onset of Obel grade 1 lameness anti-inflammatory and immunoregulatory systems are activated to modulate the inflammatory responses and leukocyte trafficking into the extravascular tissues. Similarly, L-plastin regulates leukocyte adhesion by interacting with the receptor for granulocyte-macrophage colony stimulating factor (GM-CSF) and transduces anti-apoptotic signaling in granulocytes (Chen et al., 2003; Delanote et al., 2005), and both serpin (alpha-1-antichymotrypsin 2) and nexin-1 (PN-1) are key regulators of inflammation, coagulation, apoptosis, extra-cellular matrix composition and complement activation (Kim et al., 2006; Richardson et al., 2006).

At the onset of Obel grade 1 laminitis, 2 genes whose products have antioxidant properties are up-regulated, namely SOD, and Kelch-like ECH-associated protein. Increased expression of these genes is consistent with the concurrent increase in laminar tissue concentrations of isoprostanes, which are produced locally as the result of oxidation of archidonic acid in cell membranes (Noschka et al, 2007). SOD has tissue-sparing effects by converting highly toxic superoxide (O^{2-}) into oxygen and hydrogen peroxide, leading to neutrophil apoptosis and a reduction in neutrophil-mediated tissue injury (Yasui and Baba, 2006; Yasui et al., 2005). The finding that expression of SOD is increased only at the time of Obel grade 1 laminitis is in agreement with the recent report that laminar tissue samples obtained at the 1.5 and 3 hr time points lacked SOD activity (Loftus et al., 2007); in that study, samples from later time points were not evaluated for SOD activity.

Expression of MMP-9 and its regulatory gene, TIMP-1, also were increased at the onset of Obel grade 1 lameness. Similar changes in expression of MMP-9 have been reported in previous studies, and have been associated with dysadhesion and destruction of lamellar basement membrane in laminitic horses (French and Pollitt, 2004; Kyaw-Tanner and Pollitt, 2004; Loftus et al., 2006). Presumably, the up-regulation of protease inhibitors like TIMP-1 occurs in an attempt to control the enzymatic activity of matrix metalloproteinases.

Transcripts for several other proteins involved in inflammatory processes were determined to be induced in the current study. The gene encoding for SAA, an acute phase protein commonly associated with inflammation (O'Brien and Chait, 2006; Vlasova and Moshkovskii, 2006), was up-regulated at all three time points. Similarly, transcripts for pro-inflammatory calgranulin C (Xie et al., 2007), NFAT-activation molecule 1 that regulates the NFAT transcription factor responsible for activation of cytokine gene transcription in basophils and mast cells (Marone et al., 1997), the ATP-binding cassette that influences cellular cholesterol mobilization during inflammation (Soumian et al., 2005), and a transcript variant of oxysterol-binding protein involved in regulating the proinflammatory lipid oxysterol (Massey, 2006) are increased at the onset of lameness.

Transcripts encoding for IFN regulatory factor 7, TRAF3 interacting protein 3 and IFN-γ receptor 2 also are up-regulated at the onset of lameness, and may be involved in inflammatory events initiated via activation of IFN or Toll-like receptors (Colonna, 2007; Schroder et al., 2004). For example, induction of G-CSF, MCP-3, MCP-1 and IP-10 can occur through pathways distal to the IFN and Toll-like receptors. At both 1.5 and 3 hr after administration of BWHE, B-cell specific transcripts (e.g. Ig-γ 3, Ig-γ 1 and λ-light chain Ig) decrease in the laminar tissue indicating either specific suppression of these genes in resident B-cells, apoptosis of B-cells, or

trafficking of B-cells out of the laminar tissue. Further investigations, using B-cell specific markers or in situ monitoring of immunoglobulin transcripts, would be necessary to identify which of these mechanisms is/are involved in the changes identified in these B-cell specific transcripts. Based in part on the limited relevance of such experiments to the development of acute laminitis, we have not attempted to follow this line of study further.

Several genes responsible for synthesis of anti-microbial products are induced strongly at the onset of Obel grade 1 lameness. It is feasible that these genes are induced in response to movement of bacterially-derived substances, such as endotoxin or products liberated by gram positive organisms, across a damaged intestinal mucosal barrier (Krueger et al., 1986; Weiss et al., 2000; Weiss et al., 1998). These anti-microbial genes include serotransferrin, an iron-binding protein that decreases iron availability to microorganisms by decreasing iron reutilization (Jurado, 1997), β-defensin-1, a host-derived cationic peptide with antibiotic activity that also exhibits chemoattractant activity for a range of different cell types at the sites of inflammation (Hoover et al., 2002), and elafin, an elastase-specific inhibitor with "defensin"-like antimicrobial activities that promotes inflammation when leukocyte contents are secreted in the extracellular environment (Sallenave, 2000). Transcripts for MyD-1 antigen, a protein that inhibits LPS-induced TNF-α production by monocytes (Smith et al., 2003), are also increased at the onset of lameness, perhaps indicative of the host's response to entry of endotoxin into the systemic circulation.

Collectively, the results of this study provide convincing evidence that genes associated with inflammation, activation and extravasation of leukocytes, antimicrobial activities, and destruction of the lamellar basement membrane are induced in response to administration of BWHE. The cDNA microarray used in this project allowed identification of changes in the

expression of genes not previously known to have possible relevance to the progression of laminitis in horses. Because our microarray was generated by randomly sequencing ESTs from several equine cDNA libraries, the microarray contains transcripts for which there is no clear functional annotation. Expression of seven of these transcripts was significantly changed as a result of administration of BWHE. As additional high quality gene sequences become available, it may be possible to identify these genes. By modulating the expression of genes determined to be altered by administration of laminitis-inducing substances, such as BWHE, future studies may determine the roles of specific genes in the development of laminitis.

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Table 6.1. Changes in gene expression in laminar tissue 1.5 hr after administration of BWHE.

