MECHANISMS OF EQUINE INSULIN RESISTANCE

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Abstract:

Equine metabolic syndrome, a condition characterized by obesity, insulin resistance (IR) or hyperinsulinemia, and laminitis is a common endocrine disorder of horses. The mechanisms responsible for the development of equine obesity-associated IR and hyperinsulinemia remain to be elucidated. Understanding of the pathophysiology of equine IR is critical for development of effective treatments. The purpose of this study was to improve understanding of mechanisms of equine IR.

Based on existing literature in other species, we initially hypothesized that oxidative stress due to mitochondrial dysfunction within skeletal muscle of obese horses causes IR. To address this, markers of oxidative stress, mitochondrial function and antioxidant capacity were evaluated in skeletal muscle of horses with and without IR. No markers of impaired mitochondrial function or oxidative damage were associated with hyperinsulinemia. Regulation of mitochondrial dynamics was altered with IR and hyperinsulinemia.

As oxidative stress did not appear to be the primary mechanism of equine IR, we hypothesized that skeletal muscle inflammation causes equine IR. Markers of inflammation were measured within skeletal muscle and systemic circulation. No positive associations between inflammation and IR or hyperinsulinemia were identified.

Because investigations into two key mechanisms of skeletal muscle insulin resistance did not reveal a likely pathogenesis of IR, investigations were redirected to a more global approach of evaluating dynamic testing techniques to assess sites of insulin dysregulation. We hypothesized that two commonly employed field tests, the oral sugar test (OST) and insulin response to dexamethasone test (IRDT) were comparable to the gold standard test of tissue insulin sensitivity, the hyperinsulinemic euglycemic clamp (HEC). The HEC was not correlated with fasting insulin concentration or results of the OST or IRDT, suggesting that tissue insulin sensitivity is not the primary determinant of field test results, and may not be the primary defect in insulin dysregulation of obese horses.

In summary, the mechanism of equine IR and hyperinsulinemia remains to be discovered. Additional investigations of dynamic testing in a population of hyperinsulinemic and normoinsulinemic horses may improve understanding of etiologies of insulin dysregulation in the horse and allow for further investigation into the pathophysiology of equine obesity-associated IR and hyperinsulinemia.

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LITERATURE REVIEW

Obesity is an increasingly common condition of people and companion animals, including horses.[1-4] In people, obesity is associated increased risk for cardiovascular disease,[5] type II diabetes,[6] polycystic ovary syndrome,[7] and osteoarthritis.[8] In horses, obesity is associated with infertility, poor thermoregulation, exercise intolerance, insulin resistance, and laminitis.[9] Equine metabolic syndrome (EMS) is a term coined in 2002[10] to describe an emerging clinical syndrome in middle-aged horses consisting of obesity (generalized or regional), insulin resistance or hyperinsulinemia, and laminitis. Clinical recognition of obesity and EMS continues to grow; however, the pathophysiological mechanisms leading to the development of obesity-associated IR in horses have not been elucidated. Appropriate interpretation of diagnostic test results and identification of disease pathophysiology are critical for the development of effective preventative measures and treatments.

Insulin mediated regulation of glucose homeostasis and insulin resistance

Glucose is an important energy source for mammalian cells, and glucose homeostasis is vital to survival. Blood glucose is derived from three sources: enteral absorption, gluconeogenesis and glycogenolysis. Normally, post-prandial blood glucose concentration is primarily regulated by pancreatic insulin secretion and insulin-mediated glucose uptake by insulin-sensitive tissues, with a lesser influence by insulin insensitive tissues.[11] Insulin is a peptide hormone secreted by the pancreatic β cells within the islets of Langerhans in response to hyperglycemia. Insulin is synthesized in the endoplasmic reticulum as pre-pro insulin, consisting of an A chain, a B chain, and a connecting peptide (C-peptide).[12] Pre-pro insulin is cleaved to pro-insulin, which is transported to the Golgi apparatus where pro-insulin hexamers are formed. C-peptide is removed by enzymes during secretion of the pro-insulin vesicle from the Golgi,[13] resulting in the formation of insulin. Insulin and C-peptide are co-secreted into circulation. Insulin binds to its receptor in insulin-sensitive tissues, allowing for activation of the insulin signaling cascade and glucose uptake by target tissues. Insulin-mediated glucose disposal primarily occurs in skeletal muscle, adipose, and liver, with skeletal muscle being the largest insulin sensitive tissue depot. Insulin resistance (IR) is defined as a decreased ability of insulin sensitive tissues to respond to insulin.[14] In people, whole-body IR is generally a reflection of skeletal muscle IR, as skeletal muscle is responsible for approximately 85% of glucose disposal in a euglycemic, hyperinsulinemic state.[15]

