The Equine Metabolic Syndrome:

Studies on the Pathophysiology of Obesity, Insulin Resistance, and Laminitis in Equids

Dissertation

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Abstract

Insulin resistance has been associated with predisposition to laminitis in horses and ponies. In humans and rodents, omental adipose tissue (AT) expresses pro-inflammatory cytokines and adipokines at significantly higher levels than subcutaneous or retroperitoneal AT, in correlation with the degree of insulin resistance. While this has been postulated to also be a factor contributing to insulin resistance in horses, no published data currently support or refute this hypothesis. This study characterized the expression of pro-inflammatory cytokines and chemokines in several AT depots of insulin-resistant (IR) and insulin-sensitive (IS) horses. No differences in AT expression of TNF-α, IL-1β, IL-6, PAI-1, or MCP-1 were noted between IR and IS groups for each depot. However, when data from IR and IS groups were combined for each depot, the expression of IL-1β (p=0.009) and IL-6 (p=0.023) was significantly higher in nuchal ligament AT than in other depots, suggesting that this AT depot has different biological behavior in the horse and is more likely to adopt an inflammatory phenotype than other depots examined. Importantly, these data indicate that omental AT (and other visceral fat depots) may not be as important to the pathophysiology of obesity in the horse as in other species.

Insulin resistance has been associated with predisposition to laminitis in horses and ponies and frequently accompanies nutritional obesity in this species. In humans and rodents, macrophage infiltration of AT correlates with adiposity and degree of tissue and systemic insulin resistance. These cells adopt an inflammatory phenotype, elaborate pro-
inflammatory cytokines, and perpetuate adipose tissue IR in obesity. Recent reports have established depot-specific pro-inflammatory cytokine expression profiles in equine AT, but the relationship of this expression to macrophage infiltration is unknown. A study to quantify the macrophage content of several AT depots of IR and IS horses was performed, and the results suggest a role for MCP-2 as a macrophage chemoattractant in equine AT. Further, omental AT may have a more dynamic macrophage population than other depots. Importantly, CD163 (+) macrophages may not be the most numerous or important population in equine AT, as increased depot-specific expression of pro-inflammatory cytokines (nuchal ligament) is not accompanied by an increased number of CD163 (+) cells.

The interaction of insulin and the microvascular endothelial insulin receptor (IRc) plays an important role in the normal and IR individual. While endothelial IRc signaling is normally vasodilatory, this effect is well-documented to reverse in the IR individual, resulting in vasoconstriction. Although vascular dysfunction has been reported in sepsis-associated equine laminitis, the role of the laminar microvasculature in endocrinopathic laminitis remains poorly characterized. The purpose of the next study reported here was to characterize the pattern of IRc expression in digital laminae in ponies subjected to a dietary carbohydrate challenge that mimicked abrupt exposure to pasture rich in nonstructural carbohydrates (NSC). Interestingly, laminar keratinocytes did not show significant expression of insulin receptor under basal or fed conditions. Up-regulation of insulin receptor expression in the laminar vasculature occurs acutely in response to dietary carbohydrate challenge and accompanies hyperinsulinemia in ponies. The
dramatic increase in endothelial insulin receptor expression in the laminar microvasculature in nutritionally challenged ponies, with no apparent epithelial insulin receptor present, suggests that hyperinsulinemia associated with exposure to increased dietary NSC may induce laminar epithelial injury via mechanisms that do not involve epithelial insulin signaling.

A robust laminar inflammatory response, including leukocyte infiltration and inflammatory mediator expression, has been well-characterized in laminitis induced by black walnut extract or enteral carbohydrate overload. Although inflammation has been proposed to also play a role in laminitis associated with equine metabolic syndrome, it has not been critically evaluated. The purpose of the next study reported here was to characterize the expression of inflammatory genes and leukocyte infiltration in digital laminar tissue of ponies subjected to a dietary carbohydrate challenge designed to mimic abrupt exposure to pasture rich in nonstructural carbohydrate). The results suggest that the primary inflammatory events occurring in the marked inflammatory response reported in sepsis models of laminitis, leukocyte infiltration and proinflammatory cytokine/chemokine expression, are not central events in EMS-associated laminitis. As laminar COX-2 expression has been primarily localized to vascular wall components (endothelium and smooth muscle) and laminar keratinocytes in the normal and laminitic equid in previous reports, the increased laminar mRNA concentrations of COX-2 in this study may reflect laminar epithelial dysfunction and/or vascular pathology in the affected laminae.
AMPK, a highly conserved enzymatic regulator of cellular energy status, has become a therapeutic target for human MS and EMS due to its reported ability to increase systemic insulin sensitivity. For example, the AMPK agonist metformin has recently been used clinically to treat EMS-affected equids. However, regulation of AMPK activity in both 1) tissues primarily responsible for insulin-mediated glucose disposal (liver, skeletal muscle) and 2) the target tissue for injury in EMS-related laminitis, the digital laminae, is largely unknown. The purpose of the next study was to characterize the cellular localization and activation state of AMPK in liver, skeletal muscle, and digital laminae of ponies subjected to a dietary carbohydrate challenge meant to mimic abrupt exposure to pasture rich in nonstructural carbohydrate (NSC). Western blot analysis for phospho(P)-AMPK (indicating activated AMPK) demonstrated decreased laminar P-AMPK concentrations upon challenge with a high-carbohydrate diet \((p = 0.01)\). In contrast, P-AMPK concentrations were unchanged in skeletal muscle \((p = 0.33)\), and there was a trend for increased AMPK activation in the liver in obese ponies in response to dietary CHO challenge \((p = 0.13)\). Unchanged or increased P-AMPK concentrations in the setting of increased caloric intake suggest insulin resistance in skeletal muscle and liver; the decreased laminar P-AMPK concentrations with CHO challenge indicate that laminar tissue remains insulin sensitive. Whereas skeletal muscle and liver are likely contributory to systemic insulin resistance and resulting hyperinsulinemia in EMS, laminar dysfunction/injury in EMS is more likely due to the local effects of hyperinsulinemia, and not due to local insulin resistance/energy failure.
Obesity, insulin resistance, and endocrinopathic laminitis have emerged as critical issues in equine medicine, causing significant morbidity, mortality, and economic loss to the equine industry. In obese humans and rodent models of nutritional obesity, systemic insulin resistance and hyperinsulinemia are followed temporally in a majority of individuals by decreased glucose tolerance, pancreatic β-cell failure, and type II diabetes mellitus. In stark contrast to humans, obese horses and ponies chronically remain in what is termed a “prediabetic” state in human IR, characterized by hyperinsulinemic euglycemia. Few data exist describing the biology of the equine endocrine pancreas in the chronically IR animal that may both: 1) explain this unique equine endocrine physiology and 2) characterize the animal at-risk for hyperinsulinemia-associated laminitis. The purpose of the final study reported here was to characterize the morphology and physiology of the equine endocrine pancreas in response to a dietary carbohydrate challenge. There was a trend for greater total islet surface area in pancreatic tissue from ponies fed the high NSC diet when compared to the ponies on the hay diet (p = 0.068); however, no difference was noted in β-cell surface area between diet treatments (p = 0.12). The change in serum insulin concentration was significantly greater in the high NSC-fed ponies than in controls (403.8 +/- 317.1 mIU/L vs. 1.00 +/- 4.03 mIU/L; p = 0.002); however, this variable was not correlated with total islet surface area (r = 0.32; p = 0.17) or β-cell surface area (r= 0.25; p = 0.3). Due to the relatively modest changes in pancreatic islet surface area that accompany marked increases in serum insulin concentrations in ponies fed a high NSC diet, it is important to assess both β-cell function
and insulin clearance mechanisms in future studies to delineate the mechanism(s) of hyperinsulinemia in this model.
For Natalie and Justin

‘I carry your heart (I carry it in my heart)’

T.S. Elliot
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Major Field:  Comparative and Veterinary Medicine
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<td>ACC</td>
<td>Acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>AIRg</td>
<td>Acute insulin response to glucose index</td>
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<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
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<tr>
<td>AT</td>
<td>Adipose tissue</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CGIT</td>
<td>Combined glucose and insulin tolerance test</td>
</tr>
<tr>
<td>EHC</td>
<td>Euglycemic hyperinsulinemic clamp</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>EMS</td>
<td>Equine metabolic syndrome</td>
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<tr>
<td>EMSAL</td>
<td>EMS-associated laminitis</td>
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<tr>
<td>FSIGTT</td>
<td>Frequently-sampled insulin-modified intravenous glucose tolerance test</td>
</tr>
<tr>
<td>HMS</td>
<td>Human metabolic syndrome</td>
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<tr>
<td>IDE</td>
<td>Insulin degrading enzyme</td>
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<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor-1</td>
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<td>IGF-1R</td>
<td>Insulin-like growth factor-1 receptor</td>
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<td>IL-1β</td>
<td>Interleukin-1β</td>
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<td>IL-6</td>
<td>Interleukin-6</td>
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<td>IR</td>
<td>Insulin-resistant, insulin resistance</td>
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<td>IRc</td>
<td>Insulin receptor</td>
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<td>IS</td>
<td>Insulin-sensitive</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>LKB1</td>
<td>Liver kinase B1</td>
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<td>PAL</td>
<td>Pasture-associated laminitis</td>
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<tr>
<td>PGC1α</td>
<td>Peroxisome proliferator-activated receptor γ coactivator 1-α</td>
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<td>RIA</td>
<td>Radioimmunoassay</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>RT-PCR</td>
<td>Real-time polymerase chain reaction</td>
</tr>
<tr>
<td>SI</td>
<td>Insulin sensitivity index</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor-α</td>
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</table>
Chapter 1: Introduction

Human metabolic syndrome (HMS)

*Definition and overview of HMS*

Obesity in humans has long been associated with adverse effects on health. More recently, a clustering of risk factors associated with obesity has been shown to predict the onset of cardiovascular disease and type II diabetes mellitus in affected individuals; this syndrome of risk factors, previously known as Syndrome X, the cardiometabolic syndrome, or insulin resistance syndrome, is now referred to as the metabolic syndrome (HMS) (Day 2007). The first formal definition of the metabolic syndrome was created by a working group of the World Health Organization in 1998 (Alberti and Zimmet 1998); the definition adopted by this group included evidence of systemic insulin resistance and at least 2 additional risk factors from a list of criteria including obesity, hypertension, hypertriglyceridemia, low serum high-density lipoprotein concentration, and microalbuminuria. While seemingly inclusive, this definition has proven to be difficult to apply globally to the many diverse disease states which involve insulin resistance (such as anti-retroviral therapy for HIV/AIDS, congenital lipodystrophy, polycystic ovary syndrome, Cushing’s syndrome, obesity, etc.), and exceptions to the
diagnostic criteria are easily identified, particularly by individuals working in different medical specialties seeing affected patients (e.g., cardiologists, endocrinologists, gerontologists, infectious disease specialists). Consequently, the definition of metabolic syndrome in human medicine has emerged as a somewhat controversial issue (Saely, Rein, Drexel 2007; Stolar 2007). A joint interim statement recently issued as a collaborative effort involving the International Diabetes Federation Task Force on Epidemiology and Prevention, the National Heart, Lung, and Blood Institute, the American Heart Association, the World Heart Federation, the International Atherosclerosis Society, and the International Association for the Study of Obesity in 2009 suggested that the diagnosis of metabolic syndrome be made in an individual that displayed three out of the following five criteria: hypertension, hypertriglyceridemia, lowered high-density lipoprotein cholesterol, elevated fasting plasma glucose concentration, and central/truncal obesity (Alberti and others 2009). Of these diagnostic criteria, this collaboration emphasized the importance of body/adipose tissue morphometrics (waist circumference, as a proxy for central/truncal obesity) as a useful screening tool for at-risk patients. The importance of establishing a uniform set of diagnostic criteria to guide physicians in their management of these patients is based on both the severity of the adverse sequelae of metabolic syndrome (creating urgency to identify at-risk individuals for the purpose of prevention of these events), and the large (and increasing) number of patients affected globally. Metabolic syndrome has been shown to be associated with an increased incidence of morbidity and mortality attributed to cardiovascular disease (atherosclerotic coronary artery disease and stroke), type II
diabetes mellitus, degenerative joint disease, and certain types of neoplasms (Cabarcas, Hurt, Farrar 2010; Rask-Madsen and Kahn 2012; Safar and others 2013).

From an epidemiologic perspective, obesity and its associated metabolic and cardiovascular consequences have emerged over the past 10-15 years as critically important disease states in human medicine worldwide, creating an increasing economic burden on health care systems of developed nations worldwide (Polonsky 2012). In fact, in the United States and the United Kingdom, 2/3 of all adults are reported to be either overweight or obese (Ljungvall and Zimmerman 2012; Martinson 2012; Stevens and others 2012); accompanying this finding are increasing rates of atherothrombotic cardiovascular disease, stroke, type II diabetes mellitus (and all of its associated comorbidities), degenerative joint disease, and certain types of neoplastic disease (Daviglus and others 2012; Kraschnewski and others 2013). Perhaps more alarming is the ballooning rate of obesity among young people in developed nations (Robbins and others 2012; Turchiano and others 2012); in fact, the current generation of school-age children has been suggested to have a life expectancy shorter than that of their parents, which is historically unprecedented (Bray and Bellanger 2006). Clearly, obesity is a critical health care issue in human medicine, both for the well-being of human populations and for the prioritization of the spending of increasingly limited health care funding allocations. With this economic imperative, a strong research effort has been put forth over the past 10-15 years to elucidate risk factors for systemic insulin resistance and the metabolic syndrome in humans and to characterize the role that the expanded adipose
tissue itself may play in the pathogenesis of the cardiometabolic sequelae of the syndrome.

**Insulin signaling and insulin resistance**

Recent research in humans and experimental rodent models has revealed the importance of aberrant insulin signaling in both the pathogenesis of the metabolic syndrome and the risk of adverse consequences in affected patients (Tanti and Jager 2009; Wang and Jin 2009). Not only is basal and stimulated hyperinsulinemia considered a marker/diagnostic criterion for at-risk individuals, it is considered to play direct and indirect roles in the pathophysiology of diseases associated with metabolic syndrome (Alberti and others 2009). Knowledge of insulin signaling in health and mechanisms by which it becomes dysfunctional in the insulin-resistant individual is imperative for the design of rational treatment and prevention programs.

Glucose, the primary carbohydrate fuel of cellular intermediary metabolism, crosses cell membranes via facilitated diffusion through various members of a family of glucose transport proteins (GLUT’s), of which 14 have been described in mammalian systems to date (Augustin 2010). In the post-prandial state, elevation in plasma glucose concentration following its absorption from the gastrointestinal tract is a potent secretagogue for insulin from β-cells in the islets of Langerhans of the endocrine pancreas. Secretion of insulin from the endocrine pancreas results in increased plasma concentration of this hormone and its subsequent delivery via the systemic circulation to
its target tissues (primarily skeletal muscle, adipose tissue, and liver); here insulin binds its receptor present on the cell surface (Leto and Saltiel 2012).

Binding of insulin to its receptor, a heterotetrameric receptor tyrosine kinase composed of two α and two β subunits, results in autophosphorylation of critical cytoplasmic tyrosine residues on the β-chains of the receptor and recruitment of insulin receptor substrate (IRS) proteins to the cytoplasmic side of the plasma membrane (Saltiel and Pessin 2002; Vigneri, Squatrito, Sciacca 2010). Although several IRS’s have been described (at least 4), IRS-1 and IRS-2 primarily subserve the functions of signal transduction through the insulin receptor (IRS-1 is of primary importance in skeletal muscle and adipose tissue; IRS-2 is of greater importance in liver) (Copps and White 2012). IRS’s contain multiple tyrosine and serine phosphorylation sites; in general (with a few exceptions), tyrosine phosphorylation of IRS’s by activated insulin receptor results in conformational changes in IRS’s that activate and promote their function as docking proteins, recruiting additional SH-2 domain-containing molecules involved in insulin signal transduction through two main pathways (Copps and White 2012). Serine phosphorylation of IRS’s is broadly inhibitory to their function in recruiting downstream effectors of signaling; while more than 50 canonical serine phosphorylation sites have been described on IRS-1, the functions of all of which have not been fully characterized to date, phosphorylation of some of these (such as Ser312/307) results in retention of IRS-1 in the cytosol in association with 14-3-3 proteins and enhanced IRS-1 ubiquitination and degradation. In this way, serine phosphorylation of IRS’s serves as an important site of negative feedback of insulin action; it is also a primary mechanism
responsible for acquired insulin resistance in pathologic states, such as obesity and inflammation (Tanti and Jager 2009).

As mentioned above, IRS’s recruit effectors of two intracellular signaling cascades that are activated by insulin binding to its receptor: the mitogen activated protein kinase (MAPK) pathway, responsible for the mitogenic and differentiation effects of insulin, does not play a significant role in glucose metabolism. Activation of the phosphatidylinositol-3-kinase (PI3K) pathway is responsible for the increased transcellular glucose conductance observed following insulin binding. Tyrosine phosphorylation of IRS-1 and/or IRS-2 results in recruitment of the p85 subunit of PI3K to the plasma membrane, where its primary substrate(s) phospholipids are located. Phosphorylation of PIP2 by the catalytic p110 subunit of PI3K and subsequent generation of PIP3 results in activation of phosphatidylinositol-dependent protein kinase (PDK1). PDK1 phosphorylates PKB/Akt, a kinase that then phosphorylates Akt substrate of 160 kD (AS160) (Avogaro, de Kreutzenberg, Fadini 2010; Bashan and others 2009; Frojdo, Vidal, Pirola 2009; Jensen and De Meyts 2009; Vigneri, Squatrito, Sciacca 2010). GLUT4, the primary insulin-responsive glucose transporter protein in skeletal muscle, is sequestered in cytoplasmic vesicles in the absence of insulin receptor activation; AS160 is involved (likely with many other molecules) in promoting this retention. Upon phosphorylation and inhibition of AS160, GLUT4-containing vesicles translocate to and fuse with the plasma membrane, resulting in increased cell surface expression of GLUT4. Plasma glucose then is allowed to enter skeletal muscle cells through GLUT4-facilitated diffusion, until these transporters are re-sequestered following cessation of the insulin
signal (Leto and Saltiel 2012). Other GLUTs are expressed in skeletal muscle (e.g., GLUT1, GLUT2); however, they are not insulin-responsive and their involvement in glucose transport into skeletal muscle is considered minor in comparison to that of GLUT4 (Augustin 2010). Intracellular glucose trapping and prevention of back-diffusion through GLUT4 is prevented via rapid phosphorylation of intracytoplasmic glucose to glucose-6-phosphate (which does not pass through GLUTs) by glucokinase/hexokinase. These are the essential steps that promote insulin-mediated glucose movement from the systemic circulation into the skeletal myocyte, which is primarily responsible for insulin-mediated plasma glucose disposal post-prandially.

*Energy regulation and insulin resistance: AMPK*

Adenosine-5’-monophosphate-activated protein kinase (AMPK) is a eukaryotic heterotrimeric protein complex that is highly conserved among phyla and widely distributed in most tissue types of multicellular organisms (Hardie 2007; Hardie 2012). Originally described in yeast (*Saccharomyces cerevisiae*) as the sucrose non-fermenting (SNF) protein complex, homologues have been identified in plants (enabling energy production during the dark phase of light-dark cycles) and animals (including the mammalian complex, AMPK) (Lee and others 2007). Details of the metabolic functions of AMPK will be described below; here, a concise description of the structure of the complex will be outlined.

The AMPK complex is composed of three different subunits, α, β, and γ; all three subunits are required for proper functioning of the enzyme (Hardie, Ross, Hawley 2012).
AMPK exacts its effects on intermediary metabolism through direct phosphorylation of enzymes involved in carbohydrate and lipid metabolism and transcription factors and their regulators (thereby having both direct, immediate effects and delayed, indirect effects on metabolic pathways) (Hardie 2012). This important catalytic (kinase) function of AMPK is a property of the α-subunit. A critical regulatory threonine residue, Thr172, phosphorylation of which activates the kinase activity of AMPK, is also found on the alpha subunit. Two genes encoding different α subunits, α1 and α2, are described in mammalian systems; the relative importance of each α subunit to AMPK expression in different tissue types varies and may underlie the heterogeneous activation of AMPK in different tissues. For example, the α2 subunit has been reported to be more important in skeletal muscle and liver relative to the α1 subunit (Hardie and Sakamoto 2006). In addition to its catalytic function, the α subunit also functions as a docking site for the β and γ subunits, thereby holding the complex together and ensuring proper function. Spontaneously occurring mutations in either α subunit gene of AMPK have not been linked to a described disease state in humans; experimental knockout or knockdown of either subunit has been associated with mild metabolic phenotypes in certain tissues (eg, liver and skeletal muscle (Lefort and others 2008)), but there appears to be some degree of compensatory functional redundancy between the two subunits. Experimental ablation of both α subunits results in an embryonic non-viable phenotype (Viollet and others 2003).

The β subunit of AMPK, for which there are two discrete genes described (β1 and β2), is thought to serve primarily a docking function, with binding sites for both the α-
and γ-subunits located within its structure (Hardie, Ross, Hawley 2012). The β subunit also contains a canonical carbohydrate binding domain that has been shown to bind glycogen; since AMPK has important effects on glycogen mobilization during times of energy stress (described in more detail below), this domain may serve to localize the enzyme close to its downstream targets (McBride and Hardie 2009). It has also been suggested that AMPK may function as a sensor of intracellular glycogen stores in skeletal muscle and liver, with a role in post-exercise glycogen repletion (to give an example); glycogen binding by the β subunit is thought to be important for this effect.

The γ-subunit of AMPK also serves a regulatory function, as this subunit contains the domains (Bateman domains) responsible for the binding of AMP (which has been shown to allosterically activate AMPK activity)(Hardie, Ross, Hawley 2012). Binding of AMP to the γ-subunit is thought to render Thr172 of the α-subunit a better substrate for LKB1 (its primary activating kinase) and less susceptible to dephosphorylation by protein phosphatases (particularly PP2). Four Bateman domains form two AMP binding sites which display significant cooperativity (i.e., binding of AMP in the second site is facilitated by binding of AMP in the first site); these domains also bind ATP (with lower affinity than which they bind AMP), which inhibits AMPK activation by a mechanism that has not been completely elucidated. Three γ-subunit genes have been described in mammalian systems, and naturally-occurring mutations in at least one γ-subunit gene result in congenital myocardial glycogenosis that is manifested clinically by cardiac conduction abnormalities (Wolff-Parkinson-White syndrome) and progressive dilated cardiomyopathy in humans (Hardie, Hawley, Scott 2006; Xu and Si 2010).
Through various combinations of the three subunits of AMPK, 12 possible heterotrimeric exist (with the possibility that additional heterotrimeric may result from alternative splicing events in various tissues). This marked heterogeneity likely results in significant functional differences in AMPK between tissues; while this is a challenging reality for researchers investigating the function of AMPK in various tissues, it may also be of benefit for creation of targeted therapeutics in the future (i.e., development of drugs that activate AMPK in liver and skeletal muscle but not in keratinocytes, for example).

AMPK has been described as an important global sensor of cellular energy status, with activation of the complex during times of carbon starvation or energy deficit (such as hypoglycemia, ischemia, hypoxia, etc.) (Hardie, Hawley, Scott 2006). An increase in the cellular AMP:ATP ratio (as might occur with ATP depletion) causes activation of AMPK kinase activity through several possible mechanisms: binding of AMP to the Bateman domains of the AMPK gamma regulatory subunit induces allosteric activation of the alpha subunit kinase domain (~5 fold), and this binding also enhances phosphorylation of the alpha subunit at Thr172, which also activates kinase activity (~1000 fold). AMPK is phosphorylated by several upstream regulatory kinases, the most important of which is likely LKB1. LKB1 is constitutively active itself, but it is thought that AMP-bound AMPK is a better substrate for LKB1 phosphorylation at the Thr172 residue of AMPKα (and a poor substrate for dephosphorylating phosphatases); increased cellular AMP, then, increases LKB1 phosphorylation and activation of AMPK (Kottakis and Bardeesy 2012). Other kinases, notably CaMKK’s (esp. CaMKKβ) and TAK1 have been shown to activate AMPK through phosphorylation of Thr172; however, their
physiologic importance is likely less than that of LKB1 and tissue- and situation-dependent (Hardie, Ross, Hawley 2012).

Activation of AMPK kinase activity (again, marked by phosphorylation of the alpha subunit at Thr172) results in broad changes in carbohydrate, lipid, and protein metabolism that serve to, in general, decrease energy consumption (anabolic pathways, such as glycogen, lipid, and protein synthesis) and increase energy production (catabolic pathways, such as glucose uptake, glycolysis, glycogenolysis, lipolysis, lipid oxidation) (Hardie 2011a). AMPK activation has also been shown to have significant effects on growth and differentiation, promoting decreased mitotic activity and persistence of cells in G0/resting phase, the portion of the cell cycle in which cells exist in a quiescent, non-replicative state (Faubert and others 2013; Hardie 2011b; Mantovani and Roy 2011).

As mentioned above, AMPK induces its effects on intermediary metabolism through both direct phosphorylation (which may be stimulatory or inhibitory) of enzymes and through phosphorylation (and subsequent activation or inhibition) of transcription factors involved in regulation of production of metabolic enzymes. With respect to carbohydrate metabolism, AMPK has been reported to have several important effects. AMPK phosphorylates glycogen synthase kinase 3, which phosphorylates glycogen synthase and inhibits its function, thereby decreasing glycogen storage in skeletal muscle and liver. AMPK also phosphorylates and activates phosphorylase a, which results in glycogenolysis and glucose production. AMPK phosphorylates phosphofructokinase, a rate-limiting enzyme in glycolysis, increasing its activity. These effects on carbohydrate metabolism are important in both skeletal muscle and liver in the setting of energy
restriction. However, while skeletal muscle does not export glucose for the purpose of maintaining its plasma concentration in the fasted state, this is an important function of the liver as a systemic ‘glucostat’. Hepatic expression of glucose-6-phosphatase, which dephosphorylates glucose-6 phosphate and allows its reverse diffusion through glucose transporters (GLUTs) in the hepatocyte plasma membrane, is important for this function; AMPK activation activates G6P and induces its expression, increasing hepatic glucose release. AMPK also decreases the transcription of gluconeogenic enzyme genes, such as phosphoenolpyruvate carboxykinase (PEPCK) through its phosphorylation and inhibition of TORC2 and ChREBP (Hardie 2011b; Hardie, Ross, Hawley 2012).

With respect to fat metabolism, AMPK activity inhibits fatty acid and cholesterol biosynthetic pathways and increases lipid oxidation. Acetyl-CoA carboxylase-1 and -2 are classic phosphorylation targets of AMPK, an event that inhibits their activity; as these are the rate-limiting enzymes in fatty acid synthesis from acetyl-CoA, a profound decrease in this activity results from AMPK activation. Further, the product of ACC activity is malonyl-CoA, which significantly inhibits carnitine palmitoyl transferase-1, a transport protein important for the movement of fatty acids into mitochondria for oxidation. Decreased malonyl-CoA production secondary to ACC phosphorylation releases this inhibition, encouraging lipid oxidation and energy production in the setting of AMPK activation. AMPK also phosphorylates (and inhibits) hydroxymethylglutaryl-CoA reductase, the rate-limiting step in cholesterol biosynthesis, thereby decreasing endogenous production of cholesterol and its metabolites (including several steroid hormones, such as corticosteroids) (Towler and Hardie 2007). AMPK activates
peroxisome proliferator activated receptor gamma coactivator 1-alpha (PGC-1a) through both direct phosphorylation and through increased transcription of the PGC-1a gene; PGC-1a itself increases the expression of several oxidative enzyme genes and genes involved in mitochondrial biogenesis, an effect that is particularly important in skeletal muscle (such as in response to exercise training, an effect that has been recently demonstrated in equine skeletal muscle (Eivers and others 2012)).

The role of AMPK in skeletal muscle has been shown responsible (at least in part) for the insulin-sensitizing and non-insulin dependent effects on glucose transport following exercise. AMPK activation, which may occur in exercising muscle via several mechanisms (increased AMP:ATP ratio with fatiguing exercise [LKB1], increased cytoplasmic calcium concentration [CaMKK]) (Hardie 2011c), results in phosphorylation of AS160 and translocation of GLUT4 to the cytoplasmic membrane in the absence of insulin signaling; AMPK also enhances the activity of insulin signaling at multiple levels (most importantly, IRS’s), but increased glucose disposal during exercise has been shown to be non-insulin dependent (at least in part) (Lefort and others 2008).

AMPK may display significant cross-talk with insulin (and IGF-1) signaling at multiple levels (Towler and Hardie 2007). For example, AMPK has been shown to phosphorylate IRS-1 at a serine site that increases its affinity for PI3K, an effect which increases insulin signaling (and thereby promotes insulin sensitivity). AMPK also promotes the oxidation of accumulated ectopic lipid in multiple tissues, lipids which likely signal through PKCθ to inhibit IRS-1 and insulin signaling (again, promoting increased insulin sensitivity) (Hardie, Ross, Hawley 2012). AMPK may also directly
phosphorylate and inhibit the activity of AS160, which serves to retain GLUT4 in cytoplasmic vesicles in the absence of insulin signaling; this is likely the mechanism (or at least part of the mechanism) by which AMPK elicits non-insulin-dependent GLUT4 translocation and increased glucose disposal that is observed with muscle contraction (Towler and Hardie 2007). Conversely, Akt/PKB (an important kinase effector of the PI3K pathway, signaling downstream of insulin receptor) may phosphorylate AMPK alpha subunits and inhibit their phosphorylation by LKB1. Insulin/AMPK interactions are very complex and may ultimately be determined to be quite tissue-specific in the setting of metabolic syndrome.