Fold change * (log_2)	E-value	Condensed Annotation, Abbreviated Gene Name and Annotating Species‡	EST gene ID
4.41	1.00E-56	Monocyte chemotactic protein 3 (MCP-3/CCL7) [Macaca mulatta]	MONO1_14_G03
3.77	2.00E-56	Intercellular adhesion molecule-1 (ICAM-1) [Canis familiaris]	MONO1_16_D05
3.64	2.00E-19	Monocyte chemoattractant protein-1 (MCP-1/CCL2) [Equus caballus]	MONO1_10_F05
2.98	3.00E-128	Monocyte chemotactic protein 3 (MCP-3/CCL7) [Macaca mulatta]	MONO1_14_F10
2.18	1.00E-101	IFN-γ inducible protein-10 (IP-10/CXCL10) [Bos taurus]	APL1_3_F01
1.66	1.00E-12	Serum amyloid A protein (SAA) [Canis familiaris]	APL1_4_G02
1.50	8.00E-138	cDNA DKFZp459E042 (noID1) [Pongo pygmaeus]	MONO1_14_B06
1.49	2.00E-05	Tumor necrosis factor-alpha-induced protein 3 (TNFAIP3) [Homo sapiens]	LeukoN6_3-D05
-1.49	3.00E-27	DJ561L24.2, acidic 82 kDa protein; deoxynucleotidyltransferase, terminal, interacting protein 2 (DNTTIP2) [Homo sapiens]	LeukoS6_5_C11
-2.06	1.00E-40	Immunoglobulin variable region (IgVDJ) [Homo sapiens]	LeukoS5_2_G04
-2.26	1.00E-93	Imunnoglobulin-γ 3 heavy chain (Ig-γ 3) [Equus caballus]	LeukoN4_2_F04
-3.86	1.00E-76	λ-Immunoglobulin (λ-Ig) [Equus caballus]	MONO1_17_C11
-3.99	9.00E-48	λ-Immunoglobulin (λ-Ig) [Equus caballus]	LeukoN6_3_G09
-4.24	1.00E-98	Immunoglobulin-γ 1 heavy chain (Ig-γ 1) [Equus caballus]	MLN1_3_D07

* >2-fold change in expression at 95%CV

‡ Annotations for transcripts of unclear function abbreviated as noID1, noID2, etc.

Table 6.2. Changes in gene expression in laminar tissue 3 hr after administration of BWHE.

Fold change * (log_2)	E-value	Condensed Annotation, Abbreviated Gene Name and Annotating Species‡	EST gene ID
4.59	1.00E-56	Monocyte chemotactic protein 3 (MCP-3/CCL7) [Macaca mulatta]	MONO1_14_G03
4.65	2.00E-56	Intercellular adhesion molecule-1 (ICAM-1) [Canis familiaris]	MONO1_16_D05
4.53	2.00E-19	Monocyte chemoattractant protein-1 (MCP-1/CCL2) [Equus caballus]	MONO1_10_F05
3.71	1.00E-12	Serum amyloid A protein (SAA) [Canis familiaris]	APL1_4_G02
3.40	3.00E-128	Monocyte chemotactic protein 3 (MCP-3/CCL7) [Macaca mulatta]	MONO1_14_F10
2.80	1.00E-88	Hypothetical protein LOC456757 (noID2) [Pan troglodytes]	MONO1_16_F10
2.59	1.00E-101	IFN-γ inducible protein-10 (IP-10/CXCL10) [Bos taurus]	APL1_3_F01
2.25	8.00E-138	cDNA DKFZp459E042 (noID1) [Pongo pygmaeus]	MONO1_14_B06
2.12	5.00E-124	IFN regulatory factor 1 (IRF1) [Bos taurus]	MONO1_23_E08
2.05	1.00E-138	Immediate early response gene 5 (IER5) [Homo sapiens]	APL1_6_H08
2.02	9.00E-40	Complement factor B; Properdin factor B ; Glycine-rich beta glycoprotein (GBG/PBF2) [Homo sapiens]	APL1_2_C08
1.93	0.00E+00	Granulocyte colony-stimulating factor (G-CSF) [Equus caballus]	MONO1_18_B07
1.84	1.00E-14	Leucine carboxyl methyltransferase 1; Protein-leucine O-methyltransferase (LCMT1) [Canis familiaris]	APL1_8-G11
1.63	5.00E-38	S-100P protein (S-100P) [Homo sapiens]	MONO1_14_B05
1.59	2.00E-63	Immune-responsive protein 1 (IRP1) [Mus musculus]	LeukoS6_2_A03
1.54	2.00E-05	Tumor necrosis factor-alpha-induced protein 3 (TNFAIP3) [Homo sapiens]	LeukoN6_3_D05
1.52	2.00E-80	Ras homolog gene family member, RHOH (RHOH) [Pan troglodytes]	LeukoN1_1_H01
-1.60	1.00E-141	clone CH241-121C14 (noID3) [Equus caballus]	LeukoS4_6_B12
-2.14	1.00E-93	Imunnoglobulin-γ 3 heavy chain (Ig-γ 3) [Equus caballus]	LeukoN4_2_F04
-2.68	1.00E-98	Immunoglobulin-γ 1 heavy chain [(Ig-γ 1) Equus caballus]	MLN1_3_D07
-2.87	1.00E-76	λ-Immunoglobulin (λ-Ig) [Equus caballus]	MONO1_17_C11
-3.64	9.00E-48	λ-Immunoglobulin (λ-Ig) [Equus caballus]	LeukoN6_3_G09

* >2-fold change in expression at 95%CV
‡ Annotations for transcripts of unclear function abbreviated as noID1, noID2, etc.

Table 6.3. Changes in gene expression in laminar tissue at Obel grade 1 laminitis.

Fold change * (log_2)	E-value	Condensed Annotation, Abbreviated Gene Name and Annotating Species‡	EST gene ID
3.50	7.00E-36	Calgranulin C; Calcium-binding protein in amniotic fluid 1 (CAGC/CAAF1) [Bos taurus]	LeukoN5_5_E07
3.71	1.00E-12	Serum amyloid A (SAA) [Canis familiaris]	APL1_4_G02
3.48	1.00E-14	Leucine carboxyl methyltransferase 1; Protein-leucine O-methyltransferase (LCMT1) [Canis familiaris]	APL1_8_G11
3.47	8.00E-138	cDNA DKFZp459E042 (noID1) [Pongo pygmaeus]	MONO1_14_B06
3.40	1.00E-108	MyD-1 antigen (MyD-1) [Bos taurus]	APL1_8_H02
3.22	1.00E-129	Superoxide dismutase (Mn-SOD) [Equus caballus]	LeukoS1_6_G06
3.16	1.00E-09	Inhibitory receptor PILR-α (PILR-α) [Homo sapiens]	LeukoN5_5_F02
3.03	2.00E-46	NFAT activation molecule 1 (NFAM1) [Homo sapiens]	LeukoS6_3_G05
2.86	8.00E-70	Serum amyloid A protein (SAA) [Equus caballus]	APL1_9_G04
2.80	5.00E-94	Serotransferrin; Siderophilin; β-1-metal binding globulin (STF) [Equus caballus]	APL1_8_F11
2.68	2.00E-09	Beta-defensin 1 (BD-1) [Sus scrofa]	APL1_8_A04
2.65	0.46	RIKEN clone:4932441M08 product (noID4) [Mus musculus]	LeukoN3_8_B12
2.65	5.00E-58	Serum amyloid A protein (SAA) [Canis familiaris]	APL1_8_H08
2.64	2.00E-80	Ras homolog gene family member, RHOH (RHOH) [Pan troglodytes]	LeukoN1_1_H01
2.62	9.00E-98	Metalloproteinase inhibitor 1 (TIMP-1) [Equus caballus]	APL1_4_D12
2.60	9.00E-34	Elafin; Elastase-specific inhibitor; Skin-derived antileukoproteinase (SKALP) [Homo sapiens]	MONO1_13_C03
2.58	9.00E-44	Neutrophil Gelatinase (MMP-9) [Homo sapiens]	APL1_6_E02
2.51	2.00E-18	Kelch-like ECH-associated protein 1; Cytosolic inhibitor of Nrf2 (KEAP1) [Mus musculus]	APL1_9_A07
2.32	1.00E-72	Inter-alpha-trypsin inhibitor heavy chain H2; Serum-derived hyaluronan-associated protein (SHAP) [Homo sapiens]	APL1_6_G05
2.25	6.00E-62	Serpin peptidase inhibitor, clade F; alpha-2-antiplasmin (SERPINF2) [Bos taurus]	APL1_3_E01
2.22	4.00E-78	Mitoxantrone-resistance associated gene (MRAG) [Homo sapiens]	APL1_6_C06
2.16	9.00E-40	Complement factor B; Properdin factor B; Glycine-rich beta glycoprotein; (GBG/PBF2) [Homo sapiens]	APL1_2_C08
2.11	7.00E-60	DNA sequence from clone RP4-564F22 on chromosome 20 (noID5) [Homo sapiens]	APL1_8_B10
2.04	1.00E-138	Immediate early response gene 5 (IER5) [Homo sapiens]	APL1_6_H08
2.00	6.00E-170	Secretory granule proteoglycan core protein; Platelet proteoglycan core protein; Hematopoetic proteoglycan core protein; Serglycin (P.PG) [Canis familiaris]	LeukoN5_8_A04
1.98	1.00E-56	Monocyte chemotactic protein 3 (MCP-3/CCL7) [Macaca mulatta]	MONO1_14_G03