In obese people, IR is generally considered to be an early finding of alterations in glucose homeostasis.[16] Increased pancreatic beta cell secretion of insulin compensates for impaired tissue sensitivity, resulting in high circulating concentrations of insulin (hyperinsulinemia). Over time, the pancreas loses its ability to compensate and will secrete insufficient amounts of insulin in response to hyperglycemia, resulting in a hyperglycemic, hypoinsulinemic state (type II diabetes mellitus). The progression to type II diabetes in people is common. In contrast, horses are rarely reported to develop type II diabetes.[17]

Role of obesity in IR

In people, generalized obesity associated with the development of IR.[18] Similarly, equine obesity is negatively correlated with IS[19] and associated with an increased risk of hyperinsulinemia.[20] Identification of obesity may therefore help in identification of risk for IR. In horses, a subjective scoring system exists for measurement of body fat.[21] However, due to the inherent inter-observer variation in scoring, an accurate, objective method of assessing body fat is desirable. The body mass index (BMI) is a measurement system devised in order to improve objective evaluation of generalized adiposity.[22] Equine BMI is calculated using the following formula:[22]

$$\text{BMI} = \text{Body weight (kg)}/ \text{height at withers (m)}^2$$

In a study comprised mostly of Standardbred horses, BMI was found to correlate well with body condition score (BCS);[22] however, a subsequent study found that BMI had a weaker association with BCS in a population of Thoroughbreds and ponies.[23] These findings suggest that breed influences the relationship between BMI and BCS. In addition to BMI, other morphometric measurements have been employed to identify generalized or regional obesity, including girth, abdominal girth, and length.[23,24] In a research setting, deuterium oxide has been used to evaluate total body fat. Deuterium oxide was found to correlate well with body condition score in lean or overweight animals (BCS $\leq$7), although predictive value was lost at higher body condition scores.[25]

In addition to generalized obesity, regional adiposity appears be a characteristic of IR in people. Location of fat accumulation also appears to be important. Several lines of evidence suggest the presence of visceral adipose is a stronger predictor of IR than subcutaneous adipose in people, although this remains under debate.[26] Similarly, horses with EMS often develop regional fat stores. Recognized adipose depot locations include the neck, withers, rump, and epaxial musculature. Ultrasound has been used to quantify adipose deposits along the ribs, rump, and ventral midline.[27] Excessive deposition of fat along the nuchal ligament is recognized as a "cresty neck." This fat depot

has been subjectively assessed via a cresty neck score (CNS)[23] or objectively assessed via measurement of neck circumference.[28] Both cresty neck score[23] and neck circumference[28] are correlated with insulin sensitivity.

Complications of IR: Focus on laminitis

Equine insulin resistance or impaired glucose tolerance are associated with several endocrine conditions in horses, including osteochondritis dissecans,[29] equine metabolic syndrome (EMS) and pituitary pars intermedia dysfunction (PPID).[30] Both PPID and EMS are associated with laminitis, a painful, performance limiting and life-threatening disease of the equine foot. In both PPID and EMS, insulin concentrations correlate with laminitis severity.[31] Karikoski et al. reported endocrine disorders as the most common underlying disease in horses presenting to a veterinary hospital for laminitis.[32] In that study, 90% of laminitis cases were attributed to endocrine disease and 2/3 of the horses were hyperinsulinemic.[32] Hyperinsulinemia has independently been demonstrated to induce laminitis in both horses[33] and ponies,[34] and IR is associated with the development of laminitis.[35]