The liver, while insulin-responsive in many ways, is not dependent upon insulin for many of its functions in glucose metabolism. Insulin signaling in the liver tends to inhibit glycogenolysis and gluconeogenesis, encouraging instead glycogen storage; these effects are attenuated in the setting of insulin resistance, with persistent gluconeogenesis and exacerbation of hyperglycemia (especially in patients with Type II diabetes mellitus) (Meshkani and Adeli 2009). In skeletal muscle, however, glucose uptake occurs primarily through GLUT4, which is largely insulin-responsive (Joost and Thorens 2001). In muscle, insulin resistance is associated with decreased glucose disposal and the accumulation of intramyocellular lipid (Corcoran, Lamon-Fava, Fielding 2007; Eckardt, Taube, Eckel 2011; Franklin and Kanaley 2009). While the role of AMPK in liver and skeletal muscle is likely complex and affected by multiple inputs, it has been suggested since the late 1990’s that AMPK activation could potentially reverse many of the characteristic abnormalities of carbohydrate and lipid metabolism that are observed in
insulin-resistant individuals. This has led to the development and widespread clinical use of functional AMPK agonists, such as metformin, in the treatment of humans with the metabolic syndrome and type II DM (Mehnert 2001).

Since its original description in the late 1980’s, the effects of AMPK have been considered to be primarily on energy metabolism and metabolic sensing. However, more recent roles for the enzyme, particularly with further characterization of its upstream kinases (esp. LKB1), have been described in the maintenance of epithelial cell polarity and intercellular adhesion (Fu and others 2011; Hardie 2011d; Miranda and others 2010; Zhang and others 2006). LKB1, the primary regulatory kinase of AMPK, has been associated with abnormal epithelial polarity and developmental programming; in fact, Peutz-Jeghers syndrome, a heritable condition associated with epithelial carcinogenesis in multiple tissues, is associated with a mutation in LKB1 (Kottakis and Bardeesy 2012). In recent cell culture studies, the role of AMPK in maintenance and dynamic repair of intercellular tight junctions in response to osmotic stress has been confirmed (Madin-Darby canine kidney cell lines), with accelerated tight junction assembly observed following application of an AMPK agonist (AICAR) and delayed assembly following AMPK inhibition (compound C) (Zhang and others 2006). In invertebrate models (Drosophila melanogaster), AMPK knockouts are lethal, with severe defects in cell polarity and mitosis; restitution of AMPK activity rescues the phenotype. AMPK activation results in rapid induction of cell polarity in cell cultures (Miranda and others 2010; Nakano and others 2010). AMPK is now known to phosphorylate myosin
regulatory light chain, directly linking its activity to cytoskeletal metabolism (Jansen and others 2009).

Metformin, a functional AMPK agonist, is an orally bioavailable biguanide compound that has been used clinically as an anti-hyperglycemic and insulin-sensitizing therapeutic in the treatment of human metabolic syndrome and Type II diabetes mellitus (Mehnert 2001). The pharmacokinetics and pharmacodynamics of this drug have been well-characterized in humans; however, while metformin has been used empirically to treat equids afflicted with EMS (Durham, Rendle, Newton 2008) (and while preliminary pharmacokinetic studies have been published (Hustace, Firshman, Mata 2009)), no pharmacodynamic information regarding metformin in equids has been reported to the authors’ knowledge. Additionally, it is now recognized that AMPK may have profound effects on the integrity and confluence of epithelial cells in cell culture, including their ability to remodel tight junctions following application of a cellular stressor (Zhang and others 2006). AMPK, then, may provide an attractive link between dietary carbohydrate content, insulin sensitivity, and laminar epithelial dysfunction. Investigation of the effects of AMPK activation in the digital laminae in response to dietary carbohydrate content may open new avenues of inquiry regarding the pathophysiology of endocrinopathic laminitis, ideally leading to the development of novel therapies for treating this difficult disease.

Equine metabolic syndrome (EMS)
**Definition and overview of EMS**

Equine veterinary practitioners have long known from clinical experience that overweight and obese horses are prone to developing laminitis, even if the mechanisms underlying this complication remained obscure. Obesity in horses has also been associated with peripheral insulin resistance (Frank and others 2006; Hoffman and others 2003). As the human metabolic syndrome was increasingly well-described in the medical literature, correlations were drawn between this syndrome of obese humans and that noted clinically in obese horses (Johnson 2002). Over time, the equine syndrome of obesity (particularly regional adiposity), peripheral insulin resistance, dyslipidemia, and an increased risk of endocrinopathic laminitis was coined the ‘equine metabolic syndrome.’ (Frank and others 2006; Johnson 2002).

The ACVIM Consensus Statement on EMS was published in the Journal of Veterinary Internal Medicine in 2010 (Frank and others 2010). This paper, which collated and synthesized most of the available research and current best evidence regarding the condition in horses and ponies, identified three primary criteria for the diagnosis of EMS in individual animals: increased adiposity (generalized, regional, or both, with emphasis placed on nuchal ligament and tail head adipose tissue accumulations), systemic insulin resistance (assessed in a variety of ways; see below), and a predisposition to laminitis in the absence of another risk factor for the condition, such as septic disease (Belknap, Moore, Crouser 2009) or gross enteral carbohydrate overload (Pollitt and Visser 2010; van Eps and Pollitt 2006). Other factors that have been reportedly associated with EMS, such as hyperleptinemia (Carter and others 2009a;
Cartmill and others 2003; Kearns and others 2006), seasonally increased mean arterial blood pressure (Bailey and others 2008), and altered reproductive cyclicity in mares (Waller and others 2006), were not included in the currently accepted diagnostic criteria; the possibility exists that these (and other) factors may be included following validating studies that remain to be performed in the future. While the currently accepted criteria have all been subjected to reasonable objective evaluation, there remain potential flaws in their interpretation (methodology, sensitivity, etc.) that decrease their accuracy in laminitis risk prediction. Even the definition of the outcome variable, laminitis, may be ambiguous under some circumstances.

Horses affected with equine metabolic syndrome (EMS) are typically overweight to obese, with body condition scores typically ranging from 7.0-9.0/9.0 (Carter and others 2009a; Frank and others 2006; Kronfeld and others 2006). All breeds may be affected; however, most reports indicate that breed predispositions exist, with pony breeds, Morgans, American Saddlebreds, Spanish mustangs, and warmbloods overrepresented (Johnson, Messer, Ganjam 2004). Within at-risk breeds, affected individuals often display pronounced regional adiposity, particularly over the nuchal ligament and tail head. One report suggests that morphometric assessment of the neck of insulin-resistant horses (which incorporates assessment of the degree of nuchal ligament adiposity) can differentiate these horses from insulin-sensitive horses (Frank and others 2006). Interestingly, while the degree of insulin resistance has been shown to correlate reasonably well with body weight in horses (Carter and others 2009c; Hoffman and others 2003), not all obese horses are insulin-resistant. Conversely, some relatively lean
horses may be profoundly insulin resistant, particularly if they are of a predisposed breed. Identical observations have been made in human medicine, and abnormalities in adipocyte function have been implicated in some of the more severe forms of insulin resistance (congenital lipodystrophy, for example) (Wildman and others 2008). The underlying physiologic mechanisms that predispose some breeds of horse to EMS have not yet been elucidated, but it is a tenable hypothesis that some facet of adipocyte structure and/or function is involved, at least in part.

Unlike humans with metabolic syndrome, horses with EMS do not appear to suffer from significant cardiovascular complications of their metabolic dysfunction. While a recent report suggests that insulin-resistant ponies may be hypertensive (Bailey and others 2008), no concrete evidence of cardiovascular complication has been published related to EMS. However, these horses are at increased risk for the development of laminitis (Carter and others 2009a; Treiber, Kronfeld, Geor 2006), which is suspected to share an inflammatory pathophysiology with the more severe cardiovascular complications of obese humans, specifically, atherosclerotic coronary artery disease (Maury and Brichard 2010; Piya, McTernan, Kumar 2013). Several recent studies have revealed a robust correlation between markers of EMS in ponies (namely, body condition score, plasma triglyceride concentration, and degree of insulin resistance) and the incidence of pasture-associated laminitis, one of the more common manifestations of endocrinopathic laminitis in horses (Carter and others 2009a; Treiber and others 2006). In fact, the presence of >3 clinical findings of EMS in one study correctly predicted the development of laminitis in 11 of 13 ponies when these animals
were exposed to a high-starch pasture (Treiber and others 2006). Anecdotal evidence supports the contention that EMS is perceived by many equine veterinarians to be the most common cause of laminitis in their practices (Geor 2008, personal communication).

It appears that insulin resistance in humans is often associated with increased plasma levels of certain markers of inflammation (notably TNF-α, IL-1β), linking abnormal carbohydrate metabolism in these people with chronic, low-grade inflammation (Yudkin and others 1999). This phenomenon has also been described in veterinary species, including dogs, mice, and rats, and recently, horses (Vick and others 2007; Vick and others 2008). Obesity and insulin resistance have been loosely positively correlated with plasma concentrations of TNFα and IL-1β in horses (Vick and others 2007), and several authors have suggested (based on measurement of tissue and systemic concentrations of inflammatory mediators) that EMS is associated with a systemic, low-grade inflammatory state (Burns and others 2010; Treiber and others 2009; Vick and others 2007). The cause of laminitis in insulin-resistant horses is unknown but has been postulated to be related to a state of chronic inflammation associated with insulin resistance (Johnson and others 2010); this has yet to be documented conclusively in the horse.

While much of the characterization for the diagnostic criteria for EMS has been performed in research animals, these criteria are meant to be applied to clinical patients for the purpose of guiding their medical care. Improving the accuracy of the diagnostic criteria will hopefully improve laminitis prevention, and improving the ease of diagnostic sample collection would potentially increase testing of general equine populations (and
thus data collection, which may also be useful for research purposes). Increasing the predictive value of EMS diagnostic criteria and increasing the ease of sample collection (by validating single time point blood samples, or by validating diagnostic evaluation of easily-sampled tissues, such as nuchal ligament adipose tissue, skeletal muscle, or liver) would be reasonable steps to take to improve the diagnostic criteria for EMS.

The EMS is meant to define risk of laminitis in horses and ponies; adequate assessment of this risk requires measuring the incidence of laminitis in cohorts separated by various risk factors. While small prospective studies on inbred populations of ponies have been performed evaluating the incidence of laminitis following exposure to pasture (Carter and others 2009a; Treiber and others 2006), this work has not been repeated on a larger scale (or with outbred equine populations). Additionally, the same criteria defining laminitis risk in the above-mentioned cohort of inbred ponies are extrapolated to light breed horses, populations on which this same type of study have not been performed. Large, prospective cohort studies (somewhat akin to the Framingham Cohort (Wilson and Meigs 2008), a seminal study defining risk factors for coronary artery disease and MI in humans that has spanned several decades) would lend validation to (and perhaps reveal more accurately predictive) risk factors for endocrinopathic laminitis related to obesity in equids. If performed in conjunction with certain breed registries (breeds predisposed [gaited breeds, ponies, mules, donkeys] and breeds not predisposed [quarter horses, Thoroughbreds], for example), data might be collected on individual animals over the lifetime of the animal (at shows, etc.). Familial tendencies, and ultimately identification of candidate genes through linkage analyses may lead to new knowledge of genetic
causes of abnormal CHO, lipid metabolism (mitochondrial biogenesis, IDE, IR, IR antibodies, PI3K, etc.) in horses and ponies.

Serum biomarkers that have positive correlation with metabolic risk have been fairly well characterized in human populations; while initial work incorporating serum biomarkers (such as leptin and insulin) into a ‘pre-laminitic metabolic syndrome’ has been done in ponies (Carter and others 2009a; Treiber and others 2006), these studies have not evaluated the biomarkers that are most tightly correlated with IR in humans (namely, C-reactive protein (Behre and others 2005; Yudkin and others 1999), plasminogen activator inhibitor-1 (Cao and others 2008a; Juhan-Vague, Alessi, Morange 2000), serum amyloid A (Jylhava and others 2009; van Bussel and others 2013), adiponectin (Okamoto and others 2006), resistin (Chen and others 2004; Ou and others 2012)). Identification of additional serum biomarkers of laminitis risk, such as might be done through the long-term cohort studies mentioned above, might allow construction of a panel of analytes that could be easily measured in a single serum sample (adipokines, serum inflammatory proteins, pro-inflammatory cytokines, etc.). Again, this would require evaluation in large populations of horses (light breed horses, ponies, donkeys and mules; predisposed breeds and non-predisposed breeds), including evaluation of response to dietary challenge, evaluation of season, and correlation with incidence of laminitis in response to pasture exposure. Validated serum sampling protocols would increase the ease of diagnostic testing for EMS and might thereby increase the frequency of testing performed by practitioners on field cases.
Ideally, validation of serum biomarkers or a panel of such to predict IR should be performed against a gold standard. When reviewing the equine literature on diagnostic testing of insulin and glucose dynamics, there appears to be no such gold standard (or if so, it is in name only). Insulin sensitivity is assessed by equine researchers with a diverse range of techniques (Firshman and Valberg 2007; Pratt, Geor, McCutcheon 2005); in order to more cohesively move the field forward, a consensus needs to be reached on how this testing should be done. Direct comparison of the commonly used techniques (fasting insulin and glucose concentration with minimal model proxies (Treiber and others 2005), CGIT (Eiler and others 2005), oral glucose tolerance test (Brojer and others 2012)) in the same cohort of horses (IR and IS) to a gold standard (EHC and/or FSIGTT) should be performed to evaluate their relative benefits and shortcomings. Achievement of consensus on best laboratory testing protocol to define IR in horses (that is clinically feasible) is required to advance the field.

It has become clear in human obesity and IR that the relative roles of skeletal muscle and hepatic insulin resistance may not be equivalent. In fact, hepatic insulin resistance appears to correlate better with systemic IR and adverse disease outcomes in people than does skeletal muscle IR. EHC protocols utilizing different insulin infusion rates (lower rate for assessing skeletal muscle IR; higher rate for assessing hepatic IR) have been described to evaluate the differential role of these tissues, and these may have some benefit in evaluation of equine IR (as the respective contributions of liver and skeletal muscle in this population remains unclear). Additionally, the role of ectopic lipid – which has been shown to correlate with systemic insulin resistance and risk outcome
more tightly in humans and rodents than degree, or even location, of adipose tissue – in
the pathophysiology of equine IR remains poorly described (even though a catastrophic
disease state associated with equine IR, hyperlipemia, is defined by ectopic lipid
accumulation). The relative ease of sampling of equine skeletal muscle and liver by
minimally invasive techniques and the purported importance of these tissues to the
pathophysiology of human metabolic syndrome makes this an attractive possibility for
diagnostic evaluation of equids with EMS.

Laminitis and EMS

The equine digital laminae are a unique epidermal structure in which the adhesion
of the epidermal laminae (continuous with the hoof wall) to the dermal laminae (attached
to P3) allows for the suspension of P3 (and therefore the entire musculoskeletal system
and weight of the horse) within the hoof wall. The cell type responsible for this adhesion,
and which is the site of dysadhesion leading to laminar failure in laminitis, is the laminar
basal epithelial cell (LBEC). The LBEC is likely a polarized, highly differentiated
epithelial cell with critical adherence functions on its basement membrane side,
maintaining adequate adhesion capacity to support the weight of the horse within the
hoof wall. In addition to this critical function, the equine LBEC functions similarly to
other keratinocytes described, responding to extracellular events by undergoing a
multitude of basic signaling mechanisms, including signaling related to both
inflammation and epithelial stress in sepsis-related laminitis. Although there has been a
great deal of effort put forth to find a common signaling pathway by which diverse
systemic disease entities can lead to laminitis/laminar failure, it is more likely that the reason diverse entities lead to laminar failure is that any insult to the LBEC may result in loss of a highly differentiated polarized cell type and thus dysregulation of basement membrane adhesion, culminating in LBEC layer dysadhesion and laminar failure due to the incredible stresses placed on the digital laminae at the level of the LBEC. Thus, the cause of laminar failure in PAL may bear little similarity to that occurring in the other types of laminitis regarding extracellular events or even cell signaling; however, a similar laminar failure results due to unique disarray of cytoskeletal and adhesion functions and subsequent dysadhesion of the LBEC from the underlying basement membrane (Pollitt and Visser 2010).

While equine laminitis has been associated with many diverse causes (including gastrointestinal tract disorders, retained placenta or metritis, severe infections, and excessive weight bearing) (Belknap, Moore, Crouser 2009), pasture-associated laminitis is now reported to be the most common cause of laminitis, affecting primarily horses and ponies with a phenotype of obesity (esp. regional adiposity) and insulin resistance when they are exposed to pasture forage with high NSC content (Carter and others 2009a; Treiber and others 2006; USDA-NAHMS 2000). Clinical observations and the results of epidemiologic studies have indicated that risk of pasture laminitis is highest when horses and ponies are grazing pastures with high NSC content (i.e. fructans, simple sugars, and/or starch), and theories on the pathogenesis of pasture-associated laminitis have been extrapolated from experimental models of alimentary carbohydrate overload. However, the extent to which these severe models reflect events during development of EMS-
related laminitis may be questioned. First, it is likely that the amount and rate of starch or fructan administered for induction of carbohydrate overload in these studies far exceeds the quantity of NSC ingested by grazing equids. Second, previous studies have not used animals with an EMS phenotype and thus have not addressed the interaction between diet and phenotypic factors associated with laminitis susceptibility. The studies reported in this dissertation have attempted to address these shortcomings by comparing, in groups of obese (EMS) and lean ponies, metabolic changes in liver, skeletal muscle, and laminar tissue in response to a feeding protocol that more closely mimics the increase in dietary NSC associated with pasture laminitis.

The laminar basal epithelial cell integrates, through its interaction with the lamellar basement membrane, the load bearing function of the laminae, neutralizing the downward force of gravity transmitted through the bony column of the digit and the upward ground reaction force transmitted through the hoof wall. Its importance, therefore, cannot be overstated, as its failure results in detachment and separation of the dermal and epidermal laminae and displacement of the bony column within the hoof wall. The LBEC is clearly a highly polarized cell whose function is critically dependent on its adherence both to its neighboring epithelial cells and to its anchoring basement membrane; coordination of adhesion may be required for normal distal growth of the hoof wall, which occurs in the setting of tremendous stress of the weight of the horse. It is intriguing to speculate that decreased AMPK activity (which has been recently observed in laminar tissue of ponies challenged with an increased dietary carbohydrate load, a stimulus that has been associated with induction of laminitis in IR equids) might
induce loss of cytoskeletal polarity of the laminar basal cell (such as has been observed in vitro in other epithelial cell model systems) and subsequent detachment from its basement membrane. Loss of polarity of epithelial basal cells and decreased maintenance of epithelial tight junctions may lead to mechanical disruption of the laminar interface secondary to ~constant DDFT. Recent evidence of decreased AMPK phosphorylation in model of endocrinopathic laminitis suggests that laminar decreased AMPK activity may be pathologic; however, it is unclear what role it plays in normal and abnormal laminar physiology. While the net effects of AMPK activation or inhibition on the function of the equine laminar epidermal basal cell are unknown, it is reasonable to assume that it plays a role in this highly polarized, structurally critical cell type, whose cytoskeletal interactions are crucial for support of the weight of a horse or pony against gravity.

Laminitis is associated with a diverse range of clinical diseases in horses, from sepsis to endocrinopathies characterized by insulin resistance. Insulin resistance, which has been shown to occur in obesity and in sepsis in horses, can result in hyperinsulinemia due to decreased glucose transport into insulin-responsive tissues (especially skeletal muscle). While insulin resistance and subsequent glucose starvation have been suggested in the past as potential mechanisms underlying laminar failure in this state, it has recently been shown (Asplin and others 2011) that the primary glucose transporter type expressed in laminae is GLUT1, which is neither insulin-dependent nor highly insulin-responsive. So, instead of glucose deprivation in the setting of insulin resistance, as might occur in insulin-requiring tissues, the laminae may experience an increased glucose flux in the setting of IR. Increased glucose flux is associated with ATP production and decreased
AMP:ATP ratio, conditions in which AMPK activity would be expected to be decreased (Hardie 2011d). If basal cell polarity (which is presumed to be required for their proper function) is similarly dependent on AMPK function as has been shown in other epithelial cell types in vitro (Zhang and others 2006), this scenario might very well result in laminar failure (which is what is observed clinically).

It is also possible that the actin cytoskeletal re-organization events induced by AMPK activation may be deleterious to laminar function in certain settings. Interesting work by a group working with alveolar epithelial cell cultures suggests that mechanical signaling through dystroglycan (a membrane-anchored mechanoreceptor) and plectin (which binds dystroglycan and AMPK) simultaneously activates AMPK and MAPK pathways (ERK1/2). Laminar activation of p38 MAPK has been documented in the setting of experimental CHO-induced laminitis, suggesting a pathophysiologic role for these kinases. Laminitis associated with excessive weight bearing, which can certainly be expected to activate signaling pathways initiated by mechanoreceptors, may involve both MAPK and AMPK activation. Further investigation is clearly warranted.

Hyperinsulinemia has been known to be a risk factor for the development of endocrinopathic laminitis for some time, identified by several groups independently in cross sectional studies (Frank and others 2006; Kronfeld and others 2006; Treiber and others 2006). More recently, a direct link between insulin and laminitis has been created, with the experimental induction of laminitis via iatrogenic hyperinsulinemia in normal ponies (Asplin and others 2007) and light-breed horses (de Laat and others 2010). Insulin is a pleiotropic hormone whose functions have been largely thought to be limited
to regulation of carbohydrate, lipid, and protein metabolism in times of energy surplus (i.e., in the postprandial period) (Rask-Madsen and Kahn 2012). However, signaling through insulin receptor has been shown to activate not only pathways that direct the fates of nutritional metabolites (through PI3K activation), but also modulation of mitogenesis and extracellular matrix production through activation of MAPK pathways (Vigneri, Squatrito, Sciacca 2010). Direct effects of insulin on laminar epidermal basal cells, with subsequent alterations of their production of extracellular matrix (which is critically important for the integrity of the laminar basement membrane, known to separate from laminar basal cells and thereby define structural failure accompanying laminitis), may play a role in endocrinopathic laminitis. However, recent studies showing little evidence of insulin receptor expression on laminar epidermal cells complicates acceptance of this hypothesis (Burns and others 2012). Insulin signaling through insulin receptor may have greater effect on the perfusion of the laminae, as insulin receptor signal appears to be restricted primarily to the laminar microvasculature (Burns and others 2012).

Prolonged exposure of endothelial cells to increased concentrations of insulin in vitro has been shown to alter their insulin responsiveness, inducing cellular insulin resistance over a relatively short period of time (24-48 hours) (Schulman and Zhou 2009). Interestingly, it is shortly after this time frame, at ~72 hours, that horses and ponies are observed to become laminitic during experimental hyperinsulinemia (Asplin and others 2007). Rapid downregulation of surface expression of insulin receptor has been documented on endothelial cells in response to treatment with exogenous insulin,
which may partially explain the endothelial insulin resistance observed in response to hyperinsulinemia in vivo. Signaling through insulin receptor (through serine phosphorylation of IRS’s by Akt/PKB) also tends to be self-limiting (Copps and White 2012); this mechanism for insulin resistance may be more important in the laminae, where endothelial expression of insulin receptor appears to be increased in the setting of hyperinsulinemia (Burns and others 2012). Altered vascular responsiveness in the setting of insulin resistance is thought to result from decreased insulin-mediated elaboration of NO and its vasodilatory effects on vascular smooth muscle (Muniyappa and Yavuz 2012). Attenuation of PI3K signaling downstream of insulin receptor activation results in decreased activity of endothelial NOS (eNOS) and decreased NO production. Concurrently, while defective PI3K signaling is observed from insulin-bound insulin receptor in endothelial insulin resistance (which may be acquired over a relatively short period of time), signaling through the MAPK pathway appears to be preserved. Increased production of endothelin-1, a potent vasoconstrictor, through this pathway, along with decreased vasodilatory effects of NO, is thought to result in inappropriate vasoconstriction, failure to achieve flow-dependent vasodilation, and ultimately, hypertension. Local perfusion, particularly in those tissues lacking collateral circulation, may be inadequate – it is possible that this perfusion deficit in the laminae may activate stress kinase systems, such as MAPK and AMPK (see above). Nitric oxide also has been reported to have important anti-inflammatory and anti-coagulant effects, loss of which has been associated with the propagation of atherothrombotic lesions in humans and experimental animal models of metabolic syndrome (Bashan and others 2009). In horses
with IR, pathologic changes in vasomotor tone to a tissue with poor collateral circulation (the foot) may promote hypoxia/ischemia, both of which have been reported to play roles in the pathogenesis of laminitis (Robertson, Bailey, Peroni 2009). Thrombi have also been noted in the laminar microvasculature in experimentally-induced and naturally-occurring laminitis (Noschka and others 2009).

Another intriguing possibility that remains purely speculative at this point is that insulin may be triggering signaling in laminar epithelial cells through IGF-1 receptor, not insulin receptor (which again, doesn’t appear to be present at high levels on laminar epithelial cells). Insulin has been shown to bind and activate IGF-1R at supraphysiologic concentrations in cell cultures (concentrations easily achievable in the average IR equid in response to a dietary carbohydrate challenge)(Varewijck and Janssen 2012), and IGF-1R’s distribution within the laminae appears to be much more extensive than that of the insulin receptor (Burns and others 2012). While insulin receptor appears to be limited to the laminar microvasculature, IGF-1R is present on laminar epithelial cells, endothelial cells, and fibroblasts (and likely other cell types as well). Experiments on in vitro skin culture systems using human keratinocytes have revealed an important role for IGF-1 in the normal maturation of epithelial cells in the skin, which progresses in an orderly fashion in the presence of IGF-1 and its receptor from basal cells to anucleate squamous cells; siRNA knockdown of IGF-1R in this culture system results in disordered maturation of keratinocytes and the production of histologically abnormal epidermis (Sadagurski and others 2006). The importance of IGF-1R signaling in the laminar
epidermis, particularly its effects on extracellular matrix and cytoskeletal components, is currently unclear but warrants investigation.

The inclusion of laminitis in the diagnostic criteria for EMS is required based on the definition of the syndrome; it is the outcome variable for which a diagnosis of EMS is meant to define risk. In humans, the constellation of clinical and laboratory findings that together (however they may be defined, which, as mentioned above, is quite variable in the literature) mark the metabolic syndrome are meant to define risk of a certain outcome (typically coronary artery disease and MI, but cerebrovascular disease, type II diabetes mellitus, and microangiopathic disease have been evaluated as outcomes as well) (Alberti and others 2009). In the setting of EMS, that outcome has been held to be limited to endocrinopathic laminitis; that said, it remains to be seen whether the emergence of other adverse outcomes (reproductive failure, poor athletic performance, altered life span, incidence of PPID, etc.) may be tied to the presence of EMS in the future. As it stands currently, EMS is meant to define a set of risk factors that are associated with laminitis. Therefore, clearly, inclusion of this factor in the definition of EMS means that earning this diagnosis implies that the individual in question has experienced a bout of laminitis at some point in the past, based on historical and/or clinical evidence – further, this diagnosis in sound animals is often made with insensitive detection mechanisms for prior bouts of laminitis (hoof capsule morphology, radiography). By definition, inclusion of evidence of historical or current laminitis in the diagnostic criteria for EMS excludes animals that have not yet experienced a bout of laminitis, unarguably the ideal time for prophylactic intervention. Further, our ability to diagnose laminitis in an animal that is
not currently exhibiting clinical signs of the condition is crude and limited to individuals with disease that has altered the morphology of the foot in some way(s); animals that have experienced mild bouts may be overlooked. Prospective studies (similar to those that have been conducted in inbred populations of ponies) to assess incidence of laminitis in individual animals with certain phenotypic or biochemical characteristics (i.e., regional adiposity, hyperlipidemia, IR, ectopic lipid accumulation, etc.) are required on a larger scale and over a longer time frame, including a heterogeneous population of horses.

Equine insulin resistance

Clinical assessment of IR in equids

Assessment of systemic insulin resistance is thought to be critically important to the definition of EMS, particularly given the recent direct association of increased plasma insulin concentration with laminitis. IR in equids is frequently measured both clinically and in the research setting; however, very often the methodology used in these two settings is different, making comparisons between larger, clinical, population-based studies and smaller experimental studies difficult. Measurement of fasting serum insulin concentration (in conjunction with an assessment of plasma glucose concentration) is the most common field test, based on the availability of the methodology and the ease with which a single blood sample can be collected. However, dynamic aspects of insulin and glucose dynamics may not be revealed by a single sample; a single ‘normal’ insulin concentration (which is a parameter that also currently requires additional definition in horses and ponies) often do not predict normal insulin sensitivity in response to an oral or
intravenous glucose challenge, many of which have not been directly compared in the same cohort of horses. Validation of some of the more clinically feasible tests (such as fasting serum insulin concentrations, or the combined glucose and insulin test) against the gold standards in experimental studies (the euglycemic, hyperinsulinemic clamp or the frequently-sampled intravenous insulin and glucose tolerance test with minimal model kinetic analysis) would be helpful in gaining acceptance of a standard methodology in measuring IR in horses and ponies for future study.