1.97	4.00E-171	ATP-binding cassette, sub-family A (ABC1) [Homo sapiens]	APL1_8_C10
1.97	0.00E+00	Early growth response protein 1; Krox-24 protein; Nerve growth factor-induced protein A; Transcription factor ETR103 transcript variant 2 (EGR-1) [Canis familiaris]	LeukoN5_2_F11
1.93	5.00E-38	S-100P protein (S-100P) [Homo sapiens]	MONO1_14_B05
1.92	3.00E-52	UDP-N-acetylglucosamine transporter (UDP-GlcNAc T) [Bos taurus]	APL1_4_C12
1.89	2.00E-19	Monocyte chemoattractant protein-1 (MCP-1/CCL2) [Equus caballus]	MONO1_10_F05
1.89	0.00E+00	Oxysterol-binding protein-like protein 5, transcript variant 1 (LOC532690) (OSBP5v1)[Bos taurus]	LeukoS3_3_A06
1.88	1.00E-88	Hypothetical protein LOC456757, transcript variants1-3 (noID2) [Pan troglodytes]	MONO1_16_F10
1.84	1.00E-46	Purine nucleoside phosphorylase; Inosine phosphorylase (PNP) [Homo sapiens]	LeukoN1_2_B08
1.83	6.00E-56	IFN regulatory factor 7 isoform d (IFNRF7)[Homo sapiens]	MONO1_14_C03
1.76	2.00E-18	Protein-glutamine γ-glutamyltransferase (GγGT) [Homo sapiens]	MONO1_21_H07
1.72	1.00E-41	Inorganic pyrophosphatase; Pyrophosphate phospho-hydrolase (PPase) [Bos taurus]	LeukoS6_2_E09
1.71	5.00E-178	Lactamase, β 2, transcript variant 1 (LOC486990) (Lactβ2v1) [Canis familiaris]	APL1_9_G10
1.66	2.00E-35	Alpha-1-antichymotrypsin 2; SERPINA3 (α1-ACT) [Sus scrofa]	APL1_4_D07
1.63	3.00E-40	Endoplasmin; 94 kDa glucose-regulated protein (GRP94) [Canis familiaris]	LeukoS5_3_H01
1.60	8.00E-35	Regulator of G-protein signaling 2 (RGS2) [Rattus norvegicus]	LeukoS1_3_B10
1.60	2.00E-51	Mitochondrion, complete genome (MitoX) [Equus caballus]	LeukoS4_1_E05
1.58	1.00E-11	Splicing factor 3B subunit 1; Spliceosome associated protein 155 (SF3b155) [Homo sapiens]	LeukoN3_7_B01
1.57	1.00E-120	Nexin-1 Fragment (Nexin1) [Sus scrofa]	LeukoS3_3_A03
1.53	4.00E-46	C1-inhibitor (C1i) [Homo sapiens]	APL1_3_A07
1.52	2.00E-80	Haptoglobin alpha and beta chains (Hp) [Canis familiaris]	APL1_8_A08
1.51	4.00E-140	TRAF3 interacting protein 3(TRAF3IP3) [Homo sapiens]	LeukoS6_5_B10
1.48	3.00E-162	Secretory granule proteoglycan core protein; Platelet proteoglycan core protein; Hematopoetic proteoglycan core protein; Serglycin (P.PG) [Canis familiaris]	LeukoS3_2_F09
1.48	8.00E-51	Heterogeneous nuclear ribonucleoproteins C1/C2 (hnRNPC1/2 [Mus musculus]	LeukoS3_2_F09
1.47	0.00E+00	X-box binding protein 1 (XBBP1) [Canis familiaris]	APL1_3_A05
1.47	2.00E-20	BiP protein, Fragment (BiP) [Homo sapiens]	APL1_6_D07
1.46	3.00E-24	BiP protein, Fragment (BiP) [Homo sapiens]	MLN1_5_H09
1.45	2.00E-50	IFN-γ receptor 2; IFN-γ transducer 1 (IFNγR2) [Homo sapiens]	APL1_8_H10
1.43	4.00E-80	Secretory granule proteoglycan core protein; Platelet proteoglycan core protein; Hematopoetic proteoglycan	LeukoS4_1_A05