Laminitis may be broadly divided into two general categories: endocrinopathic and inflammatory. Both types of laminitis appear to be associated with separation of the basement membrane at the dermal-epidermal junction.[36] However, mechanisms that cause lamellar breakdown vary between inflammatory models and endocrine models. There are many natural causes of inflammatory-mediated laminitis, including strangulating intestinal lesions, inflammatory gastrointestinal disease (e.g., enteritis or colitis), metritis, and pleuropneumonia.[37] Inflammatory models developed to mimic these conditions include carbohydrate overload and black walnut extract-induced laminitis. Inflammatory laminitis is characterized by perivascular leukocyte infiltration, upregulation of pro-inflammatory cytokine expression, and apoptosis with a subsequent increase in basement membrane degradation by matrix metalloproteinases (as reviewed in Katz and Bailey).[36]

The exact mechanism underlying endocrinopathic laminitis has yet to be determined,[36] but does not appear to involve an initiating inflammatory stimulus. Proposed mechanisms include alterations in glucose metabolism,[38,39] vascular tone,[40-42] or activation of insulin-like growth factor signaling pathways.[43,44]

Equine metabolic syndrome: General overview

Equine metabolic syndrome (EMS) was a term created in 2002 to describe obesity-associated laminitis in horses,[10] and was further characterized in 2010 in a consensus statement from the American College of Veterinary Internal Medicine.[45] The term was chosen due to similarities between EMS and human metabolic syndrome. Human metabolic syndrome is a cluster of risk factors for cardiovascular disease, including central obesity and two of the following four characteristics: fasting hyperglycemia, decreased high density lipoprotein cholesterol, hypertension, and hypertriglyceridema.[46]

Equine metabolic syndrome is defined by the presence of obesity, regional adiposity, insulin resistance or hyperinsulinemia, and laminitis.[45] Additional characteristics which have been reported include dyslipidemia,[28,35,47] hyperleptinemia,[28,47] seasonal (summer) hypertension,[48] and changes in estrous cyclicity in mares.[10] Owners frequently report these horses to be "easy keepers" (i.e., horses are able to maintain stable weight despite restricted intake).[10] Horses with EMS tend to be middle aged (5-18 years old) horses.[10,45] A breed predilection appears to exist with ponies, Morgans, Paso Finos, Peruvian Pasos, Arabians, Warmbloods, Saddlebreds, and Spanish Mustangs more likely to be affected.[10,45,49]

Management of EMS consists primarily of lifestyle changes, including dietary restriction and exercise. Dietary management should consist of restricted pasture access and elimination of grain from the diet. Hay intake should be limited to 1-1.5% of body weight.[45,49] Analysis of hay should be undertaken to ensure a low (<10%) non-structural carbohydrate (NSC) content.[49] Vitamin and mineral

supplementation may be needed in cases of poor quality forage.[45] Exercise may also be helpful for weight loss in horses that are not laminitic. Moderate exercise performed four days per week facilitates loss of fat mass but does not ameliorate insulin resistance in overweight to obese horses.[50] Some equids appear resistant to dietary intervention;[51] in these cases, pharmacologic treatment is warranted. Available therapies that have been evaluated in the horse include levothyroxine, metformin, and pioglitazone. Levothyroxine is a thyroxine analogue that has been demonstrated to help promote weight loss and insulin sensitivity in horses,[52] and appears to exert no clinically significant adverse effects during short term (≤48 weeks) administration.[53] At this time, levothyroxine is recommended for short term use only as the possibility of adverse effects with chronic administration has not been evaluated. Metformin is an adenosine monophosphate-dependent protein kinase (AMPK) agonist[54] that has been used in people to improve glycemic status.[55] In horses, metformin was initially demonstrated to improve insulin sensitivity.[56] Subsequent studies demonstrated limited oral bioavailability of metformin in ponies and horses.[57,58] Pioglitazone, a peroxisome-proliferator activated receptor γ (PPARγ) agonist, has been demonstrated to promote insulin sensitivity in obese people.[55] PPARγ is a regulator of adipogenesis and lipid and glucose metabolism.[59] Administration to non-obese horses for twelve days did not improve insulin sensitivity indices,[60] but this may be due in part to poor bioavailability in horses.[61] Glyburide and glibenclamide are sulfonylureas that have been used as part of a combination therapy to treat equine type II diabetes.[17,62] Sulfonylureas enhance secretion of insulin from functional pancreatic beta cells.[63] Additional research is needed to identify effective treatments for equine IR and obesity.