Several laboratory tests have been evaluated and utilized in humans and horses for the diagnosis and monitoring of insulin resistance, including fasting plasma insulin and glucose concentrations (Frank and others 2006; Jeffcott and others 1986), the oral glucose tolerance test (Jeffcott and others 1986), the intravenous glucose tolerance test (Ralston 2002), the modified intravenous insulin and glucose tolerance test (Frank and others 2006), the intravenous insulin tolerance test (Jeffcott and others 1986), the euglycemic/hyperinsulinemic clamp technique (Rijnen and van der Kolk 2003), and the frequently-sampled intravenous glucose tolerance test (Pratt, Geor, McCutcheon 2005). Of these methods, only the frequently-sampled intravenous glucose tolerance test and the euglycemic/hyperinsulinemic clamp technique are well-established methods for accurate quantitative assessment of insulin action (Radziuk 2000). The frequently-sampled intravenous glucose tolerance test is technically simple to perform, more so than the euglycemic/hyperinsulinemic clamp technique, and as such was one of the assays chosen for the studies reported in this dissertation.
Mechanisms resulting in hyperinsulinemia in the IR equid

*Insulin clearance*

Several methods have been described in the literature attempting to quantify insulin secretion in many species (including horses and ponies). Unfortunately, the methods which are technically simplest (such as measurement of insulin in peripheral venous blood following oral or IV glucose challenge) are heavily confounded by the effect of hepatic first-pass clearance on portal blood (which contains pancreatic insulin secretion). Plasma insulin concentration can be shown to readily increase in a dose-dependent fashion in response to a carbohydrate challenge (oral vs. IV), and this is presumed to result at least in part from pancreatic secretion. However, the relative contributions of altered insulin clearance and altered pancreatic secretion cannot be evaluated effectively with this sampling method and assay (even with analysis via minimal model kinetics).

Assessment of plasma C-peptide concentrations concurrently with assessments of insulin might be more useful – C-peptide is a cleavage product of pro-insulin that is secreted in equimolar amounts with insulin, but (in contrast to insulin) it is primarily excreted unchanged in the urine. Measurement of C-peptide would allow the assessment of beta-cell function independent of hepatic action on pancreatic secretions, and initial work describing this technique’s use in horses has been published (Toth and others 2010). However, the assay requires further validation in a larger number of horses and ponies, including those that are insulin-resistant. The technique is also not commercially available for analysis of equine samples, limiting its use in clinical patients. Finally,
single time point evaluations of plasma C-peptide concentrations (which would again be most useful for equine practitioners) haven’t been validated on a large scale, still requiring a glucose stimulation test to evaluate β-cell function.

Direct measurement of insulin secretion from pancreas would require sampling portal venous blood following enteral or intravenous carbohydrate challenge; this has been reported in experimental rodent models. This would be at best very challenging in horses and ponies, as surgical access to the portal vein is difficult and accompanied by significant risk to the animal. However, this remains the only way to truly accurately measure prehepatic insulin flux from the pancreas (Matveyenko, Veldhuis, Butler 2008; Porksen and others 1995; Song and others 2000).

Regarding the involvement of other hormones in insulin release from the pancreas, as described above, incretins have been shown to be critically important mediators of insulin release during increased plasma glucose concentrations (in fact, they have little effect on insulin secretion during euglycemia, increasing their attractiveness as therapeutic agents, as they are accompanied by little to no risk of hypoglycemic episodes, in contrast to insulin) (Chrysant and Chrysant 2012). Since incretins are derived from intestinal enteroendocrine cells in response to meal feeding, their effects can be bypassed through administration of a glucose challenge intravenously. This might be one way to quantitate the relative importance of incretins to the insulinemic response in certain classes of subjects (administration of both oral and IV glucose tolerance tests, with assessment of insulin responses to both; the difference between the two is the incretin effect).
Insulin degrading enzyme (IDE) is thought to be the primary enzyme responsible for insulin degradation following its release from the endocrine pancreas (Fernandez-Gamba and others 2009). While IDE is ubiquitously expressed (in insulin-responsive and non-insulin-responsive tissues, suggesting that it subserves alternate functions in different tissues), it is thought that hepatic IDE is responsible for the degradation of the majority of plasma insulin (at least 60%) (Fernandez-Gamba and others 2009). Following binding to the insulin receptor, insulin and its receptor are removed from the plasma membrane by endocytosis in a process that is enhanced by normal insulin signaling (negative feedback loop). Endosomal acidification is thought to be required for insulin release from its receptor [which can be recycled back to the plasma membrane]; IDE can then degrade free insulin. IDE is present in the cytosol, in cell membranes, and in some organelles; it is also secreted into the extracellular space (Fernandez-Gamba and others 2009). The insulin-induced activity of IDE has been shown in hepatoma cell lines to be inhibited by elevated concentrations of glucose in the culture media, suggesting a mechanism by which increased alimentation (especially increased dietary NSC) could lead to decreased liver insulin clearance and, consequently, inappropriate hyperinsulinemia (Valera Mora and others 2003). Conversely, normal insulin signaling has been shown to upregulate IDE activity; most of these studies have been performed in the setting of experimental models of Alzheimer’s disease (β-amyloid is a target of IDE whose accumulation has been shown to correlate with increased risk of AD); AD patients show decreased IDE transcript and activity in brain tissue (Messier and Teutenberg 2005). Further, IDE-knockout mice display hyperinsulinemia, decreased glucose tolerance, and type II DM,
implicating the enzyme in some of the most characteristic aspects of the metabolic syndrome.

It is certainly possible that manipulation of insulin clearance (through pharmacologic activation of IDE, or modification of the diet to increase hepatic insulin clearance, for example) might be useful in the management of EMS. However, first it must be established that altered insulin clearance is responsible for the majority of the hyperinsulinemia observed in these patients (vs. exuberant pancreatic insulin secretion in response to insulin resistance, for example). This has not been demonstrated in equids. Experimentally, particularly if money and patience were no object, comparison of serum insulin concentrations across the liver (i.e., in portal venous blood and systemic venous blood [ideally right atrial blood]) in response to both oral and IV glucose tolerance tests with frequent sampling would identify the relative contributions of pancreatic secretion and hepatic clearance of insulin to the systemic insulin concentrations observed following the testing. Due to the technical difficulty associated with this technique, evaluation of C-peptide responses in concert with insulin responses to an oral and IV glucose tolerance test might be an easier, more efficient way to arrive at the same answer.

**White adipose tissue, inflammation, and the metabolic syndrome**

White adipose tissue is no longer taken to be a metabolically inert energy storage depot, but rather an endocrinologically active, dynamic tissue that plays a critical role in the regulation of intermediary metabolism of fats and carbohydrates. Much has been
learned about the role of the adipose tissue as an endocrinologically active organ; abdominal adipose tissue (more specifically, adipose tissue within omental and other visceral depots) appears to be more insulin-resistant, to express and secrete more adipokines and pro-inflammatory cytokines, and to correlate better with cardiovascular disease risk than do other adipose tissue depots in humans (Despres and Lemieux 2006). This belies its importance in the pathogenesis of the human metabolic syndrome; however, the roles of these adipose tissue depots, if any, in the equine metabolic syndrome remain to be defined.

In horses, an association has been made in several observational studies between the degree of adiposity (measured by body condition scoring according to the method of Henneke (Henneke and others 1983), tritiated water distribution, and ultrasonographic rump fat thickness, for example) and systemic insulin resistance. As insulin resistant equids often display hyperinsulinemia (fasting and/or in response to an oral or intravenous carbohydrate challenge), and hyperinsulinemia has recently been shown to precipitate laminitis experimentally, insulin resistance is thought to be pathophysiologically linked to laminitis, hence its inclusion in the diagnostic criteria for EMS. Further studies in ponies and small numbers of light breed horses have shown a degree of correlation between increased size of the nuchal ligament adipose tissue (assessed via neck morphometric parameters and/or neck crest scoring) and degree of systemic IR (Carter and others 2009a; Frank and others 2006). However, the relationship between degree of adiposity and systemic insulin resistance is very clearly non-linear, with the ready identification of globally obese individuals who are quite insulin sensitive
and relatively lean individuals who are insulin resistant. There appears to be a prominent breed predisposition to this phenomenon, as breeds less commonly afflicted with EMS, such as American quarter horses and Thoroughbreds, appear less likely to become severely IR with obesity; conversely, lean members of EMS-predisposed breeds, such as Morgans, Paso Finos, and American Saddlebreds, may display relatively refractory IR (Frank and others 2006). While nutritional obesity may play an important role in initiating or perpetuating IR in a genetically predisposed individual, it may not be highly predictive of laminitis upon induction of obesity, as insulin resistance and laminitis are not observed in all obese animals. Functional assessment of the adipose tissue (in addition to assessment of distribution) may be more relevant in this setting, as multiple adipokines (leptin, adiponectin, and resistin, for example) have been shown to have direct effects on insulin sensitivity in other species.

**The endocrine pancreas, insulin resistance, and type II diabetes mellitus**

Following ingestion of a meal, the components of the meal are broken down into their respective nutritional elements by salivary, gastric, pancreatic, and intestinal enzymatic activity. While an average grain meal (i.e., a 10% sweet feed preparation) for horses contains protein, lipid, and carbohydrate, the majority of said ration is carbohydrate (especially starch and simple sugars). Degradation of dietary starch and sugars to hexoses (glucose, galactose, and fructose) results in their absorption by simple and facilitated diffusion (co-transport with sodium) across the gastrointestinal tract into
the portal venous blood and ultimately the systemic circulation. While increases in other diet-derived substances in blood (such as certain amino acids) can induce pancreatic insulin secretion, the post-prandial increase in plasma glucose concentration is a potent (and the most physiologically important) secretagogue for insulin from the beta-cells of the endocrine pancreas. For the past 30 years, it has been held that transport of glucose into the beta-cell (primarily through GLUT2) during times of hyperglycemia results in increased glycolysis and ATP production. An ATP-responsive potassium channel is present on the plasma membrane of the β-cell; increased cytosolic ATP concentrations, then, result in its inhibition, inducing decreased potassium conductance from the β-cell and its relative depolarization (Aguilar-Bryan and Bryan 1999; Petit and Loubatieres-Mariani 1992). Increased activation of voltage-gated calcium channels on the plasma membrane and increased calcium influx activates calcium-dependent mechanisms responsible for fusion of insulin-containing cytoplasmic vesicles (which also contain equimolar amounts of C-peptide) with the plasma membrane, exacting insulin release into the interstitial fluid and, ultimately, the pancreatic venous blood (Horie and others 1997).

Recent work in cultured islets suggests that this mechanism is likely not the only one responsible for insulin release, however. Two phases of insulin secretion are observed in response to a glycemic load, an initial, rapid phase (i.e., within the first 10 minutes following administration of a glucose bolus) and a delayed, sustained phase (i.e., beginning ~15 minutes following the first phase) (Henquin 2009; Henquin 2011). Pharmacologic inhibition of the ATP-sensitive potassium channels described above
significantly attenuates the initial rapid phase of insulin secretion but has limited effect on the sustained secretion noted later. Gut-derived hormones (particularly incretins, such as glucagon-like peptide 1 [GLP-1] and glucose-dependent insulinotropic hormone [GIP] inhibitory peptide, from the L- and K-cells of the small intestinal mucosa, respectively) are likely responsible for the second phase of sustained insulin release and have become attractive therapeutic targets for the treatment of relative insulin deficiency in humans with type II diabetes mellitus (Nicolucci and Rossi 2008). Incretins were discovered and subsequently described following the initial observation that an oral glucose load/meal would reliably elicit a 2-3 fold greater insulinemic response than a similar glucose load given intravenously (thereby bypassing the GI tract); the incretin response is severely impaired in humans with type II DM, an abnormality which can be pharmacologically reconstituted with the administration of GLP-1 analogues (such as exendin-4 homologues, like exenetide) or inhibitors of the enzyme that degrades incretins, dipeptidyl peptidase-4 (such as sitagliptin) (Nicolucci and Rossi 2008).

Factors that are likely to modify this response in a horse consuming a meal of sweet feed include first the composition of the meal, particularly its ‘glycemic index’. The glycemic index is technically a term applied to human foodstuffs and is defined as the ability of a specific dose of the food in question to raise blood sugar compared to a similar quantity of white bread. This has been modified somewhat for use in equine nutrition, and the ‘relative’ glycemic indices of certain cereal grains have been reported (for example, the glycemic response of the horse to a corn meal is higher than that to one of barley) (Ralston 2007). Since postprandial plasma glucose concentration is the most
physiologically important secretagogue for insulin, the ability of the ingested meal to raise plasma glucose concentration would be expected to have a profound effect on the insulin response to that meal (Kasai and others 2010). This is exploited therapeutically in the dietary management of horses and ponies with EMS, as attempts are made to minimize the amount of non-structural carbohydrate in their diets (Geor and Harris 2009).

The functional pancreatic beta cell mass of the individual, which has been shown to correlate to insulinemic responses to meals and to IV glucose challenge in experimental animals (Paulsen and others 2010), is an important variable to consider when evaluating an animal’s insulin response to a meal. This has not been effectively characterized in equine populations, but beta-cell failure (insufficient insulin response to carbohydrate challenge) is uncommon in this species (Johnson and others 2005). Further, beta-cell mass is virtually impossible to measure accurately in vivo (and presents challenges ex vivo); it can be measured experimentally, which may shed light on the mechanisms by which equine beta-cell function is preserved in the setting of prolonged hyperinsulinemia (where type II DM might be expected in humans) (Park and Bell 2009).

The relative insulin sensitivity of the primary insulin-responsive tissues (skeletal muscle, liver, and adipose tissue), which are responsible for the majority of post-prandial glucose disposal, has important ramifications for the insulin secretory response. Animals that are systemically insulin resistant show decreased glucose disposal from the plasma following a meal (Frank and others 2010), and they display increased plasma fatty acid concentrations in both the fasting and fed states (decreased insulin-mediated inhibition of
hormone sensitive lipase in adipose tissue, decreased inhibition of hepatic synthesis and secretion of VLDL) (Schaeffler and others 2009). Experimental glucotoxicity, lipotoxicity, or both has been shown to decrease insulin secretion from cultured islets and in vitro over the course of several hours of glucose and lipid incubation or infusion; the mechanism for this remains to be clarified, but since metabolic cycling of pyruvate metabolites (i.e., isocitrate-malate, citrate-malate, pyruvate-malate) is suspected to be involved in insulin secretion, the dramatic addition of metabolites to these pathways can be expected to affect their outcome (insulin secretion) (Prentki and Nolan 2006). Additionally, the expression and functionality of incretin receptors on beta-cells has been shown to be blunted in the setting of insulin resistance; again, the mechanism for this is unclear, but this is an active area of investigation in human medicine (Nichols and Remedi 2012). Finally, while the pancreas is not responsible for a large amount of insulin-dependent glucose disposal, it is insulin-responsive; insulin receptor signaling is thought to be involved in pancreatic insulin secretion, which may become dysfunctional in the setting of systemic insulin resistance (completely unknown in horses and ponies) (Wang and Jin 2009).
Chapter 2: Pro-inflammatory cytokine and chemokine expression profiles of various adipose tissue depots of insulin-resistant and insulin-sensitive light breed horses

Abstract

Insulin resistance has been associated with predisposition to laminitis in horses and ponies. In humans and rodents, omental adipose tissue (AT) expresses pro-inflammatory cytokines and adipokines at significantly higher levels than subcutaneous or retroperitoneal AT, in correlation with the degree of insulin resistance. While this has been postulated to also be a factor contributing to insulin resistance in horses, no published data currently support or refute this hypothesis. This study characterized the expression of pro-inflammatory cytokines and chemokines in several AT depots of insulin-resistant (IR) and insulin-sensitive (IS) horses. Eleven mares (8 Quarter horses, 2 Thoroughbreds, and 1 Standardbred), categorized as IR (insulin sensitivity [SI] = 0.58 ± 0.31 L•min⁻¹•mU⁻¹; n = 5) or IS (SI = 2.59 ± 1.21 L•min⁻¹•mU⁻¹; n = 6) based on results of an insulin-modified frequently-sampled intravenous glucose tolerance test were studied. No statistically significant differences in weight, body condition score, neck
circumference, girth, or ultrasonographic retroperitoneal fat thickness were noted between groups. Omental, retroperitoneal, and mesocolonic AT was collected concurrently via ventral midline celiotomy; nuchal ligament and tail head AT biopsies were collected via skin incision. All tissues were snap-frozen and stored at -80°C. For each depot, total RNA was extracted and cDNA analyzed via real-time quantitative PCR to quantify expression of TNF-α, IL-1β, IL-6, plasminogen activator inhibitor-1 (PAI-1), and monocyte chemoattractant protein-1 (MCP-1). Data were analyzed using a Kruskal-Wallis test with a Dunnett’s post test (p = 0.05). No differences in AT expression of TNF-α, IL-1β, IL-6, PAI-1, or MCP-1 were noted between IR and IS groups for each depot. However, when data from IR and IS groups were combined for each depot, the expression of IL-1β (p=0.009) and IL-6 (p=0.023) was significantly higher in nuchal ligament AT than in other depots, suggesting that this AT depot has different biological behavior in the horse and is more likely to adopt an inflammatory phenotype than other depots examined. Importantly, these data indicate that omental adipose tissue (and other visceral fat depots) may not be as important to the pathophysiology of obesity in the horse as in other species.

**Introduction**

Local expression of inflammatory molecules by adipose tissue depots is reported to play a central role in the onset of local and systemic insulin resistance in the obesity-related condition, metabolic syndrome, in both humans and horses (de Luca and Olefsky
2008; Maury and Brichard 2010; Vick and others 2008). Equine metabolic syndrome (EMS), (Johnson 2002) an increasingly well-characterized constellation of clinical findings including obesity (particularly regional adiposity), systemic insulin resistance, hyperinsulinemia, dyslipidemia, and increased risk of endocrinopathic laminitis, has been suggested to represent a state of systemic inflammation similar to that reported in humans (Sutherland, McKinley, Eckel 2004), (Vick and others 2007). Historically, white adipose tissue was considered a relatively metabolically inert tissue that functioned primarily as an energy storage depot. However, since the discovery and description of the adipose tissue-derived hormone leptin in 1995 (Campfield and others 1995; Halaas and others 1995; Pelleymoune and others 1995), white adipose tissue has been increasingly recognized to be not only highly metabolically active, but also endocrinologically important in regulating metabolism (Trayhurn and Wood 2004). With the development of obesity, adipose tissue is reported to adopt a distinctly inflammatory phenotype, characterized by increased gene expression and secretion of pro-inflammatory cytokines (such as tumor necrosis factor-α [TNFα], interleukin-1β [IL-1β], and IL-6) and chemokines (Clement and others 2004; Li and others 2002).

Pro-inflammatory cytokine and chemokine expression patterns have been shown to be heterogeneous among adipose tissue depots in humans and rodents, with visceral adipose tissue (omenta, retroperitoneal, and mesenteric depots) displaying greater gene expression of these substances both in lean individuals and in the setting of genetic and nutritional obesity (Lefebvre and others 1998), (O'Rourke and others 2009). Knowledge of these depot-specific differences has led to the categorization of omental and truncal
subcutaneous obesity as strong risk factors predicting atherothrombotic cardiovascular disease in obese humans (de Koning and others 2007; Lee and others 2008). Although metabolic risk (indicating risk for EMS-associated laminitis [EMSAL]) has been documented for obese ponies with prominent regional adiposity (i.e., large nuchal ligament adipose tissue accumulation) (Carter and others 2009a; Treiber and others 2006), the relationship of this risk to metabolic activity of the adipose tissue itself has not been reported in horses.

In addition to increased production of pro-inflammatory molecules, obese adipose tissue has also been reported to display altered production of the adipokines leptin and adiponectin, among others; specifically, in the obese individual, expression of leptin in white AT is increased and that of adiponectin is decreased (Stofkova 2009). Both leptin and adiponectin have systemic and tissue-level effects on metabolism and inflammation, and derangements in their production by AT in obesity may have significant consequences for the organism (Stofkova 2009).

Leptin is the prototypical adipokine, first described in 1995 as the ob gene product whose loss of function results in severe genetic obesity (Campfield and others 1995; Halaas and others 1995; Pelleymounter and others 1995). It is a 16 kDa adipocyte-derived protein whose predominant source in vivo is white adipose tissue; circulating serum concentrations of leptin correlate with adipose tissue mass and adipocyte size in multiple species (Boucher and others 2005; Carter and others 2009a; Kearns and others 2006; Stofkova 2009), and leptin has been referred to as the primary ‘nutritional rheostat’ in mammalian systems. Leptin’s physiologic functions in the normal individual include
roles in appetite suppression (result of a signal of energy excess from adipose tissue to the hypothalamus) and enhanced catabolic metabolism both by direct activity on tissue and through modulation of hypothalamic sympathetic nervous system output; leptin’s activity within the central nervous system is critical for these effects (Yeo and Heisler 2012). In the obese individual, a phenomenon of ‘leptin resistance’ has been described, in which elevated serum leptin concentrations from an expanded adipose tissue mass do not signal satiety as would be expected; obese individuals are frequently physically hyperleptinemic but ‘functionally hypoleptinemic’ (Ryan, Woods, Seeley 2012). Additionally, leptin has been reported to promote pro-inflammatory signaling, which may not be attenuated in the ‘leptin resistance’ which accompanies obesity; consequently, leptin may play an important role in the chronic inflammatory state which has been reported to characterize obesity in multiple species (Stofkova 2009).

Adiponectin is a 244 amino acid protein whose primary endogenous source is also, like leptin, white adipose tissue (with other minor sources contributing to circulating serum levels in vivo) (Okamoto and others 2006). However, unlike leptin, whose concentration in plasma is typically elevated in insulin resistant individuals, adiponectin has been shown to decrease in serum (protein) and tissue (message) in individuals with metabolic syndrome (Stofkova 2009). This adiponectin ‘deficiency’ may have serious functional consequences, as this adipokine has been shown to be potently anti-inflammatory, insulin-sensitizing, and cardioprotective (Zhang and others 2007).

Systemic insulin resistance is a defining characteristic of EMS and appears to be strongly linked to laminitis risk in affected animals (Carter and others 2009a; Treiber and
others 2006). Hyperinsulinemia, a common sequel to peripheral insulin resistance in the EMS patient, recently has been shown to precipitate clinical laminitis in ponies and light-breed horses without clinical EMS (Asplin and others 2007; de Laat and others 2010). Therefore, insulin resistance can be considered both a defining marker of EMS and, possibly, as a result of increased insulin concentration, a central mechanism in the pathophysiology of its most important complication, laminitis. Because adipose tissue-derived inflammatory molecules are reported to play a central role in the onset of insulin resistance both at the tissue level and systemically, the study reported here was performed to investigate pro-inflammatory cytokine and chemokine gene expression in different adipose tissue depots and to determine the relationship of inflammatory gene expression patterns in different depots to systemic insulin resistance in adult light-breed horses.

Materials and Methods

Experimental animals

Light breed mares owned by The Ohio State University College of Veterinary Medicine and housed at the college teaching and research farm were utilized for this project. All mares were group-housed in a dry lot and fed timothy hay ad libitum (grown and baled on-site); forage nutrient analysis was not performed, but all mares were fed identically. No clinical evidence of laminitis was observed in any of the animals used for this study. Experimental procedures were performed according to protocols approved by the OSU Institutional Animal Care and Use Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals.
Insulin-modified frequently-sampled intravenous glucose tolerance testing (FSIGTT)

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were determined using a commercially-available radioimmunoassay\(^5\) previously validated for horses, and glucose concentrations were analyzed with a colorimetric assay\(^6\). Insulin and glucose data were analyzed by minimal model parameters (Treiber and others 2005), and the calculated insulin sensitivity (SI; L•min\(^{-1}\)•mU\(^{-1}\)) was used to categorize each horse as insulin-resistant (IR; SI < 1.0 L•min\(^{-1}\)•mU\(^{-1}\)) or insulin-sensitive (IS; SI > 1.5 L•min\(^{-1}\)•mU\(^{-1}\)). Based on the SI results, 12 of the 20 tested mares (6 IR and 6 IS) were selected to undergo adipose tissue biopsy collection.

**Adipose tissue biopsy collection**

All biopsy collections were performed in July 2008. Horses were transported to the OSU Veterinary Teaching Hospital, weighed upon arrival, and housed individually in box stalls with access to timothy hay, water, and a trace mineral block *ad libitum*. A physical examination was performed on each horse upon arrival, and morphometric parameters were recorded (neck circumference (Frank and others 2006), girth circumference, ultrasonographic retroperitoneal fat thickness at the umbilicus). All animals were considered healthy based on results of physical examination, CBC, and serum biochemical analysis performed on the day of arrival. Evidence of chronic pyometra, however, was detected in 1 IR mare during hospitalization, and data from this mare therefore were excluded from the final analysis. An acclimation period of 48 hours was observed before biopsy collection. On the day of biopsy collection, a 14-ga intravenous catheter\(^7\) was aseptically inserted in the left external jugular vein for the purposes of sample collection and induction and maintenance of general anesthesia. A
A pulmonary artery catheter was aseptically inserted via the right external jugular vein for use in collecting blood gas data for a separate study. A tetanus toxoid booster was administered in the left cervical musculature before biopsy collection, and sulfamethoxazole-trimethoprim (15 mg/kg PO q12 h) and phenylbutazone (1 g PO q12 h) were administered to each animal (both continued for 5 days after biopsy collection).

After sedation with xylazine (0.5 mg/kg IV), general anesthesia was induced with ketamine (2.2 mg/kg IV) and diazepam (0.1 mg/kg IV) and maintained with guaiifenesin-based total intravenous anesthesia (guaiifenesin 5%; xylazine 0.05%; ketamine 0.2%; 1-2 ml/kg/hour). All mares were orotracheally intubated, and positive-pressure ventilation was initiated and maintained with 100% oxygen for the duration of the procedure (approximately 30 minutes). Incisional biopsies of nuchal crest and tail head adipose tissue were collected (~3 g tissue collected from each site) with the animal in lateral recumbency after aseptic preparation of the biopsy sites with chlorhexidine surgical scrub and isopropyl alcohol; incisions were closed in a single layer with 0 polydioxanone monofilament suture material in a simple continuous pattern. Each horse then was placed on a padded surgical table in dorsal recumbency and moved into a surgical suite; the abdomen was aseptically prepared and draped for ventral midline celiotomy. A small ventral midline abdominal incision was created (~8 cm in length), and adipose tissue biopsies (~3 g each) were collected from the margins of the incision (representing retroperitoneal adipose tissue), the mesocolon of the descending colon (representing mesenteric adipose tissue), and the omentum. The abdominal incision was closed in 2 layers: the linea alba was apposed with 2 polyglactin 910 in a simple
continuous pattern, and the skin was apposed with 0 polydioxanone in a simple continuous pattern. Horses then were allowed to recover from general anesthesia and returned to box stalls. All mares remained hospitalized for 10 days after biopsy collection, during which time they were continuously monitored for incisional and other complications. After this period, all mares were returned to the teaching herd. Sutures were removed manually from the nuchal ligament and tail head incisions 14 days after biopsy collection; sutures were not removed from the abdominal incisions.

A portion of each adipose tissue biopsy was flash-frozen in liquid nitrogen at the time of collection and stored at -80º C for later analysis by real-time quantitative PCR; the remainder was fixed in neutral buffered formalin and paraffin embedded.

RNA isolation, cDNA synthesis, and real-time quantitative polymerase chain reaction (RT-qPCR)

Homogenates were made from each adipose tissue sample (1.0-1.5 g) with a tissue disruptor¹⁹, and total RNA was extracted using a modified Trizol method. cDNA was synthesized from total RNA from each sample (after DNAse treatment²⁰) via reverse transcription (Retroscript²¹) and stored at -20º C until used for RT-qPCR analysis.

A thermocycler (Roche 480²²) was used to perform RT-qPCR. Amplification was quantified against external standards using fluorescent format for SYBR Green I as previously described.(Waguespack, Cochran, Belknap 2004; Waguespack and others 2004) Primers for TNFα, IL1β, IL-6, plasminogen activator-inhibitor-1 (PAI-1), monocyte chemoattractant protein-1 (MCP-1), leptin, adiponectin, and the housekeeping
genes β-actin and β₂-microglobulin were designed against equine-specific gene sequences (intron-spanning exon sequences) using computer programs as previously described (Waguespack, Cochran, Belknap 2004) and were used for amplification of the respective genes of interest. Standard curves for quantification of transcript in adipose tissue samples were generated using serial dilutions of a linearized vector containing an insert of each amplified cDNA fragment whose identity was confirmed via DNA sequencing.

Data analysis

RT-qPCR data from housekeeping genes (β-actin and β₂-microglobulin) were entered into a computer program to test each gene’s quality as a housekeeping gene for equine adipose tissue. Because both genes were determined to be satisfactory by the program, a geometric mean was obtained from the 2 genes’ data in order to generate a normalization factor for gene expression from each adipose tissue sample. Gene expression data from the genes of interest then was normalized using this factor for each individual tissue sample.

Data analysis was performed using a statistical software program. Morphometric and gene expression data were assessed for normality by the Shapiro-Wilk and D’Agostino and Pearson omnibus normality tests. Morphometric data were analyzed with a Student’s t test; gene expression data (or log transforms of non-normal data) were subjected to 2-way analysis of variance with repetition on 1 factor (depot) followed by Bonferroni’s post-test. Correlation analyses (Pearson’s rank correlation test) were
performed to identify relationships between morphometric parameters and nuchal ligament cytokine gene expression. Statistical significance was accepted at p < 0.05.