		core protein; Serglycin (P.PG) [Canis familiaris]]	
1.43	3.00E-28	ATP-binding cassette, sub-family D member 1 (ALD) [Pan troglodytes]	MONO1_17_D03
1.42	6.00E-113	L-plastin (LCP1) [Pan troglodytes]	LeukoS1_8_D10
1.42	1.00E-37	Secretory granule proteoglycan core protein; Platelet proteoglycan core protein; Hematopoetic proteoglycan core protein; Serglycin (P.PG) [Homo sapiens]	LeukoS5_2_A05
1.42	9.00E-35	DNA sequence from clone RP3-512E2 on chromosome 6 q14.3-16.1 (noID6) [Homo sapiens]	LeukoS5_5_A02
1.42	0.00E+00	RAB1A, member RAS oncogene family (RAB1A) [Bos taurus]	MONO1_10_E07
1.41	1.00E-76	Adhesion regulating molecule 1; 110 kDa cell membrane glycoprotein (Gp110) [Rattus norvegicus]	APL1_8_D07
1.40	3.1	hypothetical protein CBS 148.51; CHGG_07130 (noID7) [Chaetomium globosum]	MONO1_20_G07
1.37	3.00E-85	60S ribosomal protein L13; Breast basic conserved protein 1 (RPL13) [Homo sapiens]	MONO1_23_B06
1.36	2.00E-74	Alpha-1-antitrypsin (A1AT/Spi2) [Equus caballus]	APL1_9_G02
1.35	4.00E-32	High affinity IgE receptor γ-subunit (IgERγ) [Equus caballus]	MONO1_21_D05
-1.35	3.00E-40	Brain-expressed HHCPA78 homolog VDUP1; thioredoxin interacting protein (TXNIP) [Homo sapiens]	LeukoN4_2_H11
-1.37	9.00E-48	λ-Immunoglobulin (λ-Ig) [Equus caballus]	LeukoN6_3_G09
-1.41	3.00E-35	Odd homeobox 1 protein (OB1) [Bos taurus]	LeukoN5_1_G03
-1.41	1.00E-171	Homeodomain-only protein, transcript variant 12 (HOP) [Pan troglodytes]	LeukoN5_1_G03
-1.43	1.00E-51	MHC class I antigen gene, subclone 274K01 3.6 (MHC1)[Equus caballus]	LeukoS4_4_H12
-1.46	4.00E-98	X2 box repressor (XBR) [Homo sapiens]	LeukoS1_7_D05
-1.54	1.00E-141	Clone CH241-121C14 (noID3) [Equus caballus]	LeukoS4_6_B12
-1.55	4.00E-09	Lymphocyte antigen Ly-6H (Ly-6H) [Homo sapiens]	LeukoS4_4_E06

* >2-fold change in expression at 95%CV

‡ Annotations for transcripts of unclear function abbreviated as noID1, noID2, etc.

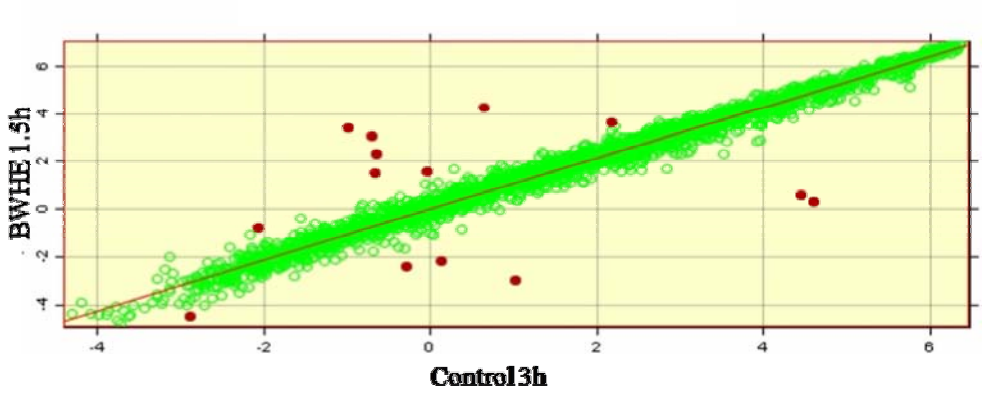

Figure 6.1. Scatter plot of gene expression at 1.5-hr after BWHE administration.

The gene expression values are given in a pairwise comparison of the experimental channel (BWHE treatment) against the control channel (Placebo treatment), with red colored spots indicating genes that are differentially expressed (>2-fold at 95% CV). The spots represent the signal intensity of the 2 channels after normalization, and compare the 2 channels using a log-log plot. Spots that are above the identity (x = y) line correspond to genes that are up-regulated, and spots below the line correspond to genes that are down-regulated in laminar tissue from BWHE treated horses in comparison to control horses. The data plotted are means of 5 individual horses in the 1.5-hr BWHE treatment group, against the pooled 3-hr control group. Each spot corresponds to a specific gene.

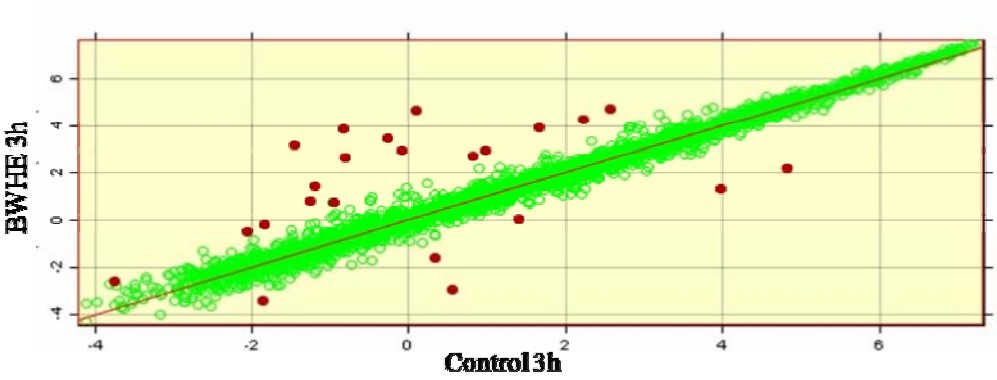

Figure 6.2. Scatter plot of gene expression at 3-hr after BWHE administration.

The data plotted are means of five individual horses in the 3-hr BWHE treatment group, against the pooled 3-hr control group. Each spot corresponds to a specific gene.

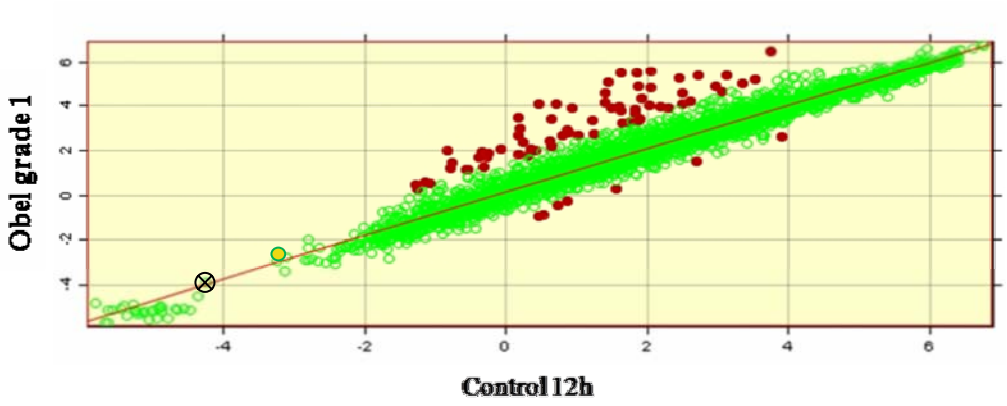

Figure 6.3. Scatter plot of gene expression at Obel grade 1 laminitis.

The data plotted are means of twelve individual 12-hr BWHE-treated horses against the pooled 12-hr control group. Each spot corresponds to a specific gene, with the exception of print buffer only (☒).