Diagnosis of IR: General overview

Insulin resistance may occur due to increased insulin degradation or neutralization, decreased binding of insulin to its receptor, or impaired downstream signaling.[64] Insulin resistance may be diagnosed by the presence of fasting hyperinsulinemia or various insulin and glucose proxies.[65] Although fasting measurements of glucose and insulin may allow for diagnosis of insulin

dysregulation, dynamic tests allow for evaluation of responses to a glucose and/or insulin challenge, which may allow for detection of abnormalities not identifiable with fasting measurements.[14] Multiple dynamic tests have been developed for evaluation of insulin sensitivity or oral glucose tolerance in people, and subsequently adapted for use in the horse. Interpretation of test results requires a working knowledge of the relative contributions of tissue insulin sensitivity, enteral glucose absorption, and insulin secretion. During an oral glucose challenge, incretin hormones may substantially alter insulin secretion. Finally, glucose tolerance may be influenced by non-insulin dependent glucose disposal.

In people, assessment of response of pancreatic beta cells to glucose or sensitivity of tissues to insulin is best achieved by use of clamps.[66,67] There are two types of glucose clamps, the hyperglycemic clamp and the hyperinsulinemic euglycemic clamp.[67,68] In the hyperglycemic clamp, an IV infusion of glucose is administered to allow for establishment of a fixed hyperglycemia. In people, this hyperglycemic state suppresses hepatic gluconeogenesis and allows for assessment of sensitivity of pancreatic beta cells to glucose.[67] The hyperinsulinemic euglycemic clamp (HEC) involves IV infusion of a supraphysiologic dose of insulin (3-6 mU/kg/min) and concurrent administration of glucose in order to maintain blood glucose within physiologic range.[68-70] The maintenance of hyperinsulinemic euglycemia suppresses pancreatic insulin secretion, and thus allows for accurate assessment of tissue insulin sensitivity. The HEC remains the gold standard for diagnosis of tissue insulin sensitivity.[14,67] In horses, the hyperinsulinemic euglycemic clamp has been used to investigate insulin sensitivity in polysaccharide storage myopathy and the effect of exercise training on insulin sensitivity.[69,70] Differences in insulin sensitivity have been identified between ponies and Warmbloods[68] and Quarter Horses and Belgian horses.[69,70]

The frequently sampled IV glucose tolerance test (FSIGTT) was initially developed as a technically more simple method to assess insulin sensitivity compared with the clamp,[71] and was considered to be an improvement over the simple IV glucose tolerance test that had previously been

adapted for use in the horse.[72] The IVGTT involves measurement of fasting blood glucose concentration, followed by measurement of glucose and insulin at 15, 30, 60, and 90 minutes and every hour for 5-6 hours thereafter.[73] The FSIGTT requires more intensive sampling, but allows for improved separation of glucose and insulin kinetics, compared with the simple IVGTT.[74,75] The FSIGTT involves administration of 300 mg/kg of glucose and blood sampling at time 0 (baseline), 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 30, 40, 50, 60, 70, 80, 90, 100, 120, 150, and 180 minutes. Glucose concentration is evaluated at all time points, while insulin concentration is evaluated at 0, 2, 4, 6, 8, 10, 14, 18, 20, 30, 40, 60, 90, 120, 150, and 180 minutes.[71] The insulin modified FSIGTT was developed in order to provide a more accurate assessment of tissue insulin sensitivity through administration of exogenous insulin.[76] This involves IV administration of 300 mg/kg of glucose in the form of dextrose, followed (20 minutes later) by administration of insulin (30 mIU/kg). In this test, blood samples are collected at 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 19, 22, 23, 24, 25, 26, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150, and 180 minutes for measurement of plasma glucose and insulin concentrations. In the insulin-modified FSIGTT, pancreatic response to glucose can be evaluated independently from tissue insulin sensitivity through minimal model analysis. An insulin sensitivity index <1.0 is generally considered to be consistent with insulin resistance.[77]

The combined glucose insulin tolerance test was developed as a simpler method of insulin sensitivity that may be employed in the field.[78] This test consists of administration of a 150 mg/kg dose of glucose (50% dextrose) followed immediately by a 0.1 U/kg dose of insulin. Blood is sampled for glucose at time 0 (baseline), 1, 5, 15, 25, 35, 45, 60, 75, 90, 105, 120, 135, and 150 minutes, and diminished insulin sensitivity is considered to exist when glucose remains elevated above baseline for >45 minutes or insulin is elevated above 20 μIU at 75 minutes.[78] Exogenous insulin allows for increased rate of glucose clearance compared to glucose alone.[78]