Results

Differences in age, body weight, body condition score, neck circumference, girth circumference, retroperitoneal adipose tissue thickness, basal insulin concentration, or basal leptin concentration between the IR and IS groups were not detected (Table 1).

Subjectively, the omental adipose tissue depot of all horses appeared grossly small at laparotomy. Of the parameters evaluated, the only statistically significant difference between the groups was peripheral insulin sensitivity (IR group, SI = 0.58 ± 0.31 x 10^{-4} L•min^{-1}•mU^{-1}, n = 5; IS group, SI = 2.59 ± 1.21 x 10^{-4} L•min^{-1}•mU^{-1}, n = 6; p = 0.008). No statistically significant difference was observed in acute insulin response to glucose (AIRg) between the groups (IR, AIRg = 821.8 +/- 585.8 mU/L/min; IS, AIRg = 394.2 +/- 162.4 mU/L/min; p = 0.18). No correlation was observed between basal insulin concentration and SI (p = 0.28).

No statistically significant effect of insulin sensitivity status on concentrations of mRNA coding for TNF-α (p = 0.29), IL-1β (p = 0.80), IL-6 (p = 0.93), MCP-1 (p = 0.23), PAI-1 (p = 0.72), or leptin (p = 0.14) (Figure 1). However, a significant difference in gene expression among depots was observed, with higher concentrations of IL-6 (p = 0.004) and IL-1β (p = 0.0005) mRNA and lower concentrations of leptin (p = 0.006) and adiponectin (p = 0.0001) measured in nuchal ligament adipose tissue compared with
other depots (Figure 2). No differences in gene expression of TNF-α (p = 0.97), MCP-1 (p = 0.14), or PAI-1 (p = 0.07) were observed among depots (Figure 2).

There was a significant correlation between the expression of IL-1β and IL-6 in the nuchal ligament (r = 0.76; p = 0.006). However, there was no statistically significant correlation between IL-1β or IL-6 expression and mean neck circumference (IL-1β: r = 0.24, p = 0.48; IL-6: r = 0.31, p = 0.36), body condition score (IL-1β: r = -0.10, p = 0.76; IL-6: r = 0.11, p = 0.74), and body weight (IL-1β: r = 0.08, p = 0.82; IL-6: r = 0.15, p = 0.64).
Table 1. Morphometric characteristics of insulin-resistant (IR) and insulin-sensitive (IS) light breed mares from which adipose tissue biopsies were collected (mean +/- SD).

<table>
<thead>
<tr>
<th>Group</th>
<th>Age (years)</th>
<th>Body weight (lb)</th>
<th>Body condition score (1-9)</th>
<th>Neck circumference (cm)</th>
<th>Girth circumference (cm)</th>
<th>Retroperitoneal fat thickness* (cm)</th>
<th>Basal insulin concentration (mIU/L)</th>
<th>Basal leptin concentration (ng/mL, HE$^\wedge$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IR (n = 5)</td>
<td>16.8 +/- 4.5</td>
<td>1122 +/- 56</td>
<td>7.2 +/- 0.7</td>
<td>90.1 +/- 4.2</td>
<td>186.3 +/- 4.3</td>
<td>3.0 +/- 0.9</td>
<td>22.9 +/- 9.9</td>
<td>4.02 +/- 1.77</td>
</tr>
<tr>
<td>IS (n = 6)</td>
<td>10.8 +/- 7.2</td>
<td>1193 +/- 133</td>
<td>6.75 +/- 1.6</td>
<td>93.2 +/- 3.1</td>
<td>189.1 +/- 6.4</td>
<td>2.7 +/- 1.7</td>
<td>15.5 +/- 11.9</td>
<td>2.60 +/- 0.85</td>
</tr>
</tbody>
</table>

*Represents thickness of the retroperitoneal adipose tissue measured ultrasonographically at the level of the umbilicus with a 7.5 mHz linear array probe. The average of 5 measurements was recorded for each horse.

$^\wedge$ HE, human equivalent
Figure 1. Gene expression of IL-1β, IL-6, TNFα, MCP-1, and PAI-1 by adipose tissue depot. Data from IR and IS groups have been stratified for each depot. No statistically significant differences in gene expression were observed between IR and IS groups for each depot. Prominent individual variability in gene expression was noted in IR and IS animals. OM, omental; RET, retroperitoneal; NUC, nuchal ligament; MES, mesocolonic; TAIL, tail head.
Figure 2. Gene expression of IL-1β, IL-6, TNFα, MCP-1, and PAI-1 by adipose tissue depot. Data from IR and IS groups have been pooled for each depot. Expression of IL-1β and IL-6 is significantly higher in the nuchal ligament adipose tissue depot (*); no other statistically significant differences were observed. OMEN, omental; RETRO, retroperitoneal; NUCHAL, nuchal ligament; MESEN, mesocolonic; TAIL, tail head.
Discussion

The study described here compares pro-inflammatory gene expression profiles of multiple visceral and subcutaneous adipose tissue depots of light-breed horses. The results suggest that the visceral adipose tissue depots of the adult horse do not have greater expression of genes encoding inflammatory cytokines when compared with subcutaneous adipose tissue depots, a finding that differs substantially from observations made in other species (Ibrahim 2009; Yang and others 2008; You and others 2005). Omental adipose tissue, which has been reported to be the primary depot responsible for adipose tissue inflammatory signaling in obese humans and laboratory animals (Cao and others 2008b; Maury and others 2007), and where fat accumulation reportedly correlates closely with cardiovascular disease risk in obese humans (Ibrahim 2009), does not appear to display a greater inflammatory gene expression profile than other adipose tissue depots in the adult horse.

Equine metabolic syndrome (EMS) has emerged in recent years as a substantial risk factor for development of endocrinopathic laminitis in horses and ponies. (Geor and Frank 2009; Johnson 2002) Whereas systemic insulin resistance, increased adiposity (particularly regional adiposity), hyperinsulinemia, and dyslipidemia characterize the syndrome as it is currently accepted (Geor and Frank 2009), only insulin resistance has thus far been identified as an independent risk factor for laminitis (Carter and others 2009a; Treiber and others 2006) and directly involved in its pathogenesis. (Asplin and others 2007; de Laat and others 2010) The horses used in this study displayed no
physical evidence of prior bouts of laminitis; neither did they have prominent nuchal ligament adipose tissue deposits. These findings suggest that these subjects should not be categorized as having EMS per se. However, because insulin resistance may be the component of EMS most directly associated with laminitis risk, its relationship to pro-inflammatory cytokine and chemokine gene expression within the adipose tissue was investigated.

In humans and experimental rodent models of obesity, adipose tissue (particularly visceral depots) expresses higher concentrations of TNFα, IL-1β, IL-6, and other pro-inflammatory cytokines and chemokines in peripherally insulin-resistant individuals than in those that are insulin-sensitive (Hotamisligil, Shargill, Spiegelman 1993; Hotamisligil and others 1995; Kern and others 2001; You and others 2005). Interestingly, the data from the present study do not identify any differences in adipose tissue inflammatory gene expression between depots from insulin-resistant and those from insulin-sensitive horses of similar body condition scores (all animals being overweight). However, differences in IL-1β and IL-6 gene expression among adipose tissue depots were statistically significant, suggesting that the nuchal crest is a more active depot in this regard. However, the lack of an observed effect of peripheral insulin sensitivity on pro-inflammatory gene expression within any specific depot suggests that the differences noted among depots may be associated with other factors related to obesity (see below).

An important confounder in the interpretation of these results is the fact that all of the horses sampled were either overweight or obese according to a widely used body condition scoring system (Henneke and others 1983). In several studies of horses,
investigators have demonstrated positive correlations between body condition score and peripheral insulin resistance (Carter and others 2009c; Frank and others 2006; Hoffman and others 2003; Vick and others 2007), plasma basal insulin concentration, plasma leptin concentration (Kearns and others 2006), pro-inflammatory cytokine gene expression and protein concentration in peripheral blood (Vick and others 2007; Vick and others 2008), and risk of laminitis (Carter and others 2009a; Treiber and others 2006). Negative correlations have been suggested between body condition score and plasma adiponectin concentration in horses (Kearns and others 2006), a finding in keeping with this adipokine’s reported anti-inflammatory and cardioprotective value in the setting of human metabolic syndrome. In humans, it is well-recognized that obese individuals often are insulin-resistant. In fact, body mass index and insulin resistance each have been interpreted as proxies for the other in certain assessments of cardiometabolic risk (Kondaki and others 2011). However, the existence of peripherally insulin-sensitive obese individuals is difficult to explain in this setting. McLaughlin and colleagues recently reported that although, predictably, obesity was more prevalent in a cohort of insulin-resistant individuals (34%) than lean body composition (16%), obesity and insulin resistance did not make equivalent contributions to combined cardiovascular disease risk in their risk assessment model. Their data reinforces the concept that although body mass index and insulin resistance are related, they are not synonymous and contribute independently to cardiovascular risk in humans (McLaughlin and others 2004). Obesity and insulin resistance clearly are correlated in humans and horses, but their respective contributions to subsequent pathology cannot be assumed to be equal. Furthermore, the
recent description of a ‘metabolically healthy but obese’ phenotype in several human study populations, in which obesity is notably not accompanied by insulin resistance, hyperglycemia, dyslipidemia, or an increased risk of cardiovascular disease, suggests uncoupling of obesity and metabolic risk in a substantial proportion of individuals. For example, a recent large survey of humans identified 31.7% of obese adults as ‘metabolically healthy’ and 23.5% of lean adults as ‘metabolically abnormal’. (Wildman and others 2008) Additionally, very recent work in mice identifies a candidate gene, brd2, as a molecular link between obesity and insulin resistance. Brd2-knockout mice develop severe obesity and hyperinsulinemia, but they display enhanced glucose tolerance, decreased adipose tissue macrophage infiltration, and increased plasma concentrations of adiponectin (Wang and others 2009). Furthermore, these mice displayed evidence of enhanced expression of pro-inflammatory cytokines that appeared unrelated to their systemic insulin sensitivity or the degree of adipose tissue macrophage infiltration. Thus, examples from multiple species have weakened the assertion that obesity and insulin resistance are inexorably related. This information may in part explain why a relationship between insulin resistance and gene expression was not identified in this study and why evidence of increased nuchal ligament inflammatory mediator expression was noted in all mares. Therefore, adiposity may factor more importantly in EMS and endocrinopathic laminitis risk than solely through its association with insulin resistance. To further test this possibility, lean cohorts of animals should be included in similar studies in the future to assess whether inflammatory signaling occurs with obesity in the horse regardless of insulin sensitivity.
University-owned mares were an attractive source population for the study reported here due to their ready availability and standardized, uniform management (feeding, housing, and preventive medicine). However, sex influences on both peripheral insulin sensitivity and biological behavior of the adipose tissue have been noted in horses (Vick and others 2006) and humans (Geer and Shen 2009). Additionally, data exists to support the contention that mares have an increased risk of (all-cause) laminitis compared with stallions or geldings (Alford and others 2001), implicating an influence of sex on this most concerning complication of equine obesity. We recognize this influence as a confounder to the interpretation of our results and acknowledge that the data reported here may not be representative of what might be noted in a population of horses including both sexes (intact and altered).

An important finding of this study, that nuchal crest fat appears to be the most reactive fat depot (with respect to inflammatory signaling) in the horse, complements clinical findings of previous investigators who have identified the accumulation of nuchal crest adipose tissue as a risk factor for laminitis associated with equine obesity (global, regional, or both). Frank and colleagues (Frank and others 2006) reported that obese horses with insulin resistance had greater mean neck circumference scores than non-obese mares, relating this morphologic change with the insulin sensitivity status and body condition of the horse. Accumulation of nuchal ligament adipose tissue has further been shown to have predictive value in assessing risk of pasture-associated laminitis in multiple cohorts of ponies, with ponies that have larger neck crests being at greater risk for developing the condition (Bailey and others 2008; Carter and others 2009a; Treiber
and others 2006). Thus, our finding of unique biological behavior of nuchal crest fat when compared to other depots supports an accumulating body of evidence that the morphology of the nuchal crest may change in the setting of EMS and may be correlated with clinical signs of the disease.

In conclusion, the current study does not support a direct relationship between the degree of insulin resistance and inflammatory mediator gene expression by adipose tissue in chronically obese horses, suggesting that other factors besides insulin resistance may mark risk for laminar injury in the obese horse. Additionally, the results of the current study indicate that the nuchal ligament adipose depot, rather than visceral adipose depots, may be more important pathophysiologically in the horse. This finding not only fits well with the clinical case of a horse or pony at risk of laminitis with enlarged nuchal adipose deposits (i.e. a “cresty neck”), but also indicates that it may be possible to focus future adipobiology studies in horses on nuchal ligament tissue biopsies (vs. obtaining abdominal or tail head samples). Additional study will be required to determine the role of the adipose tissue in EMS, including the link between changes in the adipose tissue and laminitis and its potential as a therapeutic target in animals at risk of EMS-associated laminitis.
Chapter 3: Characterization of adipose tissue macrophage infiltration in insulin-resistant and insulin-sensitive light breed horses

Abstract

Insulin resistance has been associated with predisposition to laminitis in horses and ponies and frequently accompanies nutritional obesity in this species. In humans and rodents, macrophage infiltration of adipose tissue (AT) correlates with adiposity and degree of tissue and systemic insulin resistance. These cells adopt an inflammatory phenotype, elaborate pro-inflammatory cytokines, and perpetuate adipose tissue IR in obesity. Recent reports have established depot-specific pro-inflammatory cytokine expression profiles in equine adipose tissue, but the relationship of this expression to macrophage infiltration is unknown. This study quantified the macrophage content of several AT depots of insulin-resistant (IR) and insulin-sensitive (IS) horses. Eleven mares (8 Quarter horses, 2 Thoroughbreds, and 1 Standardbred), categorized as IR (insulin sensitivity [SI] = 0.58 ± 0.31 L•min⁻¹•mU⁻¹; n = 5) or IS (SI = 2.59 ± 1.21 L•min⁻¹•mU⁻¹; n = 6) based on results of an insulin-modified frequently-sampled intravenous glucose tolerance test were studied. Omental, retroperitoneal, and mesocolonic AT was collected via ventral midline celiotomy; nuchal ligament and tail
head AT biopsies were collected via skin incision. Tissue samples were snap-frozen and fixed in formalin. For each depot, total RNA was extracted and cDNA analyzed via real-time quantitative PCR to quantify expression of monocyte chemoattractant protein-2 (MCP-2), and tissue samples were immunohistochemically stained for CD163 (macrophage surface antigen). The number of CD163 (+) cells in n = 10 40x fields per slide was recorded for each depot. Data were analyzed using one-way ANOVA for repeated measures or non-parametric equivalent (p = 0.05). The expression of MCP-2 was significantly higher in omental AT compared with other depots (p = 0.0003), and omental AT of IR horses showed greater expression than that of IS horses (p < 0.0001). The total number of CD163 (+) cells was not significantly different between depots (p = 0.06), and no effect of insulin sensitivity was observed. CD163 (+) cells were further differentiated based on their location within the tissue (interstitial vs. perivascular). There was no difference in the number of interstitial cells between depots (p = 0.48); however, omental adipose tissue contained a significantly greater number of perivascular cells than other depots (p = 0.04). These data suggest a role for MCP-2 as a macrophage chemoattractant in equine AT; omental AT may have a more dynamic macrophage population than other depots. Importantly, CD163 (+) macrophages may not be the most numerous or important population in equine AT, as increased depot-specific expression of pro-inflammatory cytokines (nuchal ligament) is not accompanied by an increased number of CD163 (+) cells.

**Introduction**
Insulin resistance has been associated with predisposition to laminitis in horses and ponies and frequently accompanies nutritional obesity in this species (Carter and others 2009c; Geor and Frank 2009). In humans and rodents, macrophage infiltration of adipose tissue (AT) correlates with adiposity and degree of tissue and systemic insulin resistance (Good and others 2006; Jiao and others 2009). In the obese individual, adipose tissue macrophages classically adopt an inflammatory phenotype, elaborate pro-inflammatory cytokines, and perpetuate adipose tissue IR in obesity (Figure 3)(Apovian and others 2008). Recent reports have established depot-specific pro-inflammatory cytokine expression profiles in equine adipose tissue (Burns and others 2010), with nuchal ligament adipose tissue exhibiting elevated expression of IL-1 and IL-6 compared to other depots. However, the relationship of this expression to macrophage infiltration is unknown. With obesity, adipocytes adopt a gene expression profile that includes the macrophage chemoattractant molecules macrophage chemoattractant Protein (MCP)-1 and MCP-2 (Chacon and others 2007; Di Gregorio and others 2005; Jiao and others 2009). While one report suggests that MCP-1 gene expression is not different among AT depots in light breed horses (Burns and others 2010), the role of MCP-2 remains poorly characterized. The aims of this study, therefore, were to quantify the macrophage content of several equine adipose tissue depots, to evaluate MCP-2 gene expression in the same depots, and to investigate the effect of systemic insulin resistance on these parameters in light breed horses.
Figure 3. With increased energy intake and obesity, excess calories are stored in adipose tissue as triglycerides (1). Adipocytes enlarge (2) and increase their expression of many genes, including macrophage chemoattractant molecules. Subsequent macrophage infiltration of adipose tissue promotes local inflammation and insulin resistance (3).
Materials and Methods

Experimental animals

Light breed mares owned by The Ohio State University College of Veterinary Medicine and housed at the college teaching and research farm were utilized for this project. All mares were group-housed in a dry lot and fed timothy hay ad libitum (grown and baled on-site); forage nutrient analysis was not performed, but all mares were fed identically. No clinical evidence of laminitis was observed in any of the animals used for this study. Experimental procedures were performed according to protocols approved by the OSU Institutional Animal Care and Use Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Insulin-modified frequently-sampled intravenous glucose tolerance testing (FSIGTT)

FSIGTT was performed on 20 mares, 12 of which were initially selected for adipose tissue biopsy collection based on FSIGTT results (a power analysis performed before initiation of the study suggested that the number of animals used in this study was appropriate for the measured variables). All tests were performed in April and June of 2008. Testing was performed as previously reported by Pratt et al. (Pratt, Geor, McCutcheon 2005). Briefly, intravenous catheters were placed in the right and left external jugular veins of each horse after aseptic preparation and subcutaneous infiltration of the sites with local anesthetic. One catheter was designated for medication administration, and 1 was designated for blood collection. The horses were confined in stalls overnight after catheter placement to minimize the effect of stress associated with
catheterization on test results. Blood initially was collected for baseline measurement of plasma insulin and glucose concentrations, followed by administration of a dose of 50% dextrose\textsuperscript{27} (300 mg/kg IV, approximately 300 ml, administered over 2-3 minutes). Completion of this infusion marked time (t) = 0. Blood samples (approximately 6-8 ml per time point) were collected in EDTA\textsuperscript{28} for measurement of insulin and glucose concentrations at 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, and 19 minutes post-dextrose infusion. A dose of regular insulin\textsuperscript{29} (0.1 IU/kg IV) was administered at t = 20 minutes, and additional blood samples were collected at 22, 24, 26, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150, and 180 minutes post-dextrose infusion. After completion of sampling, the catheters were removed and the animals returned to the herd. Insulin concentrations were determined using a commercially-available radioimmunoassay\textsuperscript{30} previously validated for horses, and glucose concentrations were analyzed with a colorimetric assay\textsuperscript{31}. Insulin and glucose data were analyzed by minimal model parameters (Treiber and others 2005), and the calculated insulin sensitivity (SI; L•min\textsuperscript{-1}•mU\textsuperscript{-1}) was used to categorize each horse as insulin-resistant (IR; SI < 1.0 L•min\textsuperscript{-1}•mU\textsuperscript{-1}) or insulin-sensitive (IS; SI > 1.5 L•min\textsuperscript{-1}•mU\textsuperscript{-1}). Based on the SI results, 12 of the 20 tested mares (6 IR and 6 IS) were selected to undergo adipose tissue biopsy collection.

\textit{Adipose tissue biopsy collection}

All biopsy collections were performed in July 2008. Horses were transported to the OSU Veterinary Teaching Hospital, weighed upon arrival, and housed individually in box stalls with access to timothy hay, water, and a trace mineral block \textit{ad libitum}. A
physical examination was performed on each horse upon arrival, and morphometric parameters were recorded (neck circumference (Frank and others 2006), girth circumference, ultrasonographic retroperitoneal fat thickness at the umbilicus). All animals were considered healthy based on results of physical examination, CBC, and serum biochemical analysis performed on the day of arrival. Evidence of chronic pyometra, however, was detected in 1 IR mare during hospitalization, and data from this mare therefore were excluded from the final analysis. An acclimation period of 48 hours was observed before biopsy collection. On the day of biopsy collection, a 14-ga intravenous catheter was aseptically inserted in the left external jugular vein for the purposes of sample collection and induction and maintenance of general anesthesia. A pulmonary artery catheter was aseptically inserted via the right external jugular vein for use in collecting blood gas data for a separate study. A tetanus toxoid booster was administered in the left cervical musculature before biopsy collection, and sulfamethoxazole-trimethoprim (15 mg/kg PO q12 h) and phenylbutazone (1 g PO q12 h) were administered to each animal (both continued for 5 days after biopsy collection).

After sedation with xylazine (0.5 mg/kg IV), general anesthesia was induced with ketamine (2.2 mg/kg IV) and diazepam (0.1 mg/kg IV) and maintained with guiafenesin-based total intravenous anesthesia (guiafenesin 5%; xylazine 0.05%; ketamine 0.2%; 1-2 ml/kg/hour). All mares were orotracheally intubated, and positive-pressure ventilation was initiated and maintained with 100% oxygen for the duration of the procedure (approximately 30 minutes). Incisional biopsies of nuchal crest and tail
head adipose tissue were collected (~3 g tissue collected from each site) with the animal in lateral recumbency after aseptic preparation of the biopsy sites with chlorhexidine surgical scrub and isopropyl alcohol; incisions were closed in a single layer with 0 polydioxanone monofilament suture material in a simple continuous pattern. Each horse then was placed on a padded surgical table in dorsal recumbency and moved into a surgical suite; the abdomen was aseptically prepared and draped for ventral midline celiotomy. A small ventral midline abdominal incision was created (~8 cm in length), and adipose tissue biopsies (~3 g each) were collected from the margins of the incision (representing retroperitoneal adipose tissue), the mesocolon of the descending colon (representing mesenteric adipose tissue), and the omentum. The abdominal incision was closed in 2 layers: the linea alba was apposed with 2 polyglactin 910 in a simple continuous pattern, and the skin was apposed with 0 polydioxanone in a simple continuous pattern. Horses then were allowed to recover from general anesthesia and returned to box stalls. All mares remained hospitalized for 10 days after biopsy collection, during which time they were continuously monitored for incisional and other complications. After this period, all mares were returned to the teaching herd. Sutures were removed manually from the nuchal ligament and tail head incisions 14 days after biopsy collection; sutures were not removed from the abdominal incisions.

A portion of each adipose tissue biopsy was flash-frozen in liquid nitrogen at the time of collection and stored at -80°C for later analysis by real-time quantitative PCR; the remainder was fixed in neutral buffered formalin and paraffin embedded.
RNA isolation, cDNA synthesis, and real-time quantitative polymerase chain reaction (RT-qPCR)

Homogenates were made from each adipose tissue sample (1.0-1.5 g) with a tissue disruptor\textsuperscript{44}, and total RNA was extracted using a modified Trizol method. cDNA was synthesized from total RNA from each sample (after DNase treatment\textsuperscript{45}) via reverse transcription (Retroscript\textsuperscript{46}) and stored at -20º C until used for RT-qPCR analysis.

A thermocycler (Roche 480\textsuperscript{47}) was used to perform RT-qPCR. Amplification was quantified against external standards using fluorescent format for SYBR Green I as previously described.\textsuperscript{(Waguespack, Cochran, Belknap 2004; Waguespack and others 2004)} Primers for monocyte chemoattractant protein-2 (MCP-2) and the housekeeping genes β-actin and β\textsubscript{2}-microglobulin were designed against equine-specific gene sequences (intron-spanning exon sequences) using computer programs as previously described (Waguespack, Cochran, Belknap 2004) and were used for amplification of the respective genes of interest. Standard curves for quantification of transcript in adipose tissue samples were generated using serial dilutions of a linearized vector\textsuperscript{48} containing an insert of each amplified cDNA fragment whose identity was confirmed via DNA sequencing.

Immunohistochemistry

Formalin-fixed adipose tissue samples were embedded in paraffin and sectioned at 5 µm for immunohistochemistry. Antibodies for macrophage/myeloid cell markers F4/80, Mac 387/calprotectin, and CD163 were tested on equine adipose tissue sections; only CD163 immunohistochemistry resulted in acceptable cellular staining. Therefore,
CD163 (macrophage surface antigen; thought to be a broad macrophage marker in the horse, but may preferentially stain cells adopting an M2, or anti-inflammatory, phenotype) immunohistochemistry results are presented. Staining of tissue samples was performed as previously reported (Burns and others 2011); briefly, sections were deparaffinized and incubated overnight in a mouse monoclonal antibody against CD163 (Cosmo; surface antigen marker of macrophages, diluted 1:100), that has previously been validated for immunohistochemistry on equine paraffin-embedded tissue (Faleiros and others 2011a; Faleiros and others 2011b; Komohara and others 2006). Detection of immunoreactivity was performed using an immunoperoxidase system (Vector Laboratories) and DAB substrate (Vector Laboratories). After IHC staining, the number of CD163 (+) cells in n = 10 (40X) light microscopy fields recorded for each depot by a single investigator who was blinded to the source of the tissue section.

Data analysis

RT-qPCR data from housekeeping genes (β-actin and β2-microglobulin) were entered into a computer program to test each gene’s quality as a housekeeping gene for equine adipose tissue. Because both genes were determined to be satisfactory by the program, a geometric mean was obtained from the 2 genes’ data in order to generate a normalization factor for gene expression from each adipose tissue sample. Gene expression data for MCP-2 then was normalized using this factor for each individual tissue sample.
Data analysis was performed using a statistical software program\textsuperscript{53}. Morphometric, leukocyte count, and gene expression data were assessed for normality by the Shapiro-Wilk and D’Agostino and Pearson omnibus normality tests. Morphometric data were analyzed with a Student’s t test; gene expression and leukocyte count data (or log transforms of non-normal data) were subjected to 2-way analysis of variance with repetition on 1 factor (depot) followed by Bonferroni’s post-test. Statistical significance was accepted at $p < 0.05$.

Results

The total MCP-2 RNA concentration was significantly higher in omental adipose tissue compared with other adipose tissue depots ($p = 0.0003$). Omental adipose tissue from IR horses displayed higher MCP-2 expression than that of IS horses ($p < 0.0001$; Figure 4). The total number of CD163(+) cells was not significantly different between depots ($p = 0.06$), and no effect of insulin sensitivity status was observed (Figure 5). CD163(+) cells were further differentiated based on their location within the adipose tissue (interstitial vs. perivascular). There was no difference in the number of interstitial cells between depots ($p = 0.48$); however, omental adipose tissue contained a significantly greater number of perivascular cells than other depots ($p = 0.04$).
Figure 4. Results of MCP-2 RT-qPCR on 5 adipose tissue depots. In the panel on the left, the data for each AT depot are stratified by insulin sensitivity status, whereas the data on the right are pooled. MCP-2 mRNA concentration was higher in omental adipose tissue than other depots, and omental adipose tissue from IR mares displayed higher expression than that from IS mares. MCP-2, monocyte chemoattractant protein-2, IR, insulin-resistant; IS, insulin-sensitive; Retro, retroperitoneal; Mesen, mesenteric; Omen, omental; Nuch, nuchal; Tail, tailhead. * signifies p<0.05.
Figure 5. Results of immunohistochemical staining of adipose tissue sections from 5 adipose tissue depots for CD163, a macrophage surface marker. The scatter plot on the left displays the data pooled for each AT depot, while the histogram on the right shows the data stratified by insulin sensitivity status. No effect of insulin sensitivity was observed on total number of CD163(+) cells in any depot; while omental adipose tissue tended to contain more CD163(+) cells than other depots, this result did not achieve statistical significance (p = 0.06). IR, insulin-resistant; IS, insulin-sensitive; Retro, retroperitoneal.


Discussion

Adipose tissue macrophages have been shown to play a prominent role in the initiation and perpetuation of the local (tissue-level) and systemic insulin resistance associated with obesity in many species (Apovian and others 2008; Huber and others 2008; Lumeng and others 2007; Lumeng, Bodzin, Saltiel 2007; Suganami, Nishida, Ogawa 2005). With the onset of nutritional obesity, white adipose tissue has been shown to be infiltrated by macrophages (Apovian and others 2008); the degree to which this occurs correlates well with the degree of tissue and systemic insulin resistance observed (Jiao and others 2009). While adipose tissue macrophages remain poorly characterized in equids (lean or obese), the data presented here suggest a role for MCP-2 as a macrophage chemoattractant in equine adipose tissue. Additionally, the increased expression of MCP-2 and elevated macrophage content noted in omental adipose tissue suggest that this depot may have a more dynamic macrophage population than the other depots evaluated.