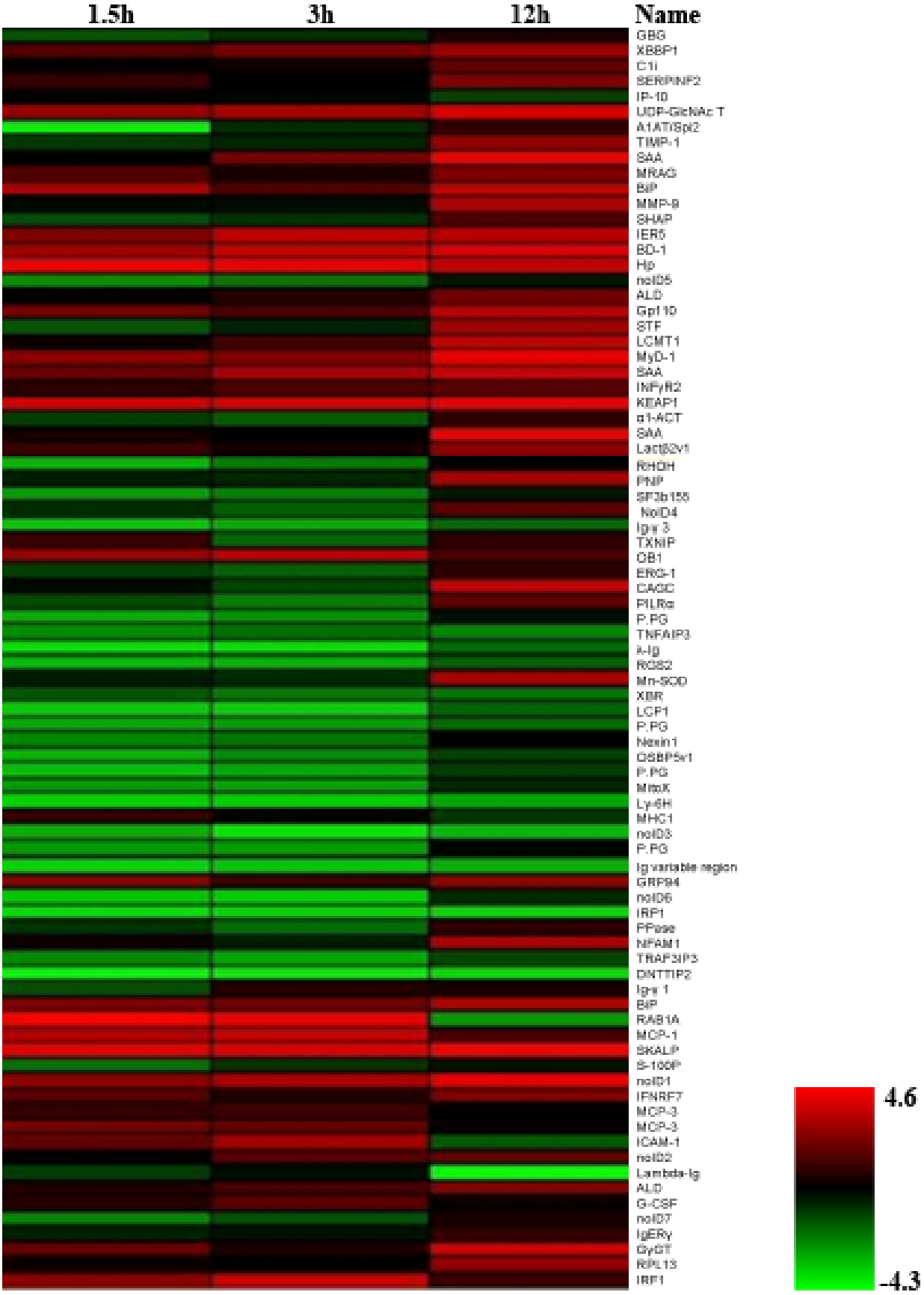
1.5h
3h
12h
Name
GBG
XBBP1
C1i
SERPINF2
IP-10
UDP-GlcNAc T
A1ATISpl2
TIMP-1
SAA
MRAG
BiP
MMP-9
SHAP
IER5
BD-1
Hp
notD5
ALD
Gpt 10
STF
LCMT1
MyD-1
SAA
INFγR2
KEAP1
α1-ACT
SAA
Lact$2V1
RHOH
PNP
SF3b155
NotD4
Ig-κ 3
TXNIP
OB1
ERG-1
CAGC
PILRα
P.PG
TNFAIP3
λ-Ig
RGS2
Mn-SOD
XBR
LCP1
P.PG
Nexin1
OSBP5v1
P.PG
MttpX
Ly-6H
MHC1
notD3
P.PG
Ig variable region
GRP94
notD6
IRP1
FPase
NFAM1
TRAF3IP3
DNTTIP2
Ig-κ 1
BiP
RAB1A
MCP-1
SKALP
S-100P
notD1
IFNRF7
MCP-3
MCP-3
ICAM-1
notD2
Lambda-Ig
ALD
G-CSF
notD7
IgERγ
GyGT
RPL13
IRF1
4.6
-4.3

Figure 6.4. Heat map of gene expression at 1.5 and 3-hr after BWHE administration and at Obel grade 1 laminitis.

The intersection of a gene (row) and sample name (column) is colored according to its expression value; red indicates high expression, green indicates low expression, and the intensity of the color is indicative of the relative level of expression. The color key represents these expression differences and assigns a fold change (log_2) value to the brightest red and green colors. All genes included in this map changed significantly in expression by at least 2-fold (95% CV) at least at one of the three time points and are presented over the three time points.

CHAPTER 7

CONCLUSION

Based on a recent national survey of horse owners, nearly 13% of all horses in the United States experienced at least one episode of laminitis in their lifetime,[1] and laminitis was the second most common reason for euthanasia in horses. In these cases, the end result was dissociation of the sensitive and insensitive laminae that bond the distal phalanx to the inner hoof wall, dislocation of the distal phalanx (coffin bone) and severe pain.

Laminitis is often linked with diseases associated with clinical evidence of endotoxemia, including diseases as diverse as intestinal strangulating obstruction, colitis and inflammatory bowel disease, grain overload, retained fetal membranes and pleuro-pneumonia.[2,3] There are several theories regarding the mechanisms responsible for initiating the structural failures that occur in horses with laminitis, including: ischemic/vascular, mechanical/traumatic, inflammatory, and toxic/enzymatic.[4] Evidence exists to support components of each theory, suggesting that the underlying pathogenesis of laminitis is complex. For example, the results of early studies of acute laminitis documented the loss of the intestinal mucosal barrier in horses with experimentally-induced laminitis, potentially allowing for the entry of endotoxins into the circulation.[5-7] However, administration of endotoxins by various routes has not induced laminitis.[8] Futhermore, administration of endotoxin to horses elicited arterial vasoconstriction within

the digit, a finding that is in contrast to the venoconstriction that has been identified as an important hemodynamic change during the development of laminitis.[8] In more recent studies, some investigators have reported changes in local expression of inflammatory mediators in the laminae of horses during the prodromal stages of laminitis, while others have focused on alterations in digital hemodynamics.