The insulin tolerance test (also referred to as the insulin response test) involves administration of a 0.1-0.4 mU/kg dose of insulin and subsequent evaluation of glucose and insulin concentrations at

30-60 minute intervals for up to 6 hours.[78,79] This test also directly assesses tissue insulin sensitivity, but may result in hypoglycemia.[80] Recent data indicates that clinical signs of hypoglycemia may be avoided by using an abbreviated (30 minute) sampling period, with dextrose administered following the 30 minute sample point.[80]

The dexamethasone suppression test (DST) was historically used as a dynamic test for pituitary pars intermedia dysfunction (PPID).[81] However, a recent study found that ponies that were predisposed to laminitis had increased insulin concentration following dexamethasone administration (0.04 mg/kg IM), compared to control ponies.[82] These ponies had normal cortisol concentrations, indicating that PPID was unlikely. The reason for a more profound relative increase in insulin concentration relative to control ponies is not known. In people, IV administration of dexamethasone enhances beta cell secretion of insulin in response to glucose,[83] likely subsequent to impaired skeletal muscle insulin signaling within skeletal muscle.[84]

The oral glucose tolerance test (OGTT) was initially used in horses in 1973 to evaluate small intestinal malabsorption but was later used to evaluate glucose tolerance in equids.[79] This test requires an overnight fast followed by administration of 1 g/kg of glucose via nasogastric tube. Blood is drawn for measurement of blood glucose at time 0, 30, 60, 90, 120, 180, 240, 300, and 360 minutes. A peak in blood glucose is observed at 90-120 minutes after administration of glucose and should return to normal within 4-6 hours.[85] More profound or prolonged hyperglycemia may indicate impaired pancreatic insulin secretion, decreased tissue insulin sensitivity or enhanced enteral absorption. Results may be affected by stress of nasogastric intubation, diet, fasting, or age of equids.[86-88]

The oral sugar test was the most recent field test of glucose tolerance to be investigated.[49] The test is performed by administering a 0.15 ml/kg oral dose of Karo syrup. Blood is drawn for assessment of insulin and glucose at time 0 (baseline), and again at 60, 75 or 90 minutes. An insulin

>60 μIU/ml at any time point is considered indicative of insulin resistance.[49] Glucose and insulin response to an oral glucose challenge is influenced by enteral absorption, pancreatic insulin secretion, and tissue insulin sensitivity.[89,90] During an oral glucose challenge, pancreatic insulin secretion is modulated by the incretin effect.[91] Incretin hormones include the glucagon-like peptide 1 and the glucose-dependent insulinotropic hormones. These hormones are released following an oral glucose challenge or following a meal, and have been shown to potentiate glucose-dependent insulin release.[91] The incretin effect is impaired in people with glucose intolerance and obesity.[92] In people, the oral glucose tolerance test has been shown to have a strong correlation with the HEC, indicating that insulin sensitivity is the primary determinant of insulin response to an oral glucose challenge.[93] The OST has been demonstrated to correlate well with the IVGTT in a population of normoinsulinemic and hyperinsulinemic horses,[94] suggesting that either insulin secretion or tissue insulin sensitivity are the primary factors in response to an oral glucose challenge in horses.

Mechanisms of obesity-associated IR: General overview

Insulin resistance may occur secondary to decreased circulating concentrations of functional insulin, altered insulin receptor binding or impaired downstream intracellular signaling. The insulin receptor is a tyrosine kinase receptor with an α and β subunit. Insulin signaling is initiated by the binding of insulin to the α subunit of the receptor on the cell surface.[12] The insulin signaling pathway is depicted in Figure 1. Following binding of insulin, the β subunit of the insulin receptor autophosphorylates at tyrosine residues. This phosphorylation allows for downstream activation of the insulin receptor substrate 1 (IRS-1) or IRS-2. Following phosphorylation of the IRS protein, the phosphoinositol-3-kinase (PI_3 kinase) is recruited to the plasma membrane. The PI_3 kinase catalyzes the conversion of phosphoinositol diphosphate (PIP_2) to phosphoinositol triphosphate (PIP_3).[95] PIP_3 then activates protein kinase D (PKD) which activates protein kinase B/Akt. Akt binds to its substrate, Akt substrate of kilodalton 160 (AS160), and phosphorylates several serine and threonine