Macrophages are not homogeneous in their tissue distribution or metabolic phenotype. In adipose tissue, macrophages have been shown to display characteristics of both a classically-activated, pro-inflammatory (M1) phenotype and an alternatively, anti-inflammatory phenotype (M2) (Maury and Brichard 2010; O'Rourke and others 2009). The M1 phenotype is associated with an inflammatory gene expression profile and insulin resistance within adipose tissue, and this is the phenotype most commonly identified in adipose tissue macrophages from obese individuals (Lumeng, Bodzin, Saltiel
2007). In contrast, adipose tissue macrophages from lean individuals most commonly are of the M2 phenotype, which is associated with improved adipose tissue insulin sensitivity and decreased tissue inflammation (Bourlier and others 2008). Importantly, CD163 (+) macrophages may not be the most numerous or important population in equine adipose tissue, as the nuchal ligament, the adipose depot whose expansion was recently reported to be associated with laminitis risk in EMS-prone equids (Carter and others 2009a; Carter and others 2009b; Frank and others 2006) and to have increased pro-inflammatory cytokine gene expression (Burns and others 2010), did not have an increased number of CD163 (+) cells when compared with other depots. Alternatively, equine adipose tissue macrophages may comprise several phenotypic populations, with classically (M1)- and alternatively-activated (M2) cells present in variable numbers. CD163 is thought to be expressed predominantly on M2 macrophages in other species (unknown in horses) (Komohara and others 2006; Zeyda and others 2007).

Further investigation into the biology of adipose tissue macrophages in horses and ponies is warranted, as their biological behavior and effects on adipose tissue metabolism have been shown to be modifiable with drug therapy in other species (O'Neill and Hardie 2013; Perman and others 2009; Yi and others 2011). Adipose tissue macrophages may be an attractive therapeutic target in the treatment of equids with EMS.

In conclusion, the data reported here suggest a role for MCP-2 as a macrophage chemoattractant in equine AT; omental AT may have a more dynamic macrophage population than other depots in this species, possibly related to analytes contained within portal venous blood. Importantly, CD163 (+) macrophages may not be the most
numerous or important population in equine AT, as increased depot-specific expression of pro-inflammatory cytokines (nuchal ligament) is not accompanied by an increased number of CD163 (+) cells.
Chapter 4: Distribution of insulin receptor in the digital laminae of ponies fed a high-carbohydrate diet: an immunohistochemical study

Abstract

The interaction of insulin and the microvascular endothelial insulin receptor (IRc) plays an important role in the normal and insulin resistant (IR) individual. While endothelial IRc signaling is normally vasodilatory, this effect is well-documented to reverse in the IR individual, resulting in vasoconstriction. Although vascular dysfunction has been reported in sepsis-associated equine laminitis, the role of the laminar microvasculature in endocrinopathic laminitis remains poorly characterized. The purpose of this study was to characterize the pattern of IRc expression in digital laminae in ponies subjected to a dietary carbohydrate challenge that mimicked abrupt exposure to pasture rich in nonstructural carbohydrates (NSC). Mixed-breed ponies (body weight 270.9 +/- 74.4 kg) received a diet of hay chop (NSC ~6% on a DM basis) for 4 weeks prior to initiation of the experimental feeding protocol. Following conditioning, ponies either remained on the control diet (n=11) or received the same diet supplemented with sweet feed and
oligofructose (total diet ~42% NSC; n=11) for a period of 7 days. Serum insulin concentrations were measured prior to and after completion of the feeding protocol. At the end of the feeding protocol, sections of numerous tissues, including dorsal digital laminae, were collected immediately following euthanasia. The samples were formalin-fixed for 48 hours, transferred to 70% ethanol, and paraffin-embedded. Laminar sections were stained immunohistochemically for IRc using a commercially-available antibody (Abcam); the number of IRc (+) cells was quantified in 40x light microscopy fields (n = 10) for each section. The total number of IRc (+) cells was greater in the laminae of challenged ponies than control ponies (p = 0.0096), and there was a significant correlation between the change in serum basal insulin concentration and number of laminar IRc (+) endothelial cells (r = 0.74; p <0.05). While the number of IRc (+) endothelial cells was significantly greater in the dermal laminae of challenged ponies (p = 0.0095), there was no difference in the number of interstitial IRc (+) cells (p = 0.82). No epithelial IRc (+) cells were observed in any laminar section, and IRc (+) cells were conspicuously absent from the deep dermal tissue (including vessels). Up-regulation of IRc expression in the laminar vasculature occurs acutely in response to dietary carbohydrate challenge and accompanies hyperinsulinemia in ponies. The dramatic increase in endothelial IRc expression in the laminar microvasculature in nutritionally challenged ponies, with no apparent epithelial IRc present, suggests that hyperinsulinemia associated with exposure to increased dietary NSC may induce laminar injury by causing a similar vasoconstriction in IR equids as described in the microvasculature of IR humans.
Introduction

Equine metabolic syndrome (EMS), a constellation of clinical findings currently accepted to include obesity, regional adiposity, systemic insulin resistance, and laminitis (historical or current) (Frank and others 2010), is an increasingly common cause of laminitis in equids in developed nations. Pasture-associated laminitis (for which EMS-affected equids may be at increased risk (Geor 2009)) is reported to be the most common cause of laminitis seen in general equine veterinary practice, accounting for approximately 46% of new clinical cases attended by equine veterinarians in the United States (USDA-NAHMS 2000). This fact, along with the perennial identification of laminitis as a research priority for the equine veterinary community (American Association of Equine Practitioners 2009), has driven a vigorous research effort toward increasing understanding of laminitis associated with metabolic diseases.

Observational studies have associated abnormalities of insulin and glucose metabolism (particularly systemic insulin resistance and resultant hyperinsulinemia) with increased risk of laminitis in ponies upon exposure to spring pasture (Treiber and others 2006), a common precipitating event in the development of the disease as reported by equine veterinary practitioners (USDA-NAHMS 2000). Additional studies in light breed horses have also reported abnormalities of insulin and glucose metabolism in animals with obesity, regional adiposity, and a predisposition to laminitis (Frank and others 2006). While useful in many respects for characterizing important risk factors for endocrinopathic laminitis and further galvanizing the EMS phenotype, these studies are
not mechanistic investigations and do not provide data supporting a pathophysiologic link(s) between the reported abnormalities in endocrine/metabolic function and laminitis.

Recently, a direct role for insulin in the pathogenesis of endocrinopathic laminitis has been suggested by the induction of laminitis in both normal ponies (Asplin and others 2007) and normal Standardbred horses (de Laat and others 2010) following 48–72 hours of experimentally-induced hyperinsulinemia. While its effects on carbohydrate metabolism are among its best-characterized effects, insulin is a pleiotropic hormone. Upon binding to its receptor (which is expressed on many cell types), insulin induces not only its well-characterized effects on transmembrane glucose uptake (mediated through activation of the PI3K signaling pathway), but also mitogenic and extracellular matrix metabolism effects (mediated through activation of the MAPK signaling pathways) (Frojdo, Vidal, Pirola 2009; Jensen and De Meyts 2009). Additionally, while the majority of insulin’s effects on intermediary metabolism are attributed to activation of intracellular signaling downstream of IRc, insulin is also known to bind and activate signal transduction through the IGF-1 receptor (IGF-1R), particularly at supraphysiologic concentrations of insulin (Vigneri, Squatrito, Sciacca 2010). Activation of the IGF-1R by insulin when insulin is present at supraphysiologic concentrations may result in activation of the anti-apoptotic and mitogenic pathways commonly associated with IGF-1R signaling; abnormal laminar keratinocyte proliferation and maturation may result and contribute to endocrinopathic laminitis. While the ability of hyperinsulinemia to induce equine laminitis has been increasingly well established, the pathway(s) through which insulin exacts its deleterious effects on the equine digital laminae are largely unknown.
Therefore, the objectives of the study reported here were 1) to identify and characterize the distribution of cells expressing IRc and IGF-1R within the digital laminae of ponies and 2) to evaluate the effects of a dietary non-structural carbohydrate challenge (one meant to mimic that which might be encountered during spring pasture exposure) on both the protein concentrations and cellular distribution of IRc and IGF-1R in the digital laminae.

**Materials and Methods**

**Animal protocol**

Twenty-two mixed breed ponies (body weight 270.9 +/- 74.4 kg; age [lean] = 9.2 +/- 3.5 years, age [obese] = 11 +/- 3.8 years) were used for this study. All animals received humane treatment in accordance with an animal care and use protocol approved by the Michigan State University Institutional Animal Care and Use Committee. Feedstuffs used in the protocol were analyzed for non-structural carbohydrate content (NSC, defined as the sum of measured starch and water-soluble carbohydrates) by a commercial laboratory^5^ (Equi-Analytical). All ponies obtained for the study were examined by a licensed veterinarian and deemed healthy based on the results of physical examination, complete blood count, and serum biochemical examination. Ponies were divided into four experimental groups based on body condition scoring results: lean, low NSC (n = 5); obese, low NSC (n = 5); lean, high NSC (n = 6); obese, high NSC (n = 6). All body condition scores were performed by two individuals; lean animals were those
assigned a body condition score of ≤ 4/9, and obese animals were those assigned a body condition score of ≥ 7/9 (Henneke and others 1983).

All ponies were housed in dirt lots and conditioned to a diet of hay chop (7% starch and ethanol soluble carbohydrate on a dry matter basis) for 4 weeks prior to initiation of the experimental feeding protocol. Ponies were fed 2.5% of their body weight in hay chop per day, divided into two feedings (7 a.m. and 6 p.m. EST). Following the conditioning period, ponies either remained on the control diet (n=10; lean and obese control groups) or received the same diet supplemented with sweet feed (1.5% body weight per day, fed three times daily at 7 a.m., 12 p.m., and 6 p.m. EST) and oligofructose\textsuperscript{55} (Beneo-ORAFTI; 2 g/kg added to hay chop ration; lean and obese challenge groups, n = 12) for a period of 7 days. The mean NSC consumption of ponies in the control groups was approximately 1.8 g/kg/day, while that of ponies in the challenged groups was approximately 8 g/kg/day. All ponies were monitored three times daily during the experimental period.

All ponies underwent insulin-modified frequently-sampled intravenous glucose tolerance testing (FSIGTT) with Minimal Model analysis (Toth and others 2009) during the first two weeks of the acclimation period. The FSIGTT’s were performed between 7 a.m. and 9 a.m. EST following a 6-8 hour period of feed withholding. Additionally, blood was collected into red top tubes\textsuperscript{56} (Becton Dickinson) for measurement of basal serum insulin concentrations on Day 0 and Day 7 of the feeding protocol (between 7 a.m. and 9 a.m. prior to feeding the morning ration); serum insulin concentrations were measured with a radioimmunoassay\textsuperscript{57} validated for equine samples (Coat-A-Count\textsuperscript{TM},
Siemens; (Freestone and others 1991)). Following the 7 day experimental period, ponies were euthanized via intravenous overdose of pentobarbital sodium and phenytoin sodium\textsuperscript{58} (Fatal-Plus\textsuperscript{TM}, Vortech; 20 mg/kg IV). The right front foot of each animal was removed by disarticulation of the metacarpophalangeal joint immediately following euthanasia, and 1.5 cm sagittal sections of the dorsal digit were cut with a band saw. After dissection of the digital laminae from the hoof wall and third phalanx, sections of laminae were snap-frozen in liquid nitrogen or fixed in 10% neutral buffered formalin; all laminar samples were processed within 15 minutes of euthanasia. Formalin-fixed samples were transferred to 70% ethanol after 48 hours, where they were stored until embedding.

\textit{Immunohistochemistry}

Formalin-fixed laminar samples were embedded in paraffin and sectioned at 5 µm for immunohistochemistry. Staining of laminar samples was performed as previously reported (Burns and others 2011); briefly, sections were deparaffinized and incubated in either a mouse monoclonal anti-IRc primary antibody\textsuperscript{59, 60} (Abcam, ab54268, used undiluted, incubated overnight at 4\textdegree C; ab69508, diluted 1:1000, incubated overnight at 4\textdegree C) or a rabbit polyclonal anti-IGF-1R primary antibody\textsuperscript{61} (Santa Cruz Biotechnology, Inc.; 1:100, incubated overnight at 4\textdegree C) that has previously been validated for immunohistochemistry on paraffin-embedded tissue (Yoon, Berger, Roser 2011). Two separate primary antibodies against IRc were tested for this IHC application to ensure specificity of the staining pattern, as no blocking peptide was commercially available to test fidelity; both antibodies yielded identical staining patterns when used for IHC on
digital laminar tissue. Detection of immunoreactivity was performed using an immunoperoxidase system (Vector Laboratories) and DAB substrate (Vector Laboratories). After IHC staining, the number of IRc (+) cells in n = 10 (40X) light microscopy fields in both 1) laminar tissue (restricted to dermal/epidermal laminae) and 2) deep dermis (dermal tissue situated between laminae and cortex of distal phalanx) was counted and recorded by a single investigator who was blinded to the source of the tissue section. The distribution of IGF-IR (+) cells was assessed via light microscopy and qualitatively described. To determine the identity of the IRc (+) cells, serial laminar tissue sections (3 µm) were stained for IRc, von Willebrand’s factor (DAKO, rabbit polyclonal, used at 1:1000; marker of endothelial cells) (Muller, Ellenberger, Schoon 2009), CD163 (Cosmo; marker of macrophages) (Faleiros and others 2011a), and calprotectin (Abcam; marker of activated myeloid and epithelial cells) (Faleiros, Nuovo, Belknap 2009), again using an immunoperoxidase system (Vector Laboratories) and DAB substrate.

**Western immunoblotting**

The concentration of IRc in laminar tissue homogenates was assessed via Western immunoblotting performed as described previously (Blikslager and others 2006). Briefly, laminar protein samples (~100 mg laminar tissue per sample) were prepared in 300 µL lysis buffer (Pierce) and quantitated via the Bradford method. Protein samples (20 µg/sample) were denatured by boiling for 5 minutes in β-ME/SDS buffer, separated on an 8% polyacrylamide gel, and transferred to a PVDF membrane. The membrane was
blocked for 1 hour with 5% milk in PBS-Tween 20 (0.1% v/v Tween-20 in PBS; PBST) at room temperature, rocking. The membrane was then incubated with primary antibody (Abcam [ab69508], mouse monoclonal, 1:1000 in 5% milk) overnight at 4º C. The membrane was washed five times with 0.1% PBST as before. Goat anti-mouse IgG-HRP secondary antibody (Pierce) was diluted 1:5000 in 5% milk and incubated with the membrane for 1 hour at room temperature, rocking. The membrane was washed 5 times with 0.1%PBST and developed for 5 min using a chemiluminescent substrate (West Femto, Pierce). The membrane was then stripped and reprobed for β-actin (primary, goat polyclonal, 1:1000, Santa Cruz; secondary, 1:20,000, Santa Cruz). Luminescence was measured using a computer software program (Carestream), and signal strength was determined using net IRc band intensity divided by β-actin band intensity.

**Statistical analyses**

Data analysis was performed using a statistical software program (GraphPad Prism). Data were assessed for normality by the Shapiro-Wilk and D’Agostino and Pearson omnibus normality tests and analyzed with either a Student’s t-test (or non-parametric equivalent) or two-way analysis of variance followed by a Bonferroni post-test, as appropriate. Correlations were assessed using Pearson’s statistic. Statistical significance was accepted at p ≤ 0.05. Data are expressed as mean +/- SD unless otherwise indicated.

**Results**
Systemic insulin response to high-carbohydrate feeding

All ponies completed the feeding protocol; four ponies were observed to become clinically laminitic during the course of the feeding protocol (Obel grade I-II/IV; one lean challenged pony, three obese challenged ponies). One of these animals was administered phenylbutazone (2.2 mg/kg PO BID) for three days prior to euthanasia in accordance with the approved animal care and use protocol associated with the project; the three remaining animals were noted to be mildly laminitic (Obel grade I/IV) on the day of euthanasia only and were not medicated.

Ponies were assigned to the experimental groups based solely on BCS with no stratification related to insulin sensitivity status. However, subsequent Minimal Model analysis indicated that systemic insulin sensitivity was markedly lower in OB when compared to LN ponies (SI, 0.50 +/- 0.26 x 10^{-4} L/min/mU in OB vs 2.57 +/- 2.18 x 10^{-4} L/min/mU in LN, p = 0.0005). Conversely, the acute insulin response to glucose (AIRg) was higher (p = 0.04) in OB (755.4 +/- 340.9 mU/L/min) than in LN (478.4 +/- 433.6 mU/L/min) ponies. There was no statistical difference in the age of the lean ponies compared to that of the obese ponies (p = 0.26).

No weight gain was observed in response to either the low NSC or high NSC feeding protocols. Basal serum insulin concentration was increased across the 7 day experimental feeding period in both LN (Day 0, 11.4 +/- 7.5 mU/L ; Day 7, 258.0 +/- 306.2 mU/L) and OB (Day 0, 14.6 +/- 6.9 mU/L; Day 7, 549.1 +/- 277.3 mU/L) ponies fed the high-NSC diet when compared with low-NSC fed animals (p = 0.007). There was
no difference in basal serum insulin concentration across the feeding protocol between
the lean and obese control groups (p > 0.05; Figure 6).
Figure 6. Effect of dietary NSC on basal serum insulin concentration in lean and obese mixed-breed ponies. Panel A, graphical representation of serum insulin concentrations of lean and obese ponies fed low- and high-NSC diets for 7 days. Panel B, effect of 7 days of high-NSC feeding on serum insulin concentrations of lean and obese ponies; the high-NSC diet caused an increase in basal serum insulin concentrations in both lean and obese ponies (p = 0.0007), but there was no effect of body condition (p = 0.12). LN, lean; OB, obese; NSC, non-structural carbohydrate; BCS, body condition score.
**Immunoblotting**

No difference in laminar IRc protein content was observed between the LN, low NSC ponies and the LN, high NSC ponies (p = 0.96). The laminar content of insulin receptor in OB, high NSC ponies, however, was lower than that of OB, low NSC ponies (p = 0.002; Figure 7).
Figure 7. Results of IRc Western immunoblotting of digital laminar protein homogenates from ponies fed either a low- or high-NSC diet for 7 days. No difference in insulin receptor expression is observed in lean ponies in response to the high-NSC diet (bottom left panel, p = 0.96); however, the laminar expression of insulin receptor is decreased in obese ponies in response to the same diet (bottom right panel, p = 0.002). NSC, non-structural carbohydrate; LN, lean; OB, obese; InsRec, insulin receptor.
**Digital laminar immunohistochemistry**

Immunohistochemical staining for IRc with two separate antibodies (ab69508 and ab54268) resulted in an identical staining pattern in digital laminar tissue; one of these antibodies (ab69508) was also found to recognize the correct size band (~95 kD, assessed with positive control 3T3 cell lysate) on Western blot (see Figure 4). The total number of IRc(+) cells was greater in the laminae of ponies fed a high NSC diet than in those fed the low-NSC diet (p = 0.01; Figures 8 and 9), and there was a positive correlation between the change in serum basal insulin concentration across the 7 day experimental period and number of laminar IRc(+) cells (r = 0.74; p < 0.0001). No IRc(+) keratinocytes were observed in any section, and IRc(+) cells were present in much lower numbers in the deep dermal tissue (including vasculature). The number of IRc(+) cells in the deep dermis did not change with diet in either lean or obese ponies. Analysis of serial section staining identified IRc(+) cells as endothelial cells, as all were also immunohistochemically vWF(+) (Figure 10). The cellular distribution of IGF-1R expression within the digital laminae was more extensive than that of IRc, with signal appearing not only within vascular elements, but also on laminar epithelial cells and cells morphologically consistent with fibroblasts (Figure 11). Distinct staining of the cytoplasmic membrane and nucleus was observed in laminar epithelial cells (Figure 11). While not specifically quantitated (due to the questionable value of quantitation of immunohistochemical stain intensity), laminar IGF-1R expression did not appear influenced by diet, body condition, or insulin sensitivity status.
Figure 8. Results of IRc immunohistochemical staining of digital laminae of lean and obese ponies fed either a low-NSC diet or a high-NSC diet for 7 days. The number of IRc (+) cells is increased in the digital laminae of both lean and obese ponies in response to a short-term dietary carbohydrate challenge. Panels A, B, and C: LN low-NSC, 40x magnifications. Panels D, E, and F: OB high-NSC, 40x magnifications. IRc, insulin receptor; LN, lean; OB, obese; NSC, non-structural carbohydrate; SEL, secondary epidermal laminae; PDL, primary dermal laminae.
Figure 9. Results of quantitation of the number of insulin-receptor positive cells in histologic sections of digital laminae (top panel) and deep laminar dermis (bottom panel) of ponies fed either a control diet or a carbohydrate-challenge diet for 7 days. An increase in IRc(+) cells was observed in the laminae of both lean and obese animals fed a high-NSC diet (p = 0.01); there was no observed effect of body condition (p = 0.6). There was no difference in IRc(+) cells observed in the deep laminar dermis (p = 0.73). IRc(+), insulin receptor positive; LMF, light microscopy fields; NSC, non-structural carbohydrate; LN, lean; OB, obese; BCS, body condition score.
Figure 10. Results of immunohistochemical staining of sequential slides of digital laminar tissue from ponies fed either a low- or high-NSC diet against IRc and von Willebrand’s factor. The top panels are tissue from a lean pony fed the high-NSC diet; the bottom panels are from an obese pony fed the high-NSC diet. Panels on the left are stained for IRc; panels on the right are stained for vWF. All IRc(+) cells are also vWF(+), identifying them as endothelial cells (arrows). IRc, insulin receptor; vWF, von Willebrand’s factor; OB, obese; CHO, high-NSC diet.
Figure 11. Results of immunohistochemical staining of digital laminar tissue from ponies fed a high-NSC diet against insulin-like growth factor-1 receptor (IGF-1R). Note the diffuse distribution of signal, including vascular elements (black arrows), laminar epithelial cells (yellow arrows), and fibroblast-like cells (white arrows). The distribution of IGF-1R was similar both between lean and obese ponies and between those fed a low- or high-NSC diet. In Panel B, note the nuclear and cell surface stain uptake within laminar epithelial cells. Panel A, 20x magnification; Panel B, 40x magnification.
Discussion

Systemic insulin resistance and resultant hyperinsulinemia have been implicated in the pathogenesis of endocrinopathic laminitis in equids (Asplin and others 2007; de Laat and others 2010). Extrapolation from studies of metabolic syndrome in other species would suggest that an insulin resistant state with high circulating insulin concentrations would result in deleterious events downstream of the insulin receptor in the laminar cellular milieu, ranging from disruption of energy metabolism and possible cellular energy failure to aberrant regulation of the extracellular matrix (critical for adhesion of the laminar epidermis to the underlying dermis (Jensen and De Meyts 2009; Towler and Hardie 2007)). These events noted in the human literature have been of great interest in the study of laminitis at the level of the laminar basal epithelial cell (LBEC), as two events reported to possibly contribute to dysadherence of this cell layer from the underlying matrix (and laminar failure) are matrix degradation and energy failure. (French and Pollitt 2004a; Loftus and others 2009; Moore, Eades, Stokes 2004) An additional event documented in studies of human insulin resistance proposed to play a role in EMS-related laminitis is endothelial dysfunction leading to vasoconstriction of microvascular beds (Geor and Frank 2009; Johnson and others 2010). In this study, the only IRc(+) cell type in the digital laminae was the microvascular endothelial cell, whereas IGF-1R was present on multiple cell types (including the LBEC). These results raise the possibility that elevated insulin concentrations may play a pathophysiologic role.
in the laminae by signaling through both IGF-1R on the LBEC and IRc on the laminar microvascular endothelial cell.

Insulin has been shown to bind and activate IGF-1R at supraphysiologic concentrations in cell cultures (Vigneri, Squatrito, Sciacca 2010); based on the results of the study reported here, IGF-1R’s cellular distribution within the laminae appears to be much more extensive than that of the IRc. While IRc appears to be limited to the laminar microvasculature, IGF-1R is present on multiple cell types, including laminar epithelial cells, endothelial cells, and dermal constituents (likely fibroblasts and tissue macrophages). Whereas IGF-1 is reported to be important in the normal maturation of epithelial cells in human skin (Sadagurski and others 2006), insulin signaling through the IGF-1R in the setting of hyperinsulinemia is thought to underlie the keratinocyte dysregulation of acanthosis nigricans and skin tag, two cutaneous manifestations of human metabolic syndrome characterized by epithelial hyperplasia and hyperpigmentation (Geffner and Golde 1988). The nuclear localization of IGF-1R in laminar epithelial cells reported here is consistent with this receptor’s documented role in mitogenesis, as nuclear translocation of surface-activated receptor has been reported in both normal and neoplastic cells (Aleksić and others 2010; Deng and others 2011). We also observed a similar surface localization of IGF-1R in the laminar keratinocyte in vivo as has been reported in cultured equine laminar keratinocytes (Bailey 2009). IGF-1R signaling events in the laminar epidermis, particularly its reported effects on regulation of extracellular matrix (Beattie, McIntosh, van der Walle 2010; Valentinis and others 1999), cytoskeletal dynamics (Saegusa and others 2009), and cellular phenotype (von der
Thusen and others 2011), may play a role in the dysregulation/dysadhesion of the LBEC from the underlying dermis; further investigation of these signaling events in the LBEC are warranted.

Insulin signaling through laminar IRc in hyperinsulinemic equids is likely exacting its most profound direct effects on laminar perfusion, as IRc signal appears restricted primarily to the laminar dermal microvascular endothelium. Endothelial insulin resistance has been associated with increased vasomotor tone in response to repeated insulin exposure, classically thought to result from decreased elaboration of nitric oxide and increased elaboration of endothelin-1. These endothelial events have been suggested to be involved in the hypertension reported in laminitis-prone ponies exposed to pasture (Bailey and others 2008). In horses with IR, changes in vasomotor tone to a tissue with poor collateral circulation (the foot) may promote hypoxia/ischemia, both of which have been suggested to play roles in the pathogenesis of laminitis (Hood and others 1993; Moore, Eades, Stokes 2004; Noschka and others 2009; Owens, Kamerling, Keowen 1995). Recent work utilizing in vitro modeling of induced vascular insulin resistance in equine digital vessel explants suggests that insulin, normally vasodilatory, may have vasoconstrictor effects in the digital vasculature of the IR equid (Venugopal and others 2011). However, the concentrations of insulin used to induce IR in the vascular explants in this study were several orders of magnitude higher than that which is likely to be encountered in the IR equid (~1.4 x 10$^6$ mIU/L)(Carter and others 2009a; Frank and others 2006); consequently, the results may not accurately reflect events occurring in vivo.
Hyperinsulinemia itself reportedly contributes to endothelial IR, as prolonged exposure of endothelial cells to increased concentrations of insulin *in vitro* has been demonstrated to alter their insulin responsiveness, with induction of cellular insulin resistance over a relatively short period of time (24-48 hours). Rapid downregulation of surface expression of IRc has been documented on endothelial cells in response to insulin treatment, which may partially explain the endothelial insulin resistance observed in response to hyperinsulinemia *in vivo* (Banskota, Carpentier, King 1986; Elliot and others 1990). Interestingly, it is shortly after the reported time required for downregulation of IRc that horses and ponies are observed to become laminitic during experimental hyperinsulinemia (Asplin and others 2007; de Laat and others 2010). A similar general decrease of tissue IRc protein concentrations as reported in these studies may be responsible for the decrease in IRc noted on Western hybridization of protein extracted from the digital tissue (laminar tissue with underlying deep dermis) in obese ponies placed on a high NSC diet. The immunohistochemical results of our study, however, do not support IRc downregulation as a general effect in the digital vasculature, as the number of endothelial cells expressing IRc in laminar microvasculature in response to hyperinsulinemia following high-NSC feeding was observed to increase. These seemingly discordant findings between immunoblotting and immunohistochemistry results most likely reflect a general decrease in cellular expression of IRc in the digital tissue, but an increase in the number of laminar microvascular endothelial cells expressing detectable IRc. Alternatively, as different antibodies generated against different epitopes of the IRc were used for IHC and WB (and they were used to detect
native vs. denatured IRc protein, respectively), it is possible that the discrepancy represents these methodological differences. Nonetheless, IRc expression in the digital laminar microvasculature appears to be uniquely regulated compared to both 1) studies of vascular IRc regulation in the face of hyperinsulinemia in other species and 2) the vasculature in the deep dermis adjacent to the laminae in the current study.

Upregulation of IRc expression on the digital microvascular endothelium in response to high-carbohydrate feeding was a somewhat unexpected result of this study. It is commonly held that exposure to insulin results in IRc downregulation on the cell surface of many cell types; however, this effect has been reported to be somewhat cell type- and tissue-specific (Maassen, Krans, Moller 1987)(Mamula and others 1990). Insulin-mediated upregulation of IRc expression has been reported in human lymphocyte cell cultures (Rouiller and Gorden 1987), among others. Further, increased cell surface expression of IRc has been reported in response to several other common physiologic stimuli, such as exposure to glucocorticoids (Lee and others 1992; McDonald and Goldfine 1988; Rouiller and others 1988) and growth arrest (Levy and Hug 1992); these factors may be involved in the regulation of IRc expression within the laminar microvasculature of the equine digit. Overall, the unique upregulation of the IRc in the laminar microvasculature may possibly play a role in the greater sensitivity of laminar tissue (compared to other tissues/organs) to injury in EMS.