The main purposes of the studies reported here were to investigate the vasoactive properties of prostanoids and isoprostanes in order to determine their possible roles in the development of equine laminitis, and to evaluate gene expression profiles in laminar tissues at three time points during the onset of laminitis. The overall goal of the studies was to improve our knowledge regarding the pathogenesis of laminitis and thereby identify potential new targets for treatment of affected horses.

In the studies reported here, we investigated the possible links between changes in inflammatory gene expression in the laminar tissues with the vasoconstrictor effects of prostanoids, thromboxane, prostaglandin E_2 and iso-prostanes on laminar microvessels of control horses and horses administered black walnut heartwood extract (BWHE). These studies were performed using an equine-specific microarray developed in our laboratory, and by examining the functional responses of laminar microvessels in small vessel myographs in Dr. Tom Robertson's laboratory.

In the first three studies, we focused on the possible roles of thromboxane, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin E_2 (PGE_2), and isoprostanes in the development laminitis induced by administration of BWHE. We determined that plasma concentrations of thromboxane, PGE_2 and $PGF_{2\alpha}$ increased transiently and coincide with the presence of severe leucopenia. Although plasma concentrations of iso-$PGF_{2\alpha}$ did not

change in either control or BWHE-administered horses, laminar tissue concentrations of iso-$PGF_{2\alpha}$ were significantly increased in BWHE horses at the onset of Obel grade 1 lameness when compared to control horses. These effects of BWHE administration are consistent with the development of systemic inflammatory events during the prodromal stages of BWHE-induced laminitis. The observation that laminar tissue concentrations of iso-$PGF_{2\alpha}$ increased in the absence of changes in plasma concentrations of the same mediator suggests that generation of reactive oxygen species (ROS) may be confined to the digit during the development of laminitis. This local generation of ROS may be due to local infiltration of the laminar tissues by activated leukocytes, or secondary to ischemia-reperfusion injury. The lack of changes in laminar tissue concentrations of iso-$PGF_{2\alpha}$ at the early time points (1.5 and 3 hours) suggest that oxidative stress occurs subsequent to the infiltration of leukocytes and coincides with venoconstriction and edema formation in the laminar tissues. This finding also provides support for a link between local inflammatory and vascular alterations. The relative lack of efficacy of non-steroidal anti-inflammatory drugs in treatment of horses with laminitis may be due to the vasoconstrictive effects of isoprostanes in the laminar tissues, as non-steroidal anti-inflammatory drugs do not affect the enzyme-independent mediated production of isoprostanes from arachidonic acid.

Studies in which laminar arteries and veins were incubated with increasing concentrations of the aforementioned eicosanoids in the absence or presence of putative prostanoid and thromboxane receptor antagonists revealed the following: In control horses, U46619, $PGF_{2\alpha}$, iso-$PGF_{2\alpha}$ and $isoPGE_2$ more potently and efficaciously constricted laminar veins when compared to laminar arteries. In BWHE-administered

horses, however, the response of laminar veins, but not laminar arteries, to the thromboxane analog (U46619) at the onset of Obel grade 1 laminitis was reduced. This diminished response may be due to a down-regulation or desensitization of the constricting E prostanoid receptors, which, based on our findings, most likely are the EP_1 receptors. A similar pattern has been reported previously for phenylephrine and 5-hydroxytryptamine, suggesting that constrictor responses of laminar veins are generally down-regulated at Obel grade 1 laminitis.[9]

PGE_2 is involved in the regulation of many physiological processes, including inflammation and local blood flow. It modulates immune responses by regulating the function of macrophages, T and B lymphocytes, and can lead to either pro- or anti-inflammatory effects.[10] PGE_2 also stimulates the release of vascular endothelial growth factor,[11,12] induces cell migration, and increases matrix metalloproteinase-2 expression and activation.[13,14] Whereas PGE_2 elicited small dilator responses in laminar veins isolated from control horses, laminar veins from BWHE-administered horses constricted in response to PGE_2. Although PGE_2 is principally regarded as a vasodilator, it can elicit venoconstriction in certain vascular beds.[15] Our findings with PGE_2 are indicative of compromised venodilator pathways of both dilatory receptor subtypes (EP_2 and EP_4) being present at Obel grade 1 laminitis.

In horses administered BWHE, laminar veins not only maintained their ability to constrict in response to iso-PGE_2 but they also constricted to a greater degree than normal in response to iso-$PGF_{2\alpha}$. These findings are consistent with the local production of isoprostanes being a potentially important factor in the venoconstriction observed during the development of laminitis. The finding that responses to iso-$PGF_{2\alpha}$ but not to iso-

PGE_2, are increased at the onset of Obel grade 1 lameness may be due in part to the loss of the vasodilatory effects of iso-$PGF_{2\alpha}$ that is present in laminar veins isolated from control horses.

When responses to $PGF_{2\alpha}$ were evaluated in the presence of putative selective prostanoid receptor antagonists, we determined that $PGF_{2\alpha}$ may elicit its vasoconstrictor effects in laminar veins via activation of TP receptors. This finding is consistent with the results of another study in which $PGF_{2\alpha}$ induced constriction of rat isolated aortic ring preparations via activation TP receptors.[16] The degree of constriction of laminar veins isolated from horses administered BWHE to $PGF_{2\alpha}$ was significantly less than that of laminar veins from control horses. Down-regulation or desensitization of the constricting prostanoid (TP, EP_1 or EP_3) receptors may account for the reduction in responses of laminar veins from BWHE administered horses to $PGF_{2\alpha}$. Our observations also are consistent with the presence of a normal $PGF_{2\alpha}$-activatable vasodilator pathway in laminar veins being compromised at Obel grade 1 laminitis, a response that is similar to that identified for iso-$PGF_{2\alpha}$. Together these results are consistent with the concept that the contractile effects of $PGF_{2\alpha}$ are mediated, at least in part, via activation of EP_1, EP_3 and TP receptors and that this pathway may be down-regulated in laminitis.

Ascribing the effects of eicosanoids to respective classically-defined receptor subtypes should be done with caution as not only do PGE and $PGF_{2\alpha}$ activate their respective receptor counterparts (i.e., EP and FP receptors, respectively), but $PGF_{2\alpha}$ can also activate EP_1, EP_3 and TP receptors in some tissues.[17] At least one of the antagonists (AH 6809) used in the present study appears to lack specificity in equine laminar vessels, rendering interpretation of its effects on $PGF_{2\alpha}$ in laminar veins problematic.