sites, which inhibits AS160 from activating the GTPase activity of Rab.[96] Rab becomes GTP loaded, allowing for movement of the glucose transporter 4 (GLUT4) vesicle from its cytosolic tether and insertion into the plasma membrane.[96] Movement of the GLUT 4 vesicle also requires remodeling of the actin cytoskeleton and fusion of the glucose vesicle with the plasma membrane.[97] In a basal state (i.e., unstimulated by insulin), <10% of GLUT4 is located at the cell surface, the rest existing intracellularly.[98,99] Stimulation by insulin results in an increase in plasma membrane insertion,[99] although the number of vesicles translocated to the membrane is dependent upon regulation of the insulin signaling pathway[100] and insulin dose.[98]

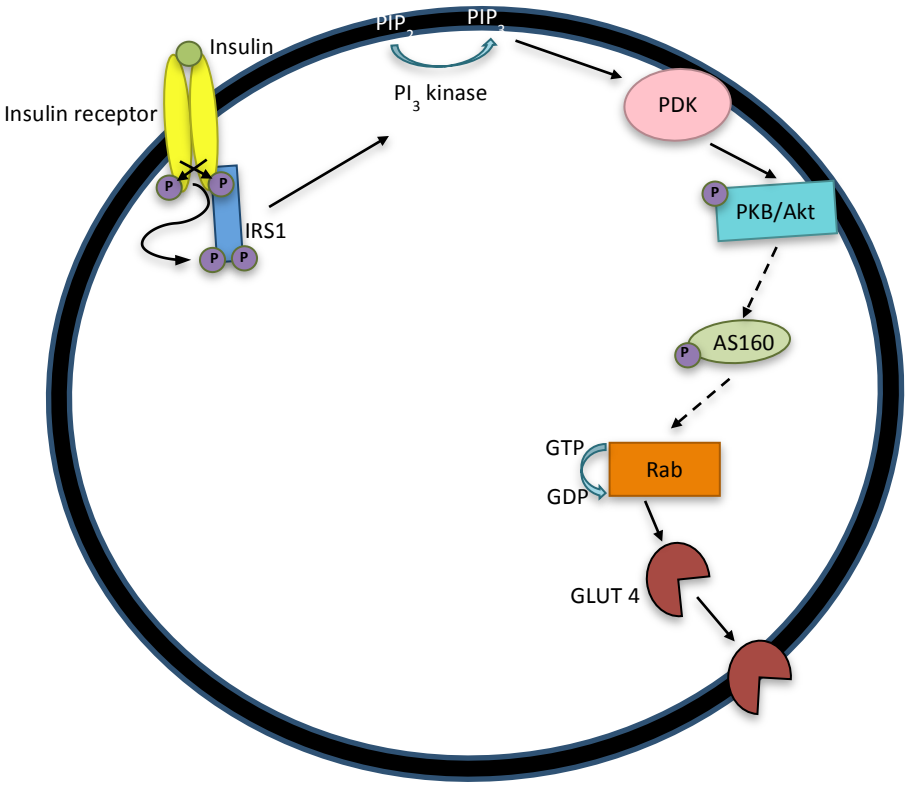

Figure 1. Schematic of the normal insulin signaling pathway. Insulin binds to the insulin receptor, phosphorylating the insulin receptor substrate-1 (IRS1) protein and activating the phosphoinsositol-3-kinase (PI_3 kinase). Protein kinase D (PKD) is recruited to the membrane and Akt is activated. Activation of Akt allows for inhibition of Akt substrate of kilodalton 160 (AS160), inhibiting its ability to activate the GTPase activity of Rab. This allows for GTP loading of a Rab and translocation of the GLUT4 vesicle to the plasma membrane (see text for further details). Dashed lines indicate inhibition, filled lines indicate activation.