The total amount of NSC consumed by the ponies in the high-NSC group during the 7-day experimental feeding period was approximately 8 g/kg body weight/day, an amount which approximates the amount administered to horses to induce laminitis via the
oligofructose model (10 g/kg) (van Eps and Pollitt 2006; van Eps and others 2012) but far less than that used to induce laminitis via the enteral carbohydrate model (17.6 g/kg corn and wood starch) (Leise and others 2011). The daily NSC dose administered to the ponies in this study was divided into three daily feedings, not administered as a single bolus, and each pony received only ~2 g/kg body weight/day of NSC as oligofructose. The experimental diet was meant to mimic the NSC consumption that might occur in a pony following acute pasture exposure (Longland and Byrd 2006) and was not composed to create a laminitis model, per se; however, it was anticipated that the diet might induce laminitis in a certain number of the animals.

In conclusion, the results of the work reported here support a role for insulin in the pathophysiology of endocrinopathic equine laminitis; however, direct effects on LBECs mediated through IRc appear unlikely due to its limited expression on this cell type. Further work is needed to more discretely characterize the mechanism(s) by which insulin exacts its effects in the equine digit; delineating the pathways mediating its effects will likely lead to logical points of intervention for the prevention and treatment of laminitis associated with EMS.
Chapter 5: Laminar inflammatory events in lean and obese ponies subjected to high carbohydrate feeding: implications for pasture-associated laminitis

Abstract

A robust laminar inflammatory response, including leukocyte infiltration and inflammatory mediator expression, has been well-characterized in laminitis induced by black walnut extract or enteral carbohydrate overload. Although inflammation has been proposed to also play a role in laminitis associated with equine metabolic syndrome, it has not been critically evaluated. The purpose of this study was to characterize the expression of inflammatory genes and leukocyte infiltration in digital laminar tissue of ponies subjected to a dietary carbohydrate challenge designed to mimic abrupt exposure to pasture rich in nonstructural carbohydrate (NSC). Following 4 weeks of conditioning consisting of a diet of hay chop (NSC ~6% on a DM basis), mixed-breed ponies (body weight 270.9 +/- 74.4 kg) were assigned to groups based on body condition scoring (lean vs. obese). Ponies either remained on the conditioning diet (CON diet; n=5 obese, n=5 lean) or received the same diet supplemented with sweet feed and oligofructose (CHO
diet [~42% NSC]; n=6 obese, n=6 lean) for a period of 7 days. At the end of the feeding protocol, dorsal digital laminar tissue samples were collected immediately following euthanasia; samples were formalin-fixed or snap frozen. Laminar immunohistochemistry was performed for CD163 and MAC387/calprotectin using commercially-available antibodies; the number of immunopositive cells was quantified at 40x magnification (n = 10 fields) for each section by a blinded observer. Real-time-quantitative PCR was used to assess laminar mRNA concentrations of pro-inflammatory cytokine/chemokine genes (TNFα, IL-1β, IL-6, IL-8, MCP-1, MCP-2) and COX-2. There was no effect of diet or body condition on the number of laminar CD163(+) or MAC387(+) cells (p >0.05); very few laminar MAC387+ cells were present in any group. There was no difference in the laminar mRNA concentrations of TNFα (p = 0.92), IL-1β (p = 0.18), IL-6 (p = 0.28), IL-8 (p = 0.21), MCP-1 (p = 0.25), or MCP-2 (p = 0.67); however, laminar mRNA concentrations of COX-2 (p = 0.01) were increased in ponies fed a high-CHO diet (vs. CON diet). These results suggest that the primary inflammatory events occurring in the marked inflammatory response reported in sepsis models of laminitis, leukocyte infiltration and proinflammatory cytokine/chemokine expression, are not central events in EMS-associated laminitis. As laminar COX-2 expression has been primarily localized to vascular wall components (endothelium and smooth muscle) and laminar keratinocytes in the normal and laminitic equid in previous reports, the increased laminar mRNA concentrations of COX-2 in this study may reflect laminar epithelial dysfunction and/or vascular pathology in the affected laminae. Ongoing immunolocalization studies will further clarify the cellular origin of the increased COX-2 expression.
**Introduction**

Laminitis is a frequent (and often life- and performance-limiting) sequel to many inflammatory diseases of equids, particularly those diseases characterized by Gram(-) sepsis or endotoxemia (such as strangulating gastrointestinal disease, septic pleuropneumonia, septic metritis, and enterocolitis) (Parsons and others 2007). Sepsis-associated laminitis has been suggested to share several pathophysiologic characteristics in common with sepsis-associated end-organ injury in humans (Belknap, Moore, Crouser 2009). Most notably amongst these commonalities, end-organ inflammation, characterized by robust pro-inflammatory cytokine and chemokine gene expression, adhesion molecule expression, and leukocyte infiltration, is reliably observed both in human sepsis and in the well-characterized experimental models of sepsis-associated laminitis, the black walnut extract model(Belknap 2010) and the enteral carbohydrate overload model(Pollitt and Visser 2010). These inflammatory processes have been proposed to play a direct role in the laminar injury and subsequent failure that occurs in these models, as the onset and acceleration of laminar inflammatory responses has been shown to correlate temporally with the onset of clinical lameness (Belknap and others 2007; Black and others 2006; Leise and others 2011).

In addition to sepsis, laminitis also frequently accompanies endocrine disease of equids, such as equine metabolic syndrome (EMS)(Frank and others 2010) and pituitary pars intermedia dysfunction (PPID)(Johnson, Messer, Ganjam 2004). In fact, laminitis associated with endocrinopathy is reported to be the type of the disease most frequently encountered in equine veterinary practice (American Association of Equine Practitioners
Obesity in several species (experimental rodent models, humans, and horses, among others) has been reported to be associated with not only expansion of general adipose tissue mass, but also increased endocrine activity of that tissue; obese individuals have increased numbers of infiltrating adipose tissue macrophages (Lumeng and others 2007; Zeyda and others 2007) and increased circulating concentrations of several pro-inflammatory cytokines, including TNFα (Ajuwon and Spurlock 2005; Borst and Bagby 2004). It has been suggested by some authors that equine endocrinopathic laminitis may represent an end-organ injury resulting from a more global ‘pro-inflammatory state’ associated with obesity and insulin resistance in these species (Vick and others 2007) and that laminitis in this setting is likely also inflammatory (de Laat and others 2011). However, the role of inflammatory events in the pathophysiology of endocrinopathic laminitis has not been rigorously investigated and is currently poorly understood. The purpose of the study reported here was to characterize the expression of pro-inflammatory cytokine and chemokine genes and the degree of leukocyte infiltration in the digital laminae of ponies subjected to a dietary carbohydrate challenge designed to mimic abrupt exposure to pasture rich in non-structural carbohydrate (NSC), a stimulus reported to be associated with new-onset laminitis in metabolically predisposed (i.e., systemically insulin resistant) equids (Carter and others 2009a; Treiber and others 2006).

*Materials and Methods*

*Animal protocol*
Twenty-two mixed breed ponies (body weight 270.9 +/- 74.4 kg) were used for this study. All animals received humane treatment in accordance with an animal care and use protocol approved by the Institutional Animal Care and Use Committee. Feedstuffs used in the protocol were analyzed for non-structural carbohydrate content (NSC, defined as the sum of measured starch and water-soluble carbohydrates) by a commercial laboratory (Equi-Analytical). Ponies were divided into four experimental groups based on body condition scoring results: lean, low NSC (n = 5); obese, low NSC (n = 5); lean, high NSC (n = 6); obese, high NSC (n = 6) as previously described (Burns and others 2012). All body condition scores were performed by two individuals (identity withheld until acceptance); for the purposes of this study, lean animals were those with a body condition score of less than or equal to 4/9, and obese animals were those with a body condition score of greater than or equal to 7/9 (Henneke and others 1983).

All ponies were housed in dirt lots and conditioned to a diet of hay chop (7% starch and ethanol soluble carbohydrate on a dry matter basis) for 4 weeks prior to initiation of the experimental feeding protocol. Ponies were fed 2.5% of their body weight in hay chop per day, divided into two feedings (7 a.m. and 6 p.m. EST). Following the four week conditioning period, ponies either remained on the control diet (n=10; lean and obese control groups) or received the same diet supplemented with sweet feed (1.5% body weight per day, fed three times daily at 7 a.m., 12 p.m., and 6 p.m. EST) and oligofructose (Beneo-ORAFITI, 2 g/kg added to hay chop ration; lean and obese challenge groups, n = 12) for a period of 7 days. The mean NSC consumption of ponies
in the control groups was approximately 1.8 g/kg/day, while that of ponies in the challenged groups was approximately 8 g/kg/day.

All ponies underwent an insulin-modified frequently-sampled intravenous glucose tolerance testing with Minimal Model analysis (Toth and others 2009) for assessment of insulin sensitivity during the first two weeks of the acclimation period. To assess the effects of the 7-day dietary treatment on insulin concentrations dynamics, blood was collected into red top tubes (Becton Dickinson) for measurement of basal serum insulin concentrations prior to (Day 0) and after completion of the feeding protocol (Day 7); serum insulin concentrations were measured with a radioimmunoassay that has been previously validated for use on equine samples (Coat-A-Count™, Siemens; (Freestone and others 1991)).

At the end of the 7 day experimental period, all ponies were humanely euthanized with an intravenous overdose of pentobarbital sodium and phenytoin sodium (Fatal-Plus™, Vortech; 20 mg/kg IV). The front feet of each animal were removed by disarticulation of the metacarpophalangeal joint immediately following euthanasia, and 1.5 cm sagittal sections of the dorsal digit were cut with a band saw. After dissection of the digital laminae away from the hoof wall and third phalanx, sections of laminar tissue were snap-frozen in liquid nitrogen and fixed in 10% neutral buffered formalin; all laminar samples were collected and fixed or frozen within 15 minutes of euthanasia. All formalin-fixed samples were transferred to 70% ethanol after 48 hours, where they were stored until paraffin embedding.
**Immunohistochemistry**

Formalin-fixed right front laminar samples were embedded in paraffin and sectioned at 5 µm for immunohistochemistry. Staining of laminar samples was performed as previously reported (Burns and others 2011); briefly, sections were deparaffinized and incubated in either a mouse monoclonal anti-CD163 primary antibody\(^f\) (Cosmo; 1:100, incubated overnight at 4º C (Faleiros and others 2011a) ), a mouse monoclonal anti-calprotectin primary antibody\(^g\) (Abcam; 1:100, incubated overnight at 4º C (Faleiros, Nuovo, Belknap 2009) ), or a goat polyclonal anti-COX-2 primary antibody\(^h\) (Santa Cruz; 1:100, incubated overnight at 4º C (Blikslager and others 2006)). Detection of immunoreactivity was performed using an immunoperoxidase system\(^i\) (Vector Laboratories) and DAB substrate\(^j\) (Vector Laboratories). After IHC staining, the number of CD163- or calprotectin-positive cells in (40X) light microscopy fields (n = 10) in primary and secondary dermal and epidermal laminar tissue was counted and recorded by a single investigator who was blinded to the source of the tissue section (identity of investigator withheld until acceptance). The distribution of laminar COX-2-positive cells was assessed by the same blinded observer via light microscopy and qualitatively described.

**RNA isolation, cDNA synthesis, and real-time quantitative polymerase chain reaction (RT-qPCR)**

Homogenates were made from each laminar tissue sample (~100 mg) with a tissue disruptor\(^k\), and total RNA was extracted using a commercially available kit\(^l\)
Messenger RNA (mRNA) was then isolated from the total RNA utilizing polyA tail hybridization (mRNA extraction kit™) and quantified (Nanodrop®). Complementary DNA (cDNA) was synthesized from the mRNA isolated from each sample via reverse transcription (Retroscript®) and stored at -20°C until used for real-time quantitative polymerase chain reaction (RT-qPCR) analysis.

A thermocycler (Roche 480ª) was used to perform RT-qPCR. Amplification was quantified against external standards using fluorescent format for SYBR Green I as previously described. (Waguespack, Cochran, Belknap 2004; Waguespack and others 2004) Primers for tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β), IL-6, IL-8, IL-10, plasminogen activator-inhibitor-1 (PAI-1), monocyte chemoattractant protein-1 (MCP-1), MCP-2, E-selectin, cyclo-oxygenase-2 (COX-2), ICAM-1, inducible nitric oxide synthetase (iNOS), and the housekeeping genes β-actin and β2-microglobulin were designed against equine-specific gene sequences (using exon sequence spanning an intron) using computer programs as previously described (Burns and others 2010; Leise and others 2011; Waguespack, Cochran, Belknap 2004); these primers were subsequently used for amplification of the respective genes of interest. Standard curves for quantification of mRNA concentration in laminar tissue samples were generated using serial dilutions of a linearized vector® containing an insert of each amplified cDNA fragment (identity previously confirmed through sequencing). All PCR assays were run in duplicate, including laminar samples, standards of known concentration, and negative control samples.
Data analysis

RT-qPCR data from two housekeeping genes (β-actin and β2-microglobulin) were entered into a commercially-available computer program to test each gene’s suitability as a housekeeping gene for the equine laminar tissue samples. Because both genes were determined to be satisfactory by the program, a geometric mean was obtained from the 2 genes’ data in order to generate a normalization factor for gene expression from each laminar tissue sample. Gene expression data from the genes of interest then was normalized using this factor for each individual sample.

Data analysis was performed using a statistical software program (GraphPad Prism). All data were assessed for normality by the Shapiro-Wilk and D’Agostino and Pearson omnibus normality tests. Basal serum insulin concentration, insulin sensitivity, and acute insulin response to glucose data were analyzed with a Student’s t test (or non-parametric equivalent as appropriate). Laminar leukocyte quantification and mRNA concentration data were analyzed with a two-way analysis of variance followed by a Bonferroni post-test, as appropriate. Statistical significance was accepted at p < 0.05. Data are expressed as mean +/- standard deviation unless otherwise indicated.

Results

Systemic insulin response to high carbohydrate feeding

All ponies completed the feeding protocol; four ponies were observed to become clinically laminitic during the course of the feeding protocol (Obel grade I-II/IV; one lean challenged pony, three obese challenged ponies). One of these animals was administered
phenylbutazone (2.2 mg/kg PO BID) for three days prior to euthanasia in accordance with the approved animal care and use protocol associated with the project; the three remaining animals were noted to be mildly laminitic (Obel grade I/IV) on the day of euthanasia only and thus were not medicated.

Ponies were assigned to the experimental groups based solely on BCS with no stratification related to insulin sensitivity status. However, subsequent Minimal Model analysis indicated that insulin sensitivity testing was markedly lower in obese when compared to lean ponies (SI, 0.50 +/- 0.26 x 10^{-4} L/min/mU in OB vs 2.57 +/- 2.18 x 10^{-4} L/min/mU in LN, p = 0.0005). Conversely, the acute insulin response to glucose (AIRg) was higher (p = 0.04) in OB (755.4 +/- 340.9 mU/L/min) than in LN (478.4 +/- 433.6 mU/L/min) ponies.

No weight gain was observed in response to either the low NSC or high NSC feeding protocols. Basal serum insulin concentration was increased across the 7 day experimental feeding period in both LN (Day 0, 11.4 +/- 7.5 mU/L; Day 7, 258.0 +/- 306.2 mU/L) and OB (Day 0, 14.6 +/- 6.9 mU/L; Day 7, 549.1 +/- 277.3 mU/L) ponies fed the high-NSC diet when compared with low-NSC fed animals (p = 0.007; see Figure 3). There was no difference in basal serum insulin concentration across the feeding protocol between the lean and obese control groups (p > 0.05).

**RT-qPCR**

There was no observed effect of diet or body condition score on laminar mRNA concentrations of TNF-α (diet, p = 0.91; BCS, p = 0.69), IL-1β (diet, p = 0.2; BCS, p =
0.3), IL-6 (diet, p = 0.17; BCS, p = 0.53), IL-8 (diet, p = 0.11; BCS, p = 0.6), IL-10 (diet, p = 0.58; BCS, p = 0.4), MCP-1 (diet, p = 0.24; BCS, p = 0.76), MCP-2 (diet, p = 0.92; BCS, p = 0.62), ICAM-1 (diet, p = 0.11; BCS, p = 0.57), PAI-1 (diet, p = 0.7; BCS, p = 0.22), or iNOS (diet, p = 0.12; BCS, p = 0.44); see Figure 6. High-carbohydrate feeding increased laminar mRNA concentrations of COX-2, but no effect of BCS was noted (diet, p = 0.02; BCS, p = 0.8). Laminar E-selectin mRNA concentrations were decreased in obese ponies compared to lean ponies, and while high-carbohydrate feeding tended to decrease laminar E-selectin mRNA concentrations in lean ponies and increase concentrations in obese ponies, this result did not achieve statistical significance (diet, p = 0.07; BCS, p = 0.02; see Figure 12).
Figure 12. Graphical representation of laminar mRNA concentrations of pro-inflammatory cytokine (panel A) and chemokine (panel B) genes from ponies fed low- and high-NSC diets for 7 days. Digital laminar concentrations of COX-2 mRNA were increased in response to high-carbohydrate feeding, an effect noted in both LN and OB ponies. Laminar concentrations of E-selectin mRNA were decreased in LN ponies in response to high-carbohydrate feeding, a result which was not observed in OB ponies. No significant difference was observed in other measured cytokines or chemokines in response to diet or body condition score effects. CON, control; CHO, high-carbohydrate diet; OB, obese; TNFα, tumor necrosis factor alpha; IL-8, interleukin-8; COX-2, cyclooxygenase-2; PAI-1, plasminogen activator inhibitor-1; MCP-1, monocyte chemoattractant protein-1; iNOS, inducible nitric oxide synthetase; ICAM, intercellular adhesion molecule-1.
Digital laminar immunohistochemistry

There was no observed effect of diet or body condition score on the number of laminar CD163-positive (diet, \( p = 0.18 \); BCS, \( p = 0.53 \)) or calprotectin-positive cells (diet, \( p = 0.32 \); BCS, \( p = 0.59 \)); see Figure 13. CD163-positive cells were readily observed in all laminar sections; in contrast, very few calprotectin-positive cells were observed in any of the laminar tissue sections, regardless of group assignment. Additionally, virtually all of the calprotectin-positive cells observed within the digital laminae were keratinocytes; very few calprotectin-positive leukocytes were noted in any laminar section, and these were all intravascular. COX-2-positive cells were observed in both the laminar epidermis and vascular elements of the dermis in a distribution similar to that previously reported (Blikslager and others 2006); while the number of laminar COX-2 cells was not quantified, subjectively, there did not appear to be any effect of diet or BCS on laminar COX-2 expression.
Figure 13. Results of quantitation of the number of CD163(+) (top panel) and MAC387(+) (bottom panel) cells in histologic sections of digital laminae of ponies fed either a control diet or a high-carbohydrate diet for 7 days. The number of laminar CD163(+) or MAC387(+) cells was not affected by high-carbohydrate feeding, and there was no observed effect on the number of either cell type of body condition score. While the morphology of all observed CD163(+) cells within the laminae was consistent with that of leukocytes, virtually all of the laminar MAC387(+) cells were keratinocytes. LMF, light microscopy fields; NSC, non-structural carbohydrate; BCS, body condition score.
Discussion

The results of the work described here do not support a prominent role for inflammatory events in the pathogenesis of laminitis associated with high-carbohydrate feeding in ponies, as no evidence of up-regulation of pro-inflammatory cytokine and chemokine gene expression or laminar leukocyte infiltration were noted following high-carbohydrate feeding for 7 days. While a criticism of this work might be that the protocol employed was not meant to create a laminitis model per se, and the findings therefore would not be appropriately extrapolated to endocrinopathic laminitis in general, it is important to note that the ponies fed the high-NSC diet did develop basal hyperinsulinemia during the feeding protocol (Burns and others 2012). Hyperinsulinemia has been described by several authors to be a primary determinant of laminitis risk in the setting of equine endocrine disease (Carter and others 2009a; Treiber and others 2006), and experimental hyperinsulinemia has been shown to precipitate laminitis in normal ponies (Asplin and others 2007) and light-breed horses (de Laat and others 2010). In that sense, then, this model may be more representative of naturally-occurring disease than protocols of previous reports, as the challenge was enteral and more closely represented pasture exposure. Additionally, four of the ponies subjected to this study protocol did become laminitic; these individuals were not outliers in any of the categories evaluated describing laminar inflammatory responses (i.e., their laminae did not contain higher concentrations of cytokine mRNA or leukocytes than non-laminitic individuals with the same diet exposure).
While the laminar mRNA concentrations of pro-inflammatory cytokines and chemokines were not observed to increase in response to high-carbohydrate feeding, laminar COX-2 mRNA concentrations were uniquely increased in the ponies of this study in response to high-carbohydrate feeding (with no observed effect of body condition). Laminar COX-2 expression was immunohistochemically localized to multiple cell types (including epidermal keratinocytes, endothelial cells, and fibroblast-like cells), and the cellular localization of laminar COX-2 expression observed in this study was in complete agreement with that described in previous reports (Blikslager and others 2006). This finding may implicate several cell types in the pathophysiology of endocrinopathic laminitis; however, the unique role of the laminar basal epithelial cell in maintenance of laminar integrity (LBE cells are responsible for maintaining adherence of the laminar epithelium to its basement membrane, dysadhesion of which is a hallmark of laminitis (Van Eps and Pollitt 2009)) makes it a compelling target of investigation.

Regulation of COX-2 gene expression has most commonly been described in the setting of acute inflammation, in which activation of COX-2 transcription has been shown to be driven primarily through activation of NFκB downstream of pro-inflammatory cytokine signaling (Chun and Surh 2004; Jung and others 2003). While the results of the study reported here would support a role for COX-2 in the laminar pathophysiology that occurs secondary to high-carbohydrate feeding, the increased laminar COX-2 expression observed in high-NSC-fed ponies does not appear to be associated with other changes characteristic of inflammation (such as laminar pro-inflammatory cytokine elaboration and leukocyte infiltration). It may be that alternative
signaling pathways known to increase COX-2 expression, such as growth factor signaling through the extracellular signal-regulated kinase (ERK) pathway (Kim and Kim 2004), may be more important in the pathogenesis of endocrinopathic equine laminitis; this hypothesis warrants further investigation, as inhibitors of several growth factor receptors are commercially available, some of which have found clinical utility in human medicine (Arteaga and others 2011; Giles 2001).

Recent studies in molecular carcinogenesis and clinical oncology have suggested an important role for COX-2 activity in promoting epithelial-to-mesenchymal transition (EMT), a phenomenon known to be involved in normal development, wound healing, and metastasis of neoplasms (Micalizzi, Farabaugh, Ford 2010). During EMT, mature epithelial cells de-differentiate and adopt a more motile/invasive phenotype, a process that involves coordinated expression of several transcription factors and activation of multiple enzymatic cascades. In several in vitro and in vivo experimental constructs, absence of COX-2 activity results in an inability or reduced ability of affected cells to undergo EMT (Kim and others 2012; Tomlinson and others 2012). EMT involving laminar basal epithelial cells would be potentially biomechanically disastrous for the equine foot, allowing separation of the laminar keratinocytes from their basement membrane; further study will identify the importance of this phenotypic change in endocrinopathic laminitis. Importantly, because of its role in potentiating EMT, COX-2 may represent a drugable target in the treatment and prevention of laminitis in at-risk horses and ponies, a very attractive target due to the commercial availability of COX-2 inhibitors that are FDA-approved for use in the horse.
Chapter 6: Effect of dietary carbohydrate challenge on activation of 5’-adenosine monophosphate activated protein kinase (AMPK) in liver, skeletal muscle, and digital laminae of lean and obese ponies

Abstract

Systemic insulin resistance and hyperinsulinemia are proposed to be important risk factors for laminitis associated with equine metabolic syndrome (EMS) in horses and ponies. AMPK, a highly conserved enzymatic regulator of cellular energy status, has become a therapeutic target for human MS and EMS due to its reported ability to increase systemic insulin sensitivity. For example, the AMPK agonist metformin has recently been used clinically to treat EMS-affected equids. However, regulation of AMPK activity in both 1) tissues primarily responsible for insulin-mediated glucose disposal (liver, skeletal muscle) and 2) the target tissue for injury in EMS-related laminitis, the digital laminae, is largely unknown. The purpose of this study was to characterize the cellular localization and activation state of AMPK in liver, skeletal muscle, and digital laminae of ponies subjected to a dietary carbohydrate challenge meant to mimic abrupt
exposure to pasture rich in nonstructural carbohydrate (NSC). Following 4 weeks of conditioning consisting of a diet of hay chop (NSC ~6% on a DM basis), mixed-breed ponies (body weight 270.9 +/- 74.4 kg) were assigned to groups based on body condition scoring (lean vs. obese). Ponies either remained on the conditioning diet (CON diet; n=5 obese, n=5 lean) or received the same diet supplemented with sweet feed and oligofructose (CHO diet [~42% NSC]; n=6 obese, n=6 lean) for a period of 7 days. At the end of the feeding protocol, samples of dorsal digital laminae, middle gluteal muscle, and liver were collected immediately following euthanasia; samples were formalin-fixed or snap frozen. AMPK was primarily localized to keratinocytes and vascular elements in the laminae and hepatocytes (vs. local vascular elements) in the liver via total AMPK immunohistochemistry. Western blot analysis for phospho(P)-AMPK (indicating activated AMPK) demonstrated decreased laminar P-AMPK concentrations upon challenge with a high-carbohydrate diet (p = 0.01). In contrast, P-AMPK concentrations were unchanged in skeletal muscle (p = 0.33), and there was a trend for increased AMPK activation in the liver in obese ponies in response to dietary CHO challenge (p = 0.13). Unchanged or increased P-AMPK concentrations in the setting of increased caloric intake suggest insulin resistance in skeletal muscle and liver; the decreased laminar P-AMPK concentrations with CHO challenge indicate that laminar tissue remains insulin sensitive. In conclusion, whereas skeletal muscle and liver are likely contributory to systemic insulin resistance and resulting hyperinsulinemia in EMS, laminar dysfunction/injury in EMS is more likely due to the local effects of hyperinsulinemia, and not due to local insulin resistance/energy failure.
Introduction

Equine laminitis is a debilitating musculoskeletal condition of the digit that affects horses and ponies of all breeds and commonly results in loss of use or life of the affected animal. (Frank and others 2010) A recent cross-sectional survey conducted by the United States Department of Agriculture demonstrated that laminitis affects up to 0.8% of all horses in the USA at any given time, with approximately 15% to 20% of horses afflicted by laminitis over the course of their lifetime. (USDA-NAHMS 2000) Furthermore, survey studies have indicated that up to 20% of affected animals fail to recover fully and 5% to 8% of animals with laminitis die or must be euthanatized as a direct result of the disease. (Frank and others 2010) Due to the devastating nature of the disease and current lack of effective therapies, equine laminitis has been identified as a high priority disease for the horse industry, with a stated need for additional research (particularly research directed at evaluation of novel therapies). (American Association of Equine Practitioners 2009)

While equine laminitis has been associated with many diverse causes (including gastrointestinal tract disorders, retained placenta or metritis, severe infections, and excessive weight bearing) (Belknap, Moore, Crouser 2009), pasture-associated laminitis is now reported to be the most common cause of laminitis, affecting primarily horses and ponies with a phenotype of obesity (esp. regional adiposity) and insulin resistance when they are exposed to pasture forage with high non-structural carbohydrate (NSC) content. (Carter and others 2009a; Treiber and others 2006; USDA-NAHMS 2000) Clinical observations and the results of epidemiologic studies have indicated that risk of pasture
laminitis is highest when horses and ponies are grazing pastures with high NSC content (i.e. fructans, simple sugars, and/or starch), and theories on the pathogenesis of pasture-associated laminitis have been extrapolated from experimental models of alimentary carbohydrate overload. However, the extent to which these severe models reflect events during development of EMS-related laminitis is questionable. First, it is likely that the amount and rate of starch or fructan administered for induction of carbohydrate overload in these studies far exceeds the quantity of NSC ingested by grazing equids (Geor and Harris 2009; Longland and Byrd 2006). Second, previous studies involving the experimental induction of laminitis with enteral carbohydrate overload have not used animals with an EMS phenotype and thus have not addressed the interaction between diet and phenotypic factors associated with laminitis susceptibility (Leise and others 2011; Pollitt and Visser 2010). The study reported here was designed with these shortcomings in mind to compare, in groups of obese (EMS) and lean ponies, metabolic changes in liver, skeletal muscle, and laminar tissue in response to a feeding protocol that more closely mimics the increase in dietary NSC associated with pasture laminitis.

Adenosine-5’-monophosphate activated kinase (AMPK) is a highly-conserved heterotrimeric enzyme widely regarded as a ‘master regulator’ of cellular energy status. The enzyme is composed of a catalytic α-subunit and regulatory β- and γ-subunits; tissue-specific expression of different allelic subunit combinations may be responsible for differential regulation of the enzyme in various tissues. (Dziewulska, Dobrzyn, Dobrzyn 2010) Activation of AMPK is increased during cellular energy stress (as manifested through an increased cellular AMP:ATP ratio), occurring primarily through
phosphorylation of a highly conserved threonine residue on the α-subunit (Thr172); this phosphorylation event results in downstream effects on intermediary metabolism that favor ATP production and limit ATP consumption (Shirwany and Zou 2010). For example, AMPK activation in the liver results in stimulation of lipid β-oxidation and inhibition of gluconeogenesis and lipid synthesis; over time, enhanced mitochondrial biogenesis also occurs, increasing use of lipid as metabolic fuel (Yang and others 2010). Globally, the metabolic changes induced by AMPK activation promote euglycemia, increase lipid oxidation, and enhance tissue and systemic insulin sensitivity. (Towler and Hardie 2007) For these reasons, AMPK has emerged as an attractive therapeutic target for the treatment of metabolic syndrome in human medicine.