Similar to findings reported for other vascular preparations, $PGF_{2\alpha}$ acts predominantly via TP receptors and the density of FP receptors in laminar veins and arteries appears to be quite small or absent as has been reported for some tissues.[18] If $PGF_{2\alpha}$ indeed acts acts predominantly via TP, then drugs that antagonize FP receptors should not be considered as potential treatments for horses with laminitis. In contrast, efforts might be better spent to inhibit the vasoconstrictive effects $PGF_{2\alpha}$ via the TP, EP_1 and EP_3 receptors or to offset the loss of the vasodilator pathway normally activated by $PGF_{2\alpha}$ at Obel grade 1 laminitis. The former approach may be particularly important given the fact that circulating concentrations of $PGF_{2\alpha}$ increase early during the development of the disease, and there is evidence of increased postcapillary resistance at the same time.[19] It is hoped that the development of more specific and chemically-distinct prostaglandin receptor antagonists will help delineate the roles of eicosanoids in the pathogenesis of laminitis.

The results of the first three studies are consistent with both systemic and local inflammatory events occurring during the prodromal stages of BWHE-induced laminitis. Because inflammation and venoconstriction occur concurrently during the development of laminitis, it is possible that the local production of prostanoids and isoprostanes, and the associated venoconstriction contribute to the increase post-capillary resistance in the laminar dermis. Because plasma concentrations of thromboxane, $PGF_{2\alpha}$ and PGE_2, as well as laminar tissue concentrations of isoprostanes increase significantly during the development of BWHE-induced laminitis, and these mediators selectively constrict laminar veins, these eicosanoids may play a important role in the prodromal stages of laminitis. Our observations are consistent with both enzymatic and non-enzymatic

production of eicosanoids playing a key role in the development of laminitis. It is possible, therefore, that these eicosanoids may be viable targets for the development of more effective therapeutic regimens for the treatment of laminitis.

In the fourth study, we pursued the molecular mechanisms involved in the development of equine laminitis using an equine-specific cDNA microarray. This microarray was used to compare expression of more than 3,000 genes in laminar tissues collected at three times during the development of acute laminitis with expression of these genes in laminar tissues of healthy control horses. Our study revealed that as early as 1.5 hours after BWHE administration, proinflammatory genes associated with leukocyte activation and emigration are up-regulated. Other genes involved in inflammatory processes, regulation of inflammation, antioxidant responses, matrix turnover and anti-microbial responses also were up-regulated at the onset of Obel grade 1 lameness. These result are in agreement with the results of our vascular studies, as inflammatory processes and increased productin of ROS may account for the increased systemic concentrations of prostanoids and local concentrations of isoprostanes, respectively.

The potential role of isoprostanes in the pathogenesis of laminitis deserves specific attention as concentrations of these important vasoactive and inflammatory mediators are increased in the laminae at Obel grade 1. Isoprostanes are formed by ROS-mediated peroxidation of arachidonic acid in cell memebranes. Hence, the expression of genes that regulate the redox balance within cells may have a strong bearing upon isoprostane production and, in turn, upon the progression of laminitis. Two genes, superoxide dismutase (SOD) and Kelch-like ECH-associated protein, whose products

have antioxidant properties, are up-regulated at the onset of Obel grade 1 laminitis, at the same time that local concentrations of isoprostanes are increased. In studies performed in other species, production of ROS by activated leucocytes or as a component of the oxidative stress that characterizes ischemia induces the production of antioxidants, such as SOD. SOD may scavenge ROS in plasma and vascular tissues and plays an important role in protecting tissues from oxidative stress. As an example, the inhibition of SOD is associated with an impairment of endothelial control of vascular tone.[20] The finding that expression of SOD is increased only at the time of Obel grade 1 laminitis is in agreement with the recent report that laminar tissue samples obtained at the 1.5 and 3 hour time points lacked SOD activity.[21,22] It is possible, therefore, that the induction of SOD and Kelch-like ECH-associated protein reflect an increase in ROS in the digit at Obel grade 1 and a resultant increase in antioxidant pathways in an attempt to ameliorate the effects of an increase in oxidative stress

As demonstrated in our microarray study, several genes responsible for synthesis of anti-microbial products are induced at the onset of Obel grade 1 lameness. It is feasible that these genes are induced in response to movement of bacterially-derived substances, such as endotoxin or products liberated by gram positive organisms, across a damaged intestinal mucosal barrier.[5-7] These anti-microbial genes provide additional support for a link between endotoxemia and the development of laminitis and might be indicative of the host's response to entry of endotoxin into the systemic circulation.

Expression of the gene for MMP-9 as well as that for TIMP-1, a protein that regulates MMP-9 activity, were increased at the onset of lameness. Similar changes in expression of MMP-9 have been reported in previous studies, and have been associated

with dysadhesion and destruction of lamellar basement membrane in laminitic horses.[23-25] Presumably, up-regulation of genes for protease inhibitors, such as TIMP-1, occurs in an attempt to control the enzymatic activity of matrix metalloproteinases. In contrast to ealier studies providing evidence for an increase in MMP-2 before the onset of clinical signs of laminitis,[25] we detected an increase in MMP-9 only at the onset of Obel grade laminitis. It has been reported that the increase in MMP-2 expression occurs before the first clinical signs of lameness, and thus provides evidence that basement membranes in the laminae are being damaged before clinical signs are apparent. In our microarray study, increased expression of MMP transcripts was evident at the time of Obel grade 1 lameness, but not at the earlier time points. It cannot be determined from our data, however, if expression of these genes occurs before the onset of clinical signs (i.e., between 3 hours after administration of BWHE and the onset of Obel grade 1). Thus, generation of MMP's might be a result of inflammation and further downstream in the casacade of events leading to structural failure of the laminar bond between hoof wall and distal phalanx and not a primary target for the prevention of laminitis in the early, preclinical stage of laminitis.

The results our studies provide convincing evidence that genes associated with inflammation, activation and extravasation of leukocytes, antimicrobial activities, and destruction of the lamellar basement membrane are induced in response to administration of BWHE. Furthermore, concentrations of prostanoids and isoprostanes are increased after administration of BWHE, and are associated with venoconstriction and impairment of vasodilatory pathways. These inflammatory mediators may contribute to the development of laminitis by increasing post-capillary resistance in the laminar dermis.

Up-regulation of proinflammatory genes as early as 1.5 hours after BWHE administration and increased expression of transcripts for SOD at Obel grade 1 correlate well with the changes in production of prostanoids and isoprostanes, and alterations in laminar microvascular function. Collectively, the results of these four studies are consisistent with inflammation being a key component in the cascade of events leading to laminitis. This central axis in the development of laminitis may offer opportunities for the development of therapeutic regimens that address both enzymatic and non-enzymatic production of inflammatory and vasoactive eicosanoids or ROS via free radical scavengers. These data suggest a new and broader therapeutic strategy to more effectively treat equine laminitis in its developmental stage.