Metformin, a functional AMPK agonist, is an orally bioavailable biguanide compound that has been used clinically as an anti-hyperglycemic and insulin-sensitizing therapeutic in the treatment of human metabolic syndrome and Type II diabetes mellitus (Mehnert 2001). The pharmacokinetics and pharmacodynamics of this drug have been well-characterized in humans, and it became the most frequently-prescribed oral antidiabetic drug in the United States within 12 weeks of its FDA approval (Bloomgarden 1996); it remains in common clinical use to this day. Relatively little is known, however, about the biological behavior of the drug or its enzymatic target (AMPK) in equids. Preliminary work in recent years has been performed to evaluate the role(s) that biochemical or genetic alterations in AMPK subunits may play in the pathophysiology of polysaccharide storage myopathy in American quarter horses (Dranchak and others 2007) and other breeds (Park and others 2003), with little evidence that AMPK is involved in a
causative way in this family of equine myopathies. Metformin has been used in the clinic to empirically treat equids afflicted with EMS (Durham, Rendle, Newton 2008), with reportedly beneficial effects. Preliminary pharmacokinetic studies of the drug in horses and ponies have been published (Hustace, Firshman, Mata 2009; Tinworth and others 2010) and suggest that the oral bioavailability of metformin in equids is significantly lower than that reported for humans; however, little pharmacodynamic information regarding metformin’s activity in equine systems (Tinworth and others 2012) or data describing AMPK activation/inhibition in target tissues of interest in EMS-affected equids has been reported to date. Very little evidence exists to support (or refute) the rational use of AMPK agonists in the insulin-resistant equid, and it was the purpose of the study reported here to describe the activation state of AMPK in two primary insulin-responsive tissues (liver and skeletal muscle) and in the digital laminae of ponies subjected to a dietary carbohydrate challenge meant to mimic acute pasture exposure. The work described here was intended to evaluate the possibility that AMPK agonism may represent a sound therapeutic target for a) improving systemic insulin sensitivity and b) treating/preventing laminitis in EMS-affected horses and ponies.

Materials and Methods

Animal protocol

Twenty-two mixed breed ponies (body weight 270.9 +/- 74.4 kg; age [lean] = 9.2 +/- 3.5 years, age [obese] = 11 +/- 3.8 years) were used for this study. All animals received humane treatment in accordance with an animal care and use protocol approved
by the Michigan State University Institutional Animal Care and Use Committee. Feedstuffs used in the protocol were analyzed for non-structural carbohydrate content (NSC, defined as the sum of measured starch and water-soluble carbohydrates) by a commercial laboratory\(^{26}\) (Equi-Analytical). All ponies obtained for the study were examined by a licensed veterinarian and deemed healthy based on the results of physical examination, complete blood count, and serum biochemical examination. Ponies were divided into four experimental groups based on body condition scoring results: lean, low NSC (n = 5); obese, low NSC (n = 5); lean, high NSC (n = 6); obese, high NSC (n = 6). All body condition scores were performed independently by two individuals; lean animals were those assigned a body condition score of \(\leq 4/9\), and obese animals were those assigned a body condition score of \(\geq 7/9\) (Henneke and others 1983).

All ponies were housed in dirt lots and conditioned to a diet of hay chop (7% starch and ethanol soluble carbohydrate on a dry matter basis) for 4 weeks prior to initiation of the experimental feeding protocol. Ponies were fed 2.5% of their body weight in hay chop per day, divided into two feedings (7 a.m. and 6 p.m. EST). Following the conditioning period, ponies either remained on the control diet (n=10; lean and obese control groups, 5 animals each) or received the same diet supplemented with sweet feed (1.5% body weight per day, fed three times daily at 7 a.m., 12 p.m., and 6 p.m. EST) and oligofructose\(^{27}\) (Beneo-ORAFTI; 2 g/kg added to hay chop ration; lean and obese challenge groups, n = 12 [6 animals each]) for a period of 7 days. The mean NSC consumption of ponies in the control groups was approximately 1.8 g/kg/day, while that of ponies in the challenged groups was approximately 8 g/kg/day. All ponies were
monitored three times daily during the experimental period and were under the supervision of a licensed veterinarian for the duration of the protocol.

All ponies underwent insulin-modified frequently-sampled intravenous glucose tolerance testing (FSIGTT) with Minimal Model analysis (Toth and others 2009) during the first two weeks of the acclimation period. The FSIGTT’s were performed between 7 a.m. and 9 a.m. EST following a 6-8 hour period of feed withholding. Additionally, blood was collected into red top tubes²⁸ (Becton Dickinson) for measurement of basal serum insulin concentrations on Day 0 and Day 7 of the feeding protocol (between 7 a.m. and 9 a.m. prior to feeding the morning ration); serum insulin concentrations were measured with a radioimmunoassay²⁴ validated for equine samples (Coat-A-Count™, Siemens; (Freestone and others 1991)). Following the 7 day experimental period, ponies were euthanized via intravenous overdose of pentobarbital sodium and phenytoin sodium³⁰ (Fatal-Plus™, Vortech; 20 mg/kg IV). The right front foot of each animal was removed by disarticulation of the metacarpophalangeal joint immediately following euthanasia, and 1.5 cm sagittal sections of the dorsal digit were cut with a band saw. After dissection of the digital laminae from the hoof wall and third phalanx, sections of laminae were snap-frozen in liquid nitrogen or fixed in 10% neutral buffered formalin; all laminar samples were processed within 15 minutes of euthanasia. Samples of skeletal muscle (middle gluteal muscle) and liver were similarly collected and snap-frozen in liquid nitrogen or fixed in 10% neutral buffered formalin. All formalin-fixed samples were transferred to 70% ethanol after 48 hours, where they were stored until paraffin embedding.
**Immunohistochemistry**

Formalin-fixed right front laminar and liver tissue samples were embedded in paraffin and sectioned at 5 µm for immunohistochemistry. Preliminary evaluation of the primary antibodies selected for immunohistochemical evaluation of both phosphorylated-AMPK and total AMPK localization in skeletal muscle tissue suggested poor staining of this tissue, and further optimization with different reagents was not performed. Additionally, the primary antibodies evaluated for immunohistochemical detection of phosphorylated AMPK performed poorly in equine tissue; therefore, the immunohistochemical work described here represents tissue localization of total AMPK. The staining procedures described below were used for the evaluation of AMPK localization within liver and digital laminar tissue. Staining of tissue samples was performed as previously reported (Burns and others 2011); briefly, sections were deparaffinized and incubated in a rabbit monoclonal anti-human AMPK-β1/2 primary antibody\(^73\) (Cell Signaling; 1:100, incubated overnight at 4º C). Detection of immunoreactivity was performed using a goat-anti-rabbit HRP conjugate secondary antibody\(^74\) (Cell Signaling Technology Inc.), an immunoperoxidase system\(^34\) (Vector Laboratories) and DAB substrate\(^35\) (Vector Laboratories). After immunohistochemical staining, the distribution of total-AMPK (+) cells in all liver and digital laminar tissue sections was evaluated via light microscopy by a single blinded observer, and the results were qualitatively described.

**Western immunoblotting**

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The concentration of phosphorylated and total AMPK in liver, skeletal muscle, and laminar tissue homogenates was assessed via Western immunoblotting performed as described previously (Blikslager and others 2006). Briefly, tissue protein samples (~100 mg tissue per sample) were prepared in 300 µL lysis buffer (Pierce) and quantitated via the Bradford method. Protein samples (20 µg/sample) were denatured by boiling for 5 minutes in β-ME/SDS buffer, separated on an 8% polyacrylamide gel, and transferred to a PVDF membrane. The membrane was blocked for 1 hour with 5% BSA in PBS-Tween 20 (0.1% v/v Tween-20 in PBS; PBST [phosphorylated AMPK blot]) or 5% dry nonfat milk in PBST at room temperature, rocking. The membrane was then incubated with primary antibody (Cell Signaling Technology, Inc., 1:1000 in blocking buffer [5% BSA or 5% nonfat milk in PBST, for phospho- and total AMPK, respectively]) overnight at 4º C. The membrane was washed five times with 0.1% PBST as before. Goat anti-rabbit IgG-HRP secondary antibody (Cell Signaling Technology, Inc.) was diluted 1:5000 in 5% milk and incubated with the membrane for 1 hour at room temperature, rocking. The membrane was washed 5 times with 0.1%PBST and developed for 5 min using a chemiluminescent substrate (West Femto, Pierce). The membrane was first probed for phosphorylated-AMPK, then stripped and reprobed sequentially for total AMPK and β-actin (XXXXX). Luminescence was measured using a computer software program (Carestream), and signal strength was determined using net phospho-AMPK band intensity divided by the total AMPK and β-actin band intensites.
RNA isolation, cDNA synthesis, and real-time quantitative polymerase chain reaction (RT-qPCR)

Homogenates were made from each tissue sample (~100 mg) with a tissue disruptor\(^1\), and total RNA was extracted using a commercially available kit\(^7\) (Agilent Technologies). Messenger RNA (mRNA) was then isolated from the total RNA utilizing poly-A tail hybridization\(^7\) (Roche Applied Science) and quantified\(^7\) (NanoDrop, Thermo Scientific). Complementary DNA (cDNA) was synthesized from the mRNA isolated from each sample via reverse transcription\(^2\) (Ambion Inc.) and stored at -20\(^\circ\) C until used for real-time quantitative polymerase chain reaction (RT-qPCR) analysis.

A thermocycler\(^2\) (Roche Applied Science) was used to perform RT-qPCR. Amplification was quantified against external standards using fluorescent format for SYBR Green I as previously described. (Waguespack, Cochran, Belknap 2004; Waguespack and others 2004) Primers for peroxisome proliferator-activated receptor-\(\gamma\) (PPAR\(\gamma\)), peroxisome proliferator-activated receptor-\(\gamma\) coactivator 1-\(\alpha\) (PGC1\(\alpha\)), AMPK\(\alpha\), phosphoenolpyruvate carboxykinase (PEPCK), and the housekeeping genes \(\beta\)-actin and \(\beta_2\)-microglobulin were designed against equine-specific gene sequences (using exon sequence spanning an intron) using computer programs as previously described (Burns and others 2010; Leise and others 2011; Waguespack, Cochran, Belknap 2004); these primers were subsequently used for amplification of the respective genes of interest. Standard curves for quantification of mRNA concentration in laminar tissue samples were generated using serial dilutions of a linearized vector\(^2\) containing an insert of each amplified cDNA fragment (identity confirmed through nucleic acid sequencing).
All PCR assays were run in duplicate, including liver, laminar, and skeletal muscle samples; standards of known concentration; and negative control samples.

**Data analysis**

RT-qPCR data from two housekeeping genes (β-actin and β₂-microglobulin) were entered into a commercially-available computer program\(^{24}\) to test each gene’s suitability as a housekeeping gene for the equine laminar tissue samples. Because both genes were determined to be satisfactory by the program, a geometric mean was obtained from the 2 genes’ data in order to generate a normalization factor for gene expression from each separate tissue sample (liver, skeletal muscle, and digital laminae from each individual pony). Gene expression data from the genes of interest then was normalized using this factor for each sample.

Data analysis was performed using a statistical software program\(^{25}\) (GraphPad Prism). All data were assessed for normality by the Shapiro-Wilk and D’Agostino and Pearson omnibus normality tests. Basal serum insulin concentration, insulin sensitivity, and acute insulin response to glucose data were analyzed with a Student’s t test (or non-parametric equivalent as appropriate). mRNA concentration data were analyzed with a two-way analysis of variance followed by a Bonferroni post-test, as appropriate. Protein concentration data were analyzed with a Student’s t test or non-parametric equivalent. Statistical significance was accepted at p < 0.05. Data are expressed as mean +/- standard deviation unless otherwise indicated.
Results

Systemic insulin response to high carbohydrate feeding

All ponies completed the feeding protocol; four ponies were observed to become clinically laminitic during the course of the feeding protocol (Obel grade I-II/IV; one lean challenged pony, three obese challenged ponies). One of these animals was administered phenylbutazone (2.2 mg/kg PO BID) for three days prior to euthanasia in accordance with the approved animal care and use protocol associated with the project; the three remaining animals were noted to be mildly laminitic (Obel grade I/IV) on the day of euthanasia only and thus were not medicated.

Ponies were assigned to the experimental groups based solely on BCS with no stratification related to insulin sensitivity status. However, subsequent Minimal Model analysis indicated that insulin sensitivity testing was markedly lower in obese when compared to lean ponies (SI, 0.50 +/- 0.26 x 10^{-4} L/min/mU in OB vs 2.57 +/- 2.18 x 10^{-4} L/min/mU in LN, p = 0.0005). Conversely, the acute insulin response to glucose (AIRg) was higher (p = 0.04) in OB (755.4 +/- 340.9 mU/L/min) than in LN (478.4 +/- 433.6 mU/L/min) ponies.

No weight gain was observed in response to either the low NSC or high NSC feeding protocols. Basal serum insulin concentration was increased across the 7 day experimental feeding period in both LN (Day 0, 11.4 +/- 7.5 mU/L; Day 7, 258.0 +/- 306.2 mU/L) and OB (Day 0, 14.6 +/- 6.9 mU/L; Day 7, 549.1 +/- 277.3 mU/L) ponies fed the high-NSC diet when compared with low-NSC fed animals (p = 0.007). There was
no difference in basal serum insulin concentration across the feeding protocol between the lean and obese control groups (p > 0.05).

**Immunohistochemistry**

The cellular and subcellular immunolocalization of the AMPK enzymatic complex was evaluated qualitatively via histochemical staining of liver and digital laminar tissue. No effect of diet or body condition score on the quantity or distribution of AMPK was readily apparent in either tissue; however, this was a subjective assessment and would be considered poorly sensitive for detection of subtle effects. Within the digital laminae, most cell types expressed AMPK, including laminar keratinocytes (including basal epithelial cells), vascular endothelial and smooth muscle cells, and dermal mesenchymal cells (presumed to be fibroblasts). Within the liver, most AMPK(+) cells were hepatic sinusoidal epithelial cells, with little staining of endothelial cells or leukocytes. In both liver and digital laminae, prominent perinuclear stain uptake was noted, and in the liver, a granular appearance to hepatocyte cytoplasmic staining was observed (consistent with AMPK’s previously reported association with glycogen; see Figure 14). While the intensity or localization of hepatic AMPK immunohistochemical staining was not observed to change with high-carbohydrate feeding, animals fed this diet did have increased cytoplasmic vacuolar change visible within hepatocytes (a finding consistent with, but not diagnostic of, lipid accumulation; Fig. 14).
Figure 14. Immunohistochemical staining of liver and digital laminae against the β-subunit of AMPK (total AMPK). Note the distinct perinuclear staining (arrows; inset) in both liver and laminae, and the granular cytoplasmic staining in liver (consistent with reported association of the complex with glycogen). All images are at 40x magnification; images on the left are from animals fed a low-carbohydrate diet, and those on the right are from animals fed the high-carbohydrate experimental diet.
Western immunoblotting

Activating phosphorylation of AMPK in digital laminar tissue was significantly decreased in lean ponies in response to high-carbohydrate feeding (p = 0.03) and displayed a trend toward decreased activation in obese ponies in response to high-carbohydrate feeding (p = 0.08). In contrast, activating phosphorylation tended to increase in liver and skeletal muscle in response to high-carbohydrate feeding in both lean (skeletal muscle, p = 0.33; liver, p = 0.84) and obese ponies (skeletal muscle, p = 0.43; liver, p = 0.13), although results from these tissues did not achieve statistical significance (see Figures 15 and 16).
Figure 15. AMPK activating phosphorylation in response to dietary carbohydrate challenge in digital laminae, liver, and skeletal muscle of obese and lean ponies. While AMPK phosphorylation decreases in laminar tissue in response to high-carbohydrate feeding (suggesting insulin sensitivity, or insulin independence of this tissue), this pattern is not observed in liver or skeletal muscle. In fact, AMPK activation tends to increase in liver and skeletal muscle, a finding which is consistent with insulin resistance in these tissues. pAMPK, phosphorylated AMPK; tAMPK, total AMPK; CON, control diet; CHO, carbohydrate challenge diet.
Western blot densitometry data from individual animal tissue samples assessing AMPK phosphorylation in laminae, liver, and skeletal muscle of lean and obese ponies. While laminar tissue shows a significant decrease in AMPK phosphorylation in response to a high-NSC diet, liver and skeletal muscle do not; this finding is consistent with insulin resistance in these tissues. Data are derived from the following ratio of immunoblot band densities: pAMPK/tAMPK/β-actin.
**RT-qPCR**

The mRNA concentration of PEPCK was significantly increased in skeletal muscle in response to high-carbohydrate feeding in both lean and obese ponies (p = 0.02); no effect was observed in digital laminae (p = 0.37) or liver (p = 0.58). No effect of diet or body condition on the gene expression of PPARγ, PGC1α, or AMPKα was observed in liver, skeletal muscle, or digital laminar tissue (p > 0.05, all analytes; Fig. 17).
Figure 17. RT-qPCR data from digital laminae, liver, and skeletal muscle for genes downstream of AMPK activation. The mRNA concentration of PEPCK was significantly increased in skeletal muscle in response to high-carbohydrate feeding in both lean and obese ponies; no effect was observed in digital laminae or liver. No effect of diet or body condition on the gene expression of PPARγ, PGC1α, or AMPKα was observed in liver, skeletal muscle, or digital laminar tissue. PPARγ, peroxisome proliferator-activated receptor-γ; PGC1α, peroxisome proliferator-activated receptor-γ coactivator 1-α; AMPKα, adenosine monophosphate activated protein kinase α-subunit; PEPCK, phosphoenolpyruvate carboxykinase.
Discussion

Adenosine-5’-monophosphate-activated protein kinase (AMPK) is a eukaryotic heterotrimeric protein complex that is highly conserved among phyla and widely distributed in most tissue types of multicellular organisms. Activation of the kinase activity results from phosphorylation of the α-subunit; the β and γ subunits are regulatory in nature (γ binding AMP, β binding carbohydrate moieties, esp. glycogen). AMPK has been described as an important global sensor of cellular energy status, with activation of the complex during times of carbon starvation or energy deficit (such as hypoglycemia, ischemia, hypoxia, etc.). Activation of AMPK kinase activity (again, marked by phosphorylation of the α subunit at Thr172) results in broad changes in carbohydrate, lipid, and protein metabolism that serve to, in general, decrease energy consumption (anabolic pathways, such as glycogen, lipid, and protein synthesis) and increase energy production (catabolic pathways, such as glucose uptake, glycolysis, glycogenolysis, lipolysis, lipid oxidation). AMPK activation has also been shown to have significant effects on growth and differentiation, promoting decreased mitotic activity, increased epithelial intercellular adhesion, and persistence of cells in G0. (Yang and others 2012)

The purpose of the study reported here was to characterize the cellular localization and activation state of AMPK in liver, skeletal muscle, and digital laminae of ponies subjected to a dietary carbohydrate challenge meant to mimic abrupt exposure to pasture rich in nonstructural carbohydrate (NSC), conditions reported to be associated with an increased risk of EMSAL (Carter and others 2009a; Treiber and others 2006).
Systemic insulin resistance and resultant hyperinsulinemia are proposed to be important risk factors for endocrinopathic laminitis associated with equine metabolic syndrome (EMS) in horses and ponies (Asplin and others 2007; de Laat and others 2010), and therapeutic interventions targeting improved systemic insulin sensitivity are in widespread clinical use in human and veterinary medicine (Durham, Rendle, Newton 2008; Frank and others 2005; Frank, Elliott, Boston 2008; Mehnert 2001). While metformin’s clinical use in equine veterinary medicine is increasing (Frank and others 2010), no experimental or clinical studies have yet provided useful pharmacodynamic data regarding the downstream effects of metformin on metabolic pathways relevant to EMS therapeutics (most importantly, those which would be expected to improve target tissue insulin sensitivity). To our knowledge, the results reported here include the first data obtained assessing tissue-specific AMPK regulation in the equid.

Since the first reports of experimental laminitis induction with prolonged hyperinsulinemia were published (Asplin and others 2007; de Laat and others 2010), much speculation has occurred regarding the most important and relevant pathophysiologic mechanisms by which laminitis develops in this setting. Initial reports suggested that laminar insulin resistance might result in chronic glucose/carbon substrate deprivation, energy failure, and dermoepidermal separation (based on in vitro experiments using laminar explants incubated in glucose-free media for prolonged periods and subjected to mechanical distraction) (French and Pollitt 2004a). However, more recent publications have established the digital laminar tissue as relatively insulin-insensitive or insulin-independent. Asplin and colleagues showed little increase in
glucose uptake by laminar tissue explants from normal horses in response to incubation with insulin, as well as evidence that GLUT1 may be the predominant glucose transporter gene expressed in both normal and chronically laminitic laminar tissue homogenates (Asplin and others 2011). Further, recent work has documented little evidence of insulin receptor expression on laminar keratinocytes of lean and obese ponies, a finding that does not appear to be affected significantly by high-carbohydrate feeding (Burns and others 2012). Finally, the results of the study reported here are not consistent with digital laminar tissue insulin resistance and consequent energy deprivation/stress; in this situation, one would expect an increased AMP:ATP ratio as cellular energy stores are depleted, with a consequent robust activation of AMPK (Towler and Hardie 2007). In fact, the opposite is observed in response to high-carbohydrate feeding.

Vascular events have also been suggested to be involved in the pathogenesis of laminitis (Hood and others 1993), with hypoperfusion and subsequent energy deprivation and hypoxia proposed as important contributors to dermoepidermal separation (Robertson, Bailey, Peroni 2009; Venugopal and others 2011). Insulin has long been known to have vasoactive effects; signaling through the PI3 kinase pathway downstream of the insulin receptor results in elaboration of nitric oxide (a potent vasodilator), while MAPK pathway activation downstream of insulin receptor activation results in increased endothelin-1 activity (a potent vasoconstrictor) (Vigneri, Squatrito, Sciacca 2010). Significantly, persistent activation of MAPK signaling may occur in insulin-resistant states, even when PI3K signaling is markedly attenuated; this may contribute to hypertension when increased production of ET-1 is unbalanced by NO elaboration.
In the insulin-resistant rat and human, decreased NO elaboration and increased ET-1 tone have been reported, which has been reported to be one mechanism by which insulin-resistant individuals develop systemic hypertension (Cardillo and others 1999; Contreras and others 2010; Eringa and others 2007; Potenza and others 2005; Taylor 2001). While systemic hypertension has been reported in ponies at risk for pasture-associated laminitis (Bailey and others 2008), the role that insulin plays in the perfusion of the equine digital laminae is poorly described. Asplin and colleagues reported that GLUT1 (which functions in an insulin-independent manner) is the primary glucose transporter expressed in both normal and pathologic equine digital laminar and coronary band tissue; this same group also found that glucose uptake by digital laminar tissue explants was unaffected by incubation with insulin (Asplin and others 2011). More recent work suggests that the distribution of insulin receptor expression in the laminar vasculature is primarily limited to digital laminar dermal capillaries, which would argue against dramatic effects on digital vasomotor tone (Burns and others 2012). Further, while the ponies of the study reported here became hyperinsulinemic in response to high-carbohydrate feeding (and many were insulin resistant systemically), evidence of hypoxia and energy stress were not observed in the digital laminae. Hypoxia is reported to result in potent AMPK activation in several species and experimental systems (Gusarova and others 2011; LaRue and Padilla 2011; Mungai and others 2011; Yan and others 2012); as mentioned above, one would expect to observe increased digital laminar concentrations of phosphorylated (activated) AMPK in response to energy deprivation if hypoperfusion and
hypoxia were present, an effect which was not observed in this study. These findings, along with the findings of previous investigations, suggest that while altered vasomotor tone may be present in the equid with EMS, it does not play a primary role in the pathogenesis of EMSAL.

In recent cell culture studies using Madin-Darby canine kidney epithelial cells, the role of AMPK activity in the maintenance and dynamic repair of intercellular tight junctions in response to osmotic stress has been demonstrated, with accelerated tight junction assembly observed following application of an AMPK agonist (AICAR) and delayed assembly following AMPK inhibition (compound C) (Miranda and others 2010; Zhang and others 2006). AMPK activation also enhances cell-cell contacts and reduces metastatic potential in melanoma cell lines (Kim and others 2012). AMPK has been shown more broadly to have important roles in determination of cell polarity both during the ontogeny and maintenance of tissue architecture (Fu and others 2011; Jansen and others 2009; Lo and others 2012), again involving regulation of intercellular contacts/adhesions. While the net effects of AMPK activation or inhibition on the function of the equine laminar epidermal basal cell are unknown, it is reasonable to assume that it likely plays a role in this highly polarized, structurally critical cell type, whose cytoskeletal interactions are crucial for support of the weight of a horse or pony against gravity (French and Pollitt 2004b; French and Pollitt 2004c). AMPK may have profound effects on the integrity and confluence of epithelial cells in cell culture, including their ability to remodel tight junctions following application of a cellular stressor (Zhang and others 2006). AMPK, then, may provide an attractive link between
dietary carbohydrate content, insulin sensitivity, and laminar epithelial dysfunction. Inhibition of laminar AMPK activation in response to high-carbohydrate feeding may affect the integrity or rapidity with which metabolism of intercellular and hemidesmosomal contacts with laminar keratinocytes occur; structural failure may then result in this tissue which exists under the constant mechanical stress of weight bearing. Investigation of the effects of AMPK activation in the digital laminae in response to dietary carbohydrate content may open new avenues of inquiry regarding the pathophysiology of endocrinopathic laminitis, ideally leading to the development of novel therapies for treating this difficult disease. If laminar AMPK activation is ultimately shown to be protective against endocrinopathic laminitis, use of AMPK agonist medications (such as metformin) may prove useful in the therapy of this disease.

The results of this study do not support carbon substrate deprivation secondary to laminar insulin resistance as a central mechanism in laminitis associated with EMS; in fact, decreased AMPK activation in the laminae associated with high-NSC feeding suggests either (or both) tissue insulin sensitivity or insulin-independence with respect to glucose uptake into this tissue. Further, decreased laminar AMPK activation argues against significant tissue ischemia in this setting, as AMPK activation would be expected in a tissue deprived of nutrients in this manner. The pattern of AMPK activation noted in liver and skeletal muscle is supportive (although not diagnostic) of tissue insulin resistance, which may contribute to and drive systemic IR and resultant hyperinsulinemia. Whereas skeletal muscle and liver are likely contributory to systemic insulin resistance and resulting hyperinsulinemia in EMS, laminar dysfunction/injury in
EMS is more likely due to the local effects of hyperinsulinemia, and not due to local insulin resistance/energy failure.
Chapter 7: Evaluation of the response of the endocrine pancreas to short-term high-carbohydrate feeding in mixed-breed ponies

Abstract

Obesity, insulin resistance, and endocrinopathic laminitis have emerged as critical issues in equine medicine, causing significant morbidity, mortality, and economic loss to the equine industry. In obese humans and rodent models of nutritional obesity, systemic insulin resistance and hyperinsulinemia are followed temporally in a majority of individuals by decreased glucose tolerance, pancreatic β-cell failure, and type II diabetes mellitus. In stark contrast to humans, obese horses and ponies chronically remain in what is termed a “prediabetic” state in human IR, characterized by hyperinsulinemic euglycemia. Few data exist describing the biology of the equine endocrine pancreas in the chronically IR animal that may both: 1) explain this unique equine endocrine physiology and 2) characterize the animal at-risk for hyperinsulinemia-associated laminitis. The purpose of the study reported here was to characterize the morphology and physiology of the equine endocrine pancreas in response to a dietary carbohydrate challenge. Twenty-
two mixed-breed ponies (body weight 266.6 ± 170.5 kg) were conditioned to a diet of chopped hay (NSC ~6% on DM basis) for 4 weeks; following conditioning, ponies either remained on the control diet (n=11), or received the same hay supplemented with sweet feed and oligofructose (total diet ~42% NSC; n=11) for 7 days. Serum insulin concentrations were measured prior to and after completion of the feeding protocol. At the end of the feeding protocol, sections of numerous tissues, including pancreas, were collected immediately following euthanasia. The samples were formalin-fixed for 48 hours, transferred to 70% ethanol, and paraffin-embedded. Immunohistochemistry was performed on pancreas sections using a commercially-available anti-insulin antibody (Abcam), and measurements of islet surface area and β-cell surface area were performed (n = 10 islets per tissue section) using a commercially-available computer software program (Image J). There was a trend for greater total islet surface area in pancreatic tissue from ponies fed the high NSC diet when compared to the ponies on the hay diet (p = 0.068); however, no difference was noted in β-cell surface area between diet treatments (p = 0.12). The change in serum insulin concentration was significantly greater in the high NSC-fed ponies than in controls (403.8 ±/− 317.1 mIU/L vs. 1.00 ±/− 4.03 mIU/L; p = 0.002); however, this variable was not correlated with total islet surface area (r = 0.32; p = 0.17) or β-cell surface area (r= 0.25; p = 0.3). Due to the relatively modest changes in pancreatic islet surface area that accompany marked increases in serum insulin concentrations in ponies fed a high NSC diet, it is important to assess both β-cell function and insulin clearance mechanisms in future studies to delineate the mechanism(s) of hyperinsulinemia in this model.
Introduction

Obesity, insulin resistance, and endocrinopathic laminitis have emerged as critical issues in equine medicine, causing significant morbidity, mortality, and economic loss to the equine industry (American Association of Equine Practitioners 2009; USDA-NAHMS 2000). In obese humans and experimental rodent models of nutritional obesity, systemic insulin resistance and hyperinsulinemia are followed temporally in a majority of individuals by decreased glucose tolerance, pancreatic β-cell failure, and type II diabetes mellitus (Frojdo, Vidal, Pirola 2009). The mechanisms by which this occurs are not completely understood; however, investigations in recent years have revealed that progressive changes in both the function and morphology (particularly increased insulin production/secretion and β-cell hyperplasia) of the endocrine pancreas occur over time in response to conditions known to increase risk for type II diabetes mellitus, such as genetic or nutritionally-induced obesity (Mercado and Castells 2006; Paulsen and others 2010), high-carbohydrate feeding (Alonso and others 2007), and high-fat feeding (Mercado and Castells 2006). Failure of increased pancreatic insulin secretion and β-cell hyperplasia in response to these stimuli is considered a hallmark of type II diabetes mellitus (Heit, Karnik, Kim 2006).

In stark contrast to humans, obese horses and ponies with systemic insulin resistance chronically remain in what is termed a “prediabetic” state in human IR, characterized by hyperinsulinemic euglycemia (Frank and others 2010). While increased pancreatic insulin secretion has been suggested to be responsible for the hyperinsulinemia
resulting from systemic insulin resistance in equids (and evidence exists for increased pancreatic insulin secretion in response to glucose infusion in insulin-resistant equids (Burns and others 2012)), the contribution of altered hepatic insulin clearance to increased basal insulin concentrations in these animals has been poorly characterized to date (Toth and others 2010). While endocrine pancreatic function (and dysfunction) would seem critically important to the pathophysiology of EMS, surprisingly little information exists describing the biology of the equine endocrine pancreas in the chronically IR equid. The purpose of the study reported here was to characterize basic parameters describing morphology and physiology of the equine endocrine pancreas in response to a dietary carbohydrate challenge, generating data that may both: 1) begin to explain the mechanisms underlying the unique endocrine physiology of equine IR that is so different from that reported in humans and 2) further characterize the equid at-risk for hyperinsulinemia-associated laminitis.

**Materials and Methods**

*Animal protocol*

Twenty-two mixed breed ponies (body weight 270.9 +/- 74.4 kg; age [lean] = 9.2 +/- 3.5 years, age [obese] = 11 +/- 3.8 years) were used for this study. All animals received humane treatment in accordance with an animal care and use protocol approved by the Michigan State University Institutional Animal Care and Use Committee. Feedstuffs used in the protocol were analyzed for non-structural carbohydrate content (NSC, defined as the sum of measured starch and water-soluble carbohydrates) by a
commercial laboratory\textsuperscript{26} (Equi-Analytical). All ponies obtained for the study were examined by a licensed veterinarian and deemed healthy based on the results of physical examination, complete blood count, and serum biochemical examination. Ponies were divided into four experimental groups based on body condition scoring results: lean, low NSC (n = 5); obese, low NSC (n = 5); lean, high NSC (n = 6); obese, high NSC (n = 6). All body condition scores were performed independently by two individuals; lean animals were those assigned a body condition score of \( \leq 4/9 \), and obese animals were those assigned a body condition score of \( \geq 7/9 \) (Henneke and others 1983).

All ponies were housed in dirt lots and conditioned to a diet of hay chop (7% starch and ethanol soluble carbohydrate on a dry matter basis) for 4 weeks prior to initiation of the experimental feeding protocol. Ponies were fed 2.5% of their body weight in hay chop per day, divided into two feedings (7 a.m. and 6 p.m. EST). Following the conditioning period, ponies either remained on the control diet (n=10; lean and obese control groups, 5 animals each) or received the same diet supplemented with sweet feed (1.5% body weight per day, fed three times daily at 7 a.m., 12 p.m., and 6 p.m. EST) and oligofructose\textsuperscript{27} (Beneo-ORAFTI; 2 g/kg added to hay chop ration; lean and obese challenge groups, n = 12 [6 animals each]) for a period of 7 days. The mean NSC consumption of ponies in the control groups was approximately 1.8 g/kg/day, while that of ponies in the challenged groups was approximately 8 g/kg/day. All ponies were monitored three times daily during the experimental period and were under the supervision of a licensed veterinarian for the duration of the protocol.
All ponies underwent insulin-modified frequently-sampled intravenous glucose tolerance testing (FSIGTT) with Minimal Model analysis (Toth and others 2009) during the first two weeks of the acclimation period. The FSIGTT’s were performed between 7 a.m. and 9 a.m. EST following a 6-8 hour period of feed withholding. Additionally, blood was collected into red top tubes (Becton Dickinson) for measurement of basal serum insulin concentrations on Day 0 and Day 7 of the feeding protocol (between 7 a.m. and 9 a.m. prior to feeding the morning ration); serum insulin concentrations were measured with a radioimmunoassay (Coat-A-Count™, Siemens; (Freestone and others 1991)). Following the 7 day experimental period, ponies were euthanized via intravenous overdose of pentobarbital sodium and phenytoin sodium (Fatal-Plus™, Vortech; 20 mg/kg IV). Samples of numerous tissues, including pancreas, were collected immediately following euthanasia. Tissue samples were either snap-frozen in liquid nitrogen or formalin-fixed for 48 hours; formalin-fixed tissues were then transferred to 70% ethanol and subsequently paraffin-embedded. All pancreatic samples were processed within 15 minutes of euthanasia.

**Immunohistochemistry**

Immunohistochemistry was performed on pancreatic tissue sections using a commercially-available anti-insulin antibody (Abcam), and measurements of islet surface area and β-cell surface area were performed (n = 10 islets per tissue section) using a commercially-available computer software program (Image J). Formalin-fixed pancreatic tissue samples were embedded in paraffin and sectioned at 5 µm for
immunohistochemistry. Staining of tissue samples was performed as previously reported (Burns and others 2011); briefly, sections were deparaffinized and incubated in a mouse monoclonal anti-insulin primary antibody\(^77\) (Abcam; 1:100, incubated overnight at 4º C). Detection of immunoreactivity was performed using a goat-anti-mouse HRP conjugate secondary antibody (Cell Signaling Technology Inc.), an immunoperoxidase system\(^34\) (Vector Laboratories) and DAB substrate\(^35\) (Vector Laboratories). After immunohistochemical staining, measurements of total islet surface area, β-cell surface area, and number of islets per surface area of pancreas were made using a commercially-available computer software program\(^78\) (Image J), and α-δ cell surface area was calculated (= total islet surface area - β-cell surface area) for each tissue section by a single blinded observer. Ten randomly-selected islets were evaluated per pancreatic tissue section, measurements of which were averaged to create a result for each tissue section.

**Enzyme-linked immunosorbent assay (ELISA)**

Evaluation of pancreatic insulin content was evaluated with a commercially-available ELISA previously validated for use with equine plasma, serum, and cell culture supernatants\(^82\) (Mercodia). Briefly, pancreatic protein lysates (~100 mg pancreatic tissue per sample) were prepared in 300 µL lysis buffer\(^39\) (Pierce) and quantitated via the Bradford method. Protein lysates were diluted 1:4000-1:8000 in order to generate results lying on the linear portion of the standard curve associated with the assay; 25 µl of lysate was loaded for each sample. Negative control samples (mammalian protein extraction
reagent [MPER] and digital laminar protein lysate from one pony of this cohort; 24 μl of sample, respectively) and positive control samples (serum from a lean, insulin-sensitive and an obese, insulin-resistant pony; 25 μl of sample, respectively) were included in-run with the pancreatic lysate samples. Further, ELISA results for the included serum samples were compared with previously-obtained insulin concentrations for the same samples measured by radio-immunoassay (RIA) as external calibrators. All data obtained were corrected for the protein concentration of the lysate.

Data analysis

Data analysis was performed using a statistical software program (GraphPad Prism). All data were assessed for normality by the Shapiro-Wilk and D’Agostino and Pearson omnibus normality tests. Basal serum insulin concentration, insulin sensitivity, and acute insulin response to glucose data were analyzed with a Student’s t test (or non-parametric equivalent as appropriate). Pancreatic histomorphometry and insulin content data were analyzed with a two-way analysis of variance followed by a Bonferroni post-test, as appropriate. Pooled histomorphometry and pancreatic insulin content data comparing control-fed and high-carbohydrate fed animals (irrespective of body condition score) were analyzed with a Student’s t-test. Statistical significance was accepted at p < 0.05. Data are expressed as mean +/- standard deviation unless otherwise indicated.
Results

Immunohistochemical staining readily identified β-cells in equine endocrine pancreas (Figure 18). There was no significant effect of body condition score (p = 0.5) or high-carbohydrate feeding (p = 0.15) on pancreatic insulin content measured by ELISA. There was a trend for greater total islet surface area in pancreatic tissue from ponies fed the high NSC diet when compared to the ponies on the hay diet (p = 0.07); however, no difference was noted in β-cell surface area between diet treatments (p = 0.13; Figure 19). The change in serum insulin concentration was significantly greater in the high NSC-fed ponies than in controls (403.8 +/- 317.1 mIU/L vs. 1.00 +/- 4.03 mIU/L; p = 0.002); however, this variable was not correlated with total islet surface area (r = 0.32; p = 0.17) or β-cell surface area (r= 0.25; p = 0.3). There was no significant correlation detected between total islet surface area and pancreatic insulin content, β-cell surface area and pancreatic insulin content, or β-cell surface area and insulin sensitivity (all, p > 0.05). There was no observed effect of body condition score on pancreatic histomorphometry.
Figure 18. Section of equine pancreas immunohistochemically stained for insulin. In all pancreatic sections evaluated, β-cells (stained for insulin) formed a peripheral rim around the more central α-δ cells. The section depicted above is from an obese pony fed the high-carbohydrate challenge diet; ponies fed this diet tended to have greater total islet surface area than control-fed ponies, but the β-cell surface area in pancreatic sections was not different.
Figure 19. Results of measurements of pancreatic β-cell surface area and total islet surface area in sections of pancreas from ponies fed a control diet or a high-carbohydrate challenge diet for 7 days. While ponies fed the high-carbohydrate diet tended to have larger islet surface area (panel on right), no significant effect of diet on β-cell surface area was observed. CON, control; CHAL, high-carbohydrate diet.
Discussion

While alterations in pancreatic islet morphology are well-characterized in the states of obesity and systemic insulin resistance in other species (Heit, Karnik, Kim 2006), particularly in response to high-glucose diets or infusions (Alonso and others 2007), the results of the study reported here do not support the hypothesis that these changes occur in the obese, insulin resistant equid. Pancreatic β-cell hyperplasia has been reported to occur in the setting of the obese, chronically insulin resistant human and rodent prior to the onset of secretory failure and type II diabetes mellitus (Augstein and Salzsieder 2009; Meier and others 2008; Paulsen and others 2010). However, this morphological change in the pancreatic islets has also been reported to occur in response to parenteral glucose infusions over the course of as little as 6 days (Alonso and others 2007). Since both lean and obese ponies fed a high-carbohydrate diet for 7 days and chronically overweight/obese ponies who were also systemically insulin resistant were assessed in this study (representative of acute and chronic stimulus for β-cell hyperplasia, respectively), effects on pancreatic morphology were anticipated. No association was found between body condition score or systemic insulin sensitivity and pancreatic histomorphometry in the ponies of this study; similarly, there was no association between pancreatic insulin content measured by ELISA and pancreatic histomorphometry, body condition score, or systemic insulin sensitivity. The results presented here do not support morphologic changes in the equine endocrine pancreas following short-term high-carbohydrate feeding.
Due to the relatively modest changes in pancreatic islet surface area that accompany marked increases in serum insulin concentrations in ponies fed a high NSC diet, it is important to assess both β-cell function and insulin clearance mechanisms in future studies to delineate the mechanism(s) of hyperinsulinemia in this model. Systemic insulin resistance and subsequent basal and post-prandial hyperinsulinemia have been increasingly well-characterized in equids over the past decade (Firshman and Valberg 2007; Pratt, Geor, McCutcheon 2005; Pratt, Geor, McCutcheon 2006). Hyperinsulinemia in this setting has been broadly assumed to be the result of pancreatic hypersecretion in response to hyperglycemia (Carter and others 2009c; Hoffman and others 2003; Treiber and others 2005); however, this has not been quantified well to date. Plasma insulin concentration is regulated not only by the rate of pancreatic insulin secretion (which is initially increased in the setting of systemic insulin resistance secondary to β-cell hyperplasia), but also by the rate of hepatic insulin clearance. Attempts have been made to clarify the role of hepatic insulin clearance in the setting of hyperinsulinemia in horses via assessment of plasma C-peptide concentrations following a frequently-sampled intravenous insulin-modified glucose tolerance test (Toth and others 2010). However, while the results suggest that insulin clearance decreases as pancreatic secretion of insulin increases in response to dextrose infusion (supporting a role for altered clearance in hyperinsulinemia), this study was performed on a small number of horses, and the conclusions drawn should therefore be considered preliminary. Further study, including a more specific assessment of pancreatic insulin secretion (possibly through sampling of portal venous blood following administration of an insulin secretagogue), needs to be
performed to further clarify this issue, which is important as it speaks directly to the pathophysiology of EMS in horses and ponies. Further, understanding the physiology of the equine endocrine pancreas in the setting of chronic systemic insulin resistance may shed light on why it is that IR in horses (analogous to ‘pre-diabetes’ in humans) uncommonly progress to overt type II diabetes mellitus. Comparative, translational research on this subject may ultimately yield benefits for both veterinary and human medicine.
Chapter 8: Discussion and conclusions

Equine metabolic syndrome is increasingly well-characterized in the equine veterinary medical literature, and it has become established as a common clinical syndrome encountered by equine veterinarians in clinical practice. While the veterinary community now has clinical familiarity with the syndrome, the pathophysiology of both systemic insulin resistance in horses and the most significant complication associated with EMS, laminitis (EMSAL), remain elusive. The experiments described in this dissertation were undertaken in an attempt to further clarify the roles of inflammation in both the digital laminae and in primary insulin-responsive tissues in equine insulin resistance (particularly the adipose tissue); to describe insulin signaling within the digital laminae, with a view toward establishing a mechanistic basis for laminar injury that occurs in the setting of hyperinsulinemia; to describe tissue energy regulation in the digital laminae and insulin-responsive tissues in equids at-risk for EMSAL; and to evaluate the morphology of the endocrine pancreas of equids at risk for EMSAL. Taken collectively, this work suggests that while equine IR and EMS display many similarities to human metabolic syndrome, both tissue-level and systemic physiologic differences exist; these differences identify the need for research efforts directed specifically at the EMS-affected equid, as extrapolation from the human and experimental rodent literature describing metabolic syndrome may be misleading.
White adipose tissue is now held to be not only a storage depot for excessive dietary energy, but broadly endocrinologically active in many species. In the setting of nutritional obesity in humans and rodents, white adipose tissue becomes progressively more insulin resistant, accumulates infiltrative macrophages, and expresses and secretes increasing amounts of many pro-inflammatory cytokine and chemokine molecules; these changes correlate well with the degree of obesity and systemic insulin resistance of the subject. Additionally, pronounced regional differences in adipose tissue physiology have been documented, with omental/truncal adipose tissue displaying a more inflammatory expression profile and greater tissue insulin resistance. While regional adiposity (accumulation in the nuchal ligament) has long been noted anecdotally by equine veterinarians, and nuchal adiposity has recently been correlated with insulin resistance and laminitis risk, the biological behavior of various adipose tissue depots of equids has not been investigated with rigor. The study reported in Chapter 2, comparing expression of pro-inflammatory cytokine and chemokine gene expression in several adipose tissue depots between insulin sensitive and insulin resistant light breed horses, suggests that omental adipose tissue is not as metabolically important (or inflammatory) in this species as it is in humans. However, the difference in the number of perivascular leukocytes observed in omental adipose tissue compared with other depots in the study described in Chapter 3 may suggest a role for this depot’s unique access to nutrients from the gastrointestinal tract in its physiology. Nuchal ligament adipose tissue (which showed the highest expression of pro-inflammatory cytokines) may be the correlate ‘high-risk’ depot in equids, information which will be useful moving forward with future
adipobiology studies in horses, as this depot is technically easier and less invasive to sample than visceral depots. Further, while no effect of systemic insulin sensitivity status was observed regarding inflammatory gene expression in any adipose tissue depot, it may be that the degree of adiposity (and possibly regional adiposity) in general is more predictive of an inflammatory adipose tissue phenotype than systemic insulin sensitivity status alone.

Hyperinsulinemia, previously known to be a predictive risk factor for endocrinopathic laminitis, has recently been shown to be involved in the pathophysiology of the disease with recent publications documenting laminitis in response to 72 hours of experimental hyperinsulinemia (Asplin and others 2007; de Laat and others 2010). The mechanisms by which insulin might mediate laminar injury are unclear but have been suggested to include local energy/glucose deprivation due to laminar insulin resistance (French and Pollitt 2004a), altered mitogenesis of keratinocytes due to insulin signaling through either/both insulin receptor and insulin-like growth factor-1 receptor (IGF-1R) (Bailey 2009; de Laat and others 2013), and glycotoxicity (de Laat and others 2012). The study described in Chapter 4 of this dissertation describes the immunolocalization of insulin receptor and IGF-1R in the digital laminae of ponies and the effects of high-carbohydrate feeding on that distribution. The results suggest that the effects of insulin on laminar keratinocytes are either 1) indirect, with direct effects on another cell type altering keratinocyte function in a paracrine or endocrine mechanism or 2) resulting from signaling through receptors other than insulin receptor, as no evidence of insulin receptor expression on laminar keratinocytes was observed. Insulin has been shown to activate
signaling through IGF-1R at supraphysiologic concentrations that are attainable in the IR equid (Varewijck and Janssen 2012); since the laminar distribution of IGF-1R is more diffuse than that of insulin receptor (including keratinocytes, vascular elements, and dermal interstitial mesenchymal cells), it is possible that local activation of laminar IGF-1R by insulin in the digit may contribute to the pathophysiology of EMSAL.

While the expression of insulin receptor was noted to be increased in the laminar dermal microvasculature following high-carbohydrate feeding, the vessels involved were not considered likely to have significant effects on vasomotor tone within the laminae (their morphology was consistent with venules or capillaries). Since insulin is known to have vasoactive properties in other species, and insulin resistance in other species is often accompanied by pathologic systemic and local hypertension, it has been suggested that local digital ischemia may play a role in EMSAL when animals are exposed to dietary risk that precipitates hyperinsulinemia. Based on the results of the study reported here, this mechanism appears to be unlikely. Further evaluation of vasomotor tone to the digit in vivo in response to hyperinsulinemia induced by diet should be pursued to further clarify this mechanism.

Laminitis in equids can occur as a complication of multiple diverse types of disease, including sepsis-associated laminitis, support limb laminitis, and EMSAL. Sepsis-associated disease has been modeled well experimentally through black walnut extract induced laminitis and enteral carbohydrate or oligofructose overload laminitis; laminitis in both of these settings has been shown to be highly inflammatory, with
profound increases in elaboration of pro-inflammatory cytokines and chemokines and local leukocyte infiltration during the developmental stage and at the onset of clinical lameness. While EMSAL has been suggested to be similarly inflammatory, the same types of rigorous analyses have not been performed with laminar tissue from EMSAL-affected equids as have been done for sepsis-associated laminitis. The study reported in Chapter 5 was undertaken to characterize the degree of laminar inflammation present in ponies exposed to high-carbohydrate feeding; while gene expression of cyclooxygenase-2 was increased in the laminae of high-carbohydrate-fed ponies, no other evidence of laminar inflammation was detected in this study (including cytokines, chemokines, and infiltrative leukocytes). The degree of inflammation observed in EMSAL appears to be dramatically different to that observed in experimental sepsis-associated laminitis; consequently, the pathophysiologic mechanisms important to these forms of the disease should be assumed to be different. Interestingly, even while fulminant local inflammation does not appear to be responsible for laminar damage in EMSAL, treatment of affected horses with an anti-inflammatory drug (COX-2 inhibitor) may ultimately prove useful in the management of this form of the disease, and not merely for its analgesic properties. COX-2 has been identified in the literature (developmental biology and oncology) as an important mediator of the epithelial-to-mesenchymal transition, during which epithelial cells de-differentiate and dysadhere from neighboring cells. In the setting of neoplastic disease, EMT is thought to be predictive of a more invasive phenotype of carcinoma cells, with earlier and more aggressive distant metastasis in vivo. Within the digital laminae, induction of an EMT within the laminar basal epithelial cell
would be anticipated to have catastrophic consequences for the architecture of the tissue, as dysadhesion of the LBEC’s both from each other and from their basement membrane would be followed quickly by displacement of the digit within the hoof capsule under the weight of the animal. The possibility of laminar EMT in EMSAL should be investigated further, as therapeutic strategies currently exist to alter this phenotype of epithelial cells (including COX inhibition and AMPK agonism) and are in clinical use in human medicine.

Energy dysregulation has been assumed to in some way contribute to the pathogenesis of EMSAL, as EMS itself may be taken to be a systemic manifestation of disordered energy metabolism. Tissue insulin resistance and vasoconstriction have been previously thought to cause local energy deprivation; however, recent evidence suggesting the insulin-independence of the digital laminar keratinocytes has brought this hypothesis into question. The study reported in Chapter 6 was designed to investigate the energy status of the digital laminae and primary insulin responsive tissues (liver and skeletal muscle) in ponies exposed to a dietary carbohydrate challenge, with activation of AMPK used as a marker of energy deprivation (a large body of comparative literature would support the use of this highly conserved regulator of cellular energy metabolism in this way). The findings of this study suggest that the digital laminae are neither insulin-resistant nor energy deprived in equids fed a high-carbohydrate diet (a diet considered to carry increased risk of EMSAL), as decreased phosphorylation of AMPK was observed under these circumstances. Decreased AMPK activation also fails to support hypoxia as an important mechanism in EMSAL, as one would expect increased AMPK
phosphorylation in hypoxic conditions. AMPK is also known to play an important role in mitigating EMT (mentioned previously under the discussion of Chapter 5); AMPK activation enhances intercellular adhesions, promotes differentiation of epithelial cells, and renders a more stable epithelial architecture, all of which would be anticipated to be beneficial if EMT plays a role in the pathophysiology of EMSAL. Again, AMPK agonists are commercially available, and use of these agents to treat EMSAL-affected equids may be shown useful following additional studies.

In conclusion, while the findings of the work described in this dissertation differed (occasionally dramatically) from what was anticipated based on review of the human and experimental rodent literature on metabolic syndrome, the information gleaned will be useful in clarifying the pathophysiology of the equine metabolic syndrome and its frequent sequel, laminitis.
References


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Komohara Y, Hirahara J, Horikawa T, Kawamura K, Kiyota E, Sakashita N, Araki N, Takeya M. 2006. AM-3K, an anti-macrophage antibody, recognizes CD163, a


1 Abbocath® 14-gauge; Abbott Medical Health, Abbott Park, IL
2 Dextrose 50%; Vedco, St. Joseph MO
3 BD Vacutainer K$_2$EDTA tubes; BD (Becton Dickinson), Franklin Lakes NJ
4 Humulin R®; Eli Lilly and Company, Indianapolis IN
5 Coat-A-Count® insulin RIA, Siemens Medical Solutions Diagnostics, Los Angeles CA
6 Thermo-Trace Ltd, Melbourne, Australia
7 Angiocath™, BD, Franklin Lakes NJ
8 Tetanus toxoid; Fort Dodge Animal Health/Wyeth, Madison NJ
9 Sulfamethoxazole-trimethoprim 960-mg tablets; Mutual Pharmaceutical Co. Inc., Philadelphia PA
10 Phenylzone Paste®; Schering Plough Animal Health, Union NJ
11 Xylazine HCl, 100 mg/mL; IVX Animal Health, Inc., St. Joseph MO
12 Ketaset®; Fort Dodge Animal Health/Wyeth, Madison NJ
13 Diazepam Inj, 5 mg/ml; Abbott Laboratories, Abbott Park IL
14 Guifenesin 5%; Butler Animal Health Supply, Dublin OH
15 Chlorhexidine 2% surgical scrub; First Priority, Inc., Elgin IL
16 Isopropyl alcohol 70%; Butler Animal Health Supply, Dublin OH
17 PDS®, Ethicon Inc., Somerville NJ
18 Vicryl®, Ethicon Inc., Somerville NJ
19 Tissue-Ruptor®; QIAGEN, Valencia CA
20 DNAse I; Thermo Fisher Scientific Inc., Rockford IL
21 Retroscript®; Ambion, Inc, Austin TX
22 Roche 480®; Roche Applied Science, Indianapolis IN
23 TOPO TA Cloning Kit; Invitrogen, Carlsbad CA
24 geNorm®; Ghent University, Ghent, Belgium
25 GraphPad Prism 5®; GraphPad Software, La Jolla CA
26 Abbocath® 14-gauge; Abbott Medical Health, Abbott Park, IL
27 Dextrose 50%; Vedco, St. Joseph MO
28 BD Vacutainer K$_2$EDTA tubes; BD (Becton Dickinson), Franklin Lakes NJ
29 Humulin R®; Eli Lily and Company, Indianapolis IN
30 Coat-A-Count® insulin RIA; Siemens Medical Solutions Diagnostics, Los Angeles CA
31 Thermo-Trace Ltd, Melbourne, Australia
32 Angiocath™, BD, Franklin Lakes NJ
33 Tetanus toxoid; Fort Dodge Animal Health/Wyeth, Madison NJ
34 Sulfamethoxazole-trimethoprim 960-mg tablets; Mutual Pharmaceutical Co. Inc., Philadelphia PA
35 Phenylzone Paste®; Schering Plough Animal Health, Union NJ
36 Xylazine HCl, 100 mg/mL; IVX Animal Health, Inc., St. Joseph MO
37 Ketaset®; Fort Dodge Animal Health/Wyeth, Madison NJ
38 Diazepam Inj, 5 mg/ml; Abbott Laboratories, Abbott Park IL
39 Guifenesin 5%; Butler Animal Health Supply, Dublin OH
40 Chlorhexidine 2% surgical scrub; First Priority, Inc., Elgin IL
41 Isopropyl alcohol 70%; Butler Animal Health Supply, Dublin OH
42 PDS®, Ethicon Inc., Somerville NJ
43 Vicryl®, Ethicon Inc., Somerville NJ
44 Tissue-Ruptor®; QIAGEN, Valencia CA
45 DNAse I; Thermo Fisher Scientific Inc., Rockford IL
46 Retroscript®; Ambion, Inc, Austin TX
47 Roche 480®; Roche Applied Science, Indianapolis IN
48 TOPO TA Cloning Kit; Invitrogen, Carlsbad CA
49 Clone AM3K, Cosmo Bio Co., Ltd, Tokyo, Japan
50 Vectastain® ABC system, Vector Laboratories, Burlingame CA
51 DAB peroxidase substrate kit, Vector Laboratories, Burlingame CA
52 geNorm©; Ghent University, Ghent, Belgium
53 GraphPad Prism 5®; GraphPad Software, La Jolla CA
54 Equi-Analytical Laboratories, Ithaca NY
55 BENEObraf™, Tienen, Belgium
56 BD VacutainerTM red top blood collection tubes; BD (Becton Dickinson), Franklin Lakes NJ
57 Coat-A-Count® insulin RIA, Siemens Medical Solutions Diagnostics, Los Angeles CA
58 Fatal Plus®, Vortech Pharmaceuticals Ltd., Dearborn MI
59 ab54268, Abcam®, Cambridge MA
60 ab69508, Abcam®, Cambridge MA
61 sc713, Santa Cruz Biotechnology, Inc., Dallas TX
62 Vectastain® ABC system, Vector Laboratories, Burlingame CA
63 DAB peroxidase substrate kit, Vector Laboratories, Burlingame CA
64 A0082, Dako North America, Inc., Carpinteria CA
65 Clone AM3K, Cosmo Bio Co., Ltd, Tokyo, Japan
66 ab22506, Abcam®, Cambridge MA
67 M-PER, Thermo Fisher Scientific, Inc., Rockford IL
68 Vectastain® ABC system, Vector Laboratories, Burlingame CA
69 SuperSignal® West Femto, Thermo Fisher Scientific, Inc., Rockford IL
70 sc1616, Santa Cruz Biotechnology, Inc., Dallas TX
71 sc2961, Santa Cruz Biotechnology, Inc., Dallas TX
72 Carestream Health, Inc., Rochester NY
73 cs4150, Cell Signaling Technology Inc., Danvers MA
74 cs7074, Cell Signaling Technology Inc., Danvers MA
75 cs2535, Cell Signaling Technology Inc., Danvers MA
76 cs5831, Cell Signaling Technology Inc., Danvers MA
77 Absolutely RNA Miniprep kit, Agilent Technologies, Stratagene Products Division, La Jolla CA
mRNA extraction kit, Roche Applied Science, Indianapolis IN

NanoDrop Spectrophotometer, Thermo Scientific, Wilmington DE

ab6995, Abcam®, Cambridge MA


Mercodia Equine Insulin ELISA, Mercodia AB, Uppsala Sweden