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APPENDIX I

Context of these trials

The experiments reported in this appendix were conducted to examine the facility of using a thermography camera to record the local temperature distribution in the hoof, either as absolute or relative to initial temperature, during black walnut heartwood extract induction of laminitis in horses. This trial was preliminary in nature and provided a test bed for the technology and a chance to examine a data set. As the methods had not previously been proven and the number of animals for which the measurements were taken was relatively small (only 5 horses per treatment), we do not feel that these data justified separate publication in the main scientific literature. However, there are both interesting and significant findings among this data. Therefore, we feel if warrants inclusion in this dissertation.

MATERIALS AND METHODS

Animals utilized and their treatment

Ten mixed breed horses ranging in age from 4 to 12 years old (mean 9 years) were used in this study. Each horse lacked clinical evidence of lameness and survey radiographs of the forelimb digits were within normal limits. Horses were randomly assigned to control or black walnut heartwood extract (BWHE) groups. Horses in the control group (5 horses) received 6 L of water via nasogastric tube, and were euthanatized after the collection of the final (12 hour) blood sample. Horses in the BWHE group (5 horses) received BWHE via nasogastric tube and were euthanatized at the onset of Obel grade 1 laminitis (clinical signs consisting of weight shifting

and bounding digital pulses without evidence of lameness at a walk) or at 12 hours after intubation, if signs of Obel grade 1 laminitis had not developed by that time. Each horse was evaluated prior to intubation and every hour thereafter for attitude, heart rate, respiratory rate, capillary refill time, hoof temperature, digital pulses, and evidence of lameness consistent with Obel grade 1 laminitis. Blood samples were obtained via a jugular catheter at 0, 1, 2, 3, 4, 6, 8, 10, and 12 hours. Catheter patency was maintained by flushing the catheter with 20 ml heparinized saline (5 IU heparin/ml) after each blood collection. All protocols were approved by the University of Georgia Institutional Animal Care and Use Committee. The horses were euthanatized using a penetrating captive bolt, as approved by the Report of the American Veterinary Medical Association's Panel on Euthanasia (2001).

Thermographic evaluation of digits

Thermographs of the digits were taken using a high-resolution, remotely sensed forward-looking infrared camera (ThermaCAM® E4, FLIR Systems AB, Danderyd, Sweden). Images were recorded onto the camera prior to blood sampling for each timepoint, downloaded into a personal computer, and analyzed using a software program (ThermaCAM™ QuickView Program version 2.0) which allowed measurement of the mean temperature in a consistently assigned region of the digit.

Briefly, any debris in the scanning field was removed, and approximately 2 minutes allowed to elapse before scanning to ensure that any transient change in the heat pattern generated by debris removal had dissipated before obtaining the thermographs. For all readings, target areas were scanned approximately 0.5m from the horse. Environmental temperature and relative humidity were recorded at each sample time. Although the environment in which the

studies were performed was not temperature controlled, all scans were performed in the same enclosed barn at approximately the same time of day, with no lighting. While there was some fluctuation in temperature and humidity, these patterns were consistent among the horses. The horses were scanned before the administration of water or BWHE, and at each sample time.

After all experiments were completed, a consistent region on each image from each sample time from each horse was compared to corresponding regions in the control group (see details below). This region was selected to encompass the entire area of interest and was consistently repeated at all sampling times for each animal. Temperature was recorded in °C.

Data and statistical analyses

The results of the thermographic study were analyzed using a Student's t test to compare temperature differences between the control horses and BWHE horses. A value of $P < 0.05$ was deemed to be significant.

RESULTS

Hoof Wall Surface Temperature

Hoof wall surface temperature differed significantly between control and BWHE horses at 10 and 12 hours after administration of equal amounts of BWHE or water (Figure 1).

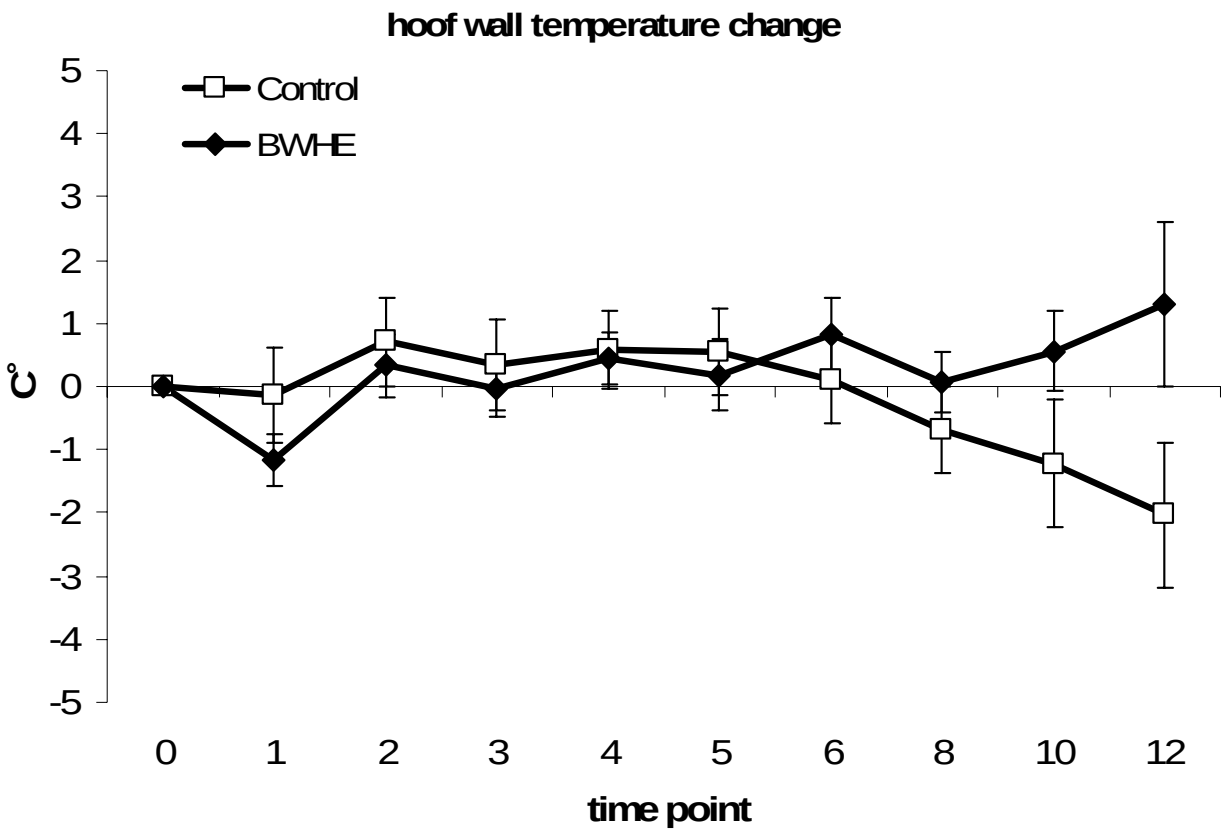

Figure A.1. Mean ± SEM hoof wall temperature in control (□) or BWHE-administered (♦) horses.

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