Interference with any part of this signaling pathway would theoretically cause insulin resistance. However, the IRS1 appears to be a key regulatory site.[101] Serine or threonine (inhibitory) phosphorylation of a number of residues can prevent tyrosine phosphorylation of the IRS1 and subsequent activation of the PI_3 kinase. The IRS1 is a site of inhibitory phosphorylation by stress kinases, including c-Jun kinase (JNK), protein kinase C (PKCθ), mitogen activated protein kinase 4 kinase (MAP4K4), and inhibitor of nuclear factor kappa B kinase β (IKKβ).[102,103] Stress kinases may be activated by a variety of intra- and extracellular signals, including di-acylglycerol (DAG), ROS, and inflammation.[102-104]

In horses, insulin resistance has been associated with decreased GLUT-4 insertion within the membrane of skeletal muscle.[105] Interestingly, the amount of GLUT-4 inserted in the membrane did not change in either IR or IS horses following *in vitro* stimulation of muscle with insulin.[105] Evaluation of phosphorylation patterns of AS160 indicate that the defect in insulin signaling is likely downstream of AS160.[105] The mechanism responsible for the alteration in membrane-bound GLUT-4 remains to be elucidated.

Mechanisms of obesity-associated IR: oxidative stress

Obesity-associated insulin resistance has been linked with increased cellular oxidative stress, both in insulin-sensitive tissues and systemic circulation. Oxidative stress is the disruption in the balance of antioxidant defenses and reactive oxygen species (ROS) exposure, such that there is increased exposure of the cell to ROS. Reactive oxygen species are formed by the reduction of molecular oxygen or the oxidation of water. Reactive oxygen species include the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), nitric oxide ($NO\cdot$), peroxynitrite ($ONOO^-$), the hydroxyl radical ($\cdot OH$), and hypochlorous acid (HOCl). The hydroxyl radical is formed following reaction of H_2O_2 with transition metals via the Fenton reaction (as reviewed in Valko et al[106]). Reactive oxygen

species are important in normal cell signaling.[107] However, excessive ROS exposure may result in transient or permanent structural modifications within the cell. Excessive ROS production may be due to production by the mitochondrial electron transport chain, or other enzyme systems including NADPH oxidase, nitric oxide synthase, or xanthine oxidase.

In a normal physiologic state, mitochondria are responsible for a majority of the cellular ROS production, with 2% of the oxygen delivered to the electron transport chain becoming superoxide.[106] The majority of superoxide production occurs at mitochondrial Complex I (NADH-dehydrogenase) and III (cytochrome b/c).[108] Impairment of mitochondrial function can have a profound effect on ROS production. Mitochondrial dysfunction may be associated with decreased electron transport chain activity[109] or decreased mitochondrial density.[109,110] Decreased mitochondrial density may be due to small size of mitochondria[109] or low numbers of mitochondria.[110]

Mitochondrial size and copy number are regulated by mitochondrial dynamics and mitochondrial biogenesis. Mitochondrial dynamics is a term used to describe the relationship between mitochondrial morphology and function. In response to changes in cellular environment, mitochondria may undergo fission or fusion, and these changes are mediated primarily by GTPases. Fission is the process of mitochondrial division without replication of mitochondrial DNA, and is primarily mediated by the outer membrane proteins fission 1 (Fis1) and dynamin-related protein 1 (Drp1). In contrast, fusion is the joining of mitochondria that allows for DNA recombination, transfer of products of metabolism, and rescue of the membrane potential. Mitochondrial fusion is protective against ROS-induced matrix damage.[111] Fusion is mediated by the outer membrane proteins, mitofusins (Mfn) 1 and 2 and the inner membrane protein, optic atrophy protein 1 (OPA1). A balance between fission and fusion is critical for mitochondrial health. Excessive fission leads to small, fragmented mitochondria, whereas excessive fusion leads to long reticulated mitochondrial networks.[112] The maintenance of a normal mitochondrial network is important for mitochondrial function, and downregulation of Mfn2[113] or Fis1[114] results in impaired oxidative phosphorylation.

Alterations in mitochondrial dynamics are also linked to ROS production. Hyperglycemia-induced mitochondrial ROS production requires mitochondrial fission.[115] Type II diabetes has been associated with an alteration in regulation of mitochondrial dynamics, with a shift towards fission.[112,116] In addition to its role in regulating mitochondrial fusion, Mfn2 also has an important role in regulation of metabolism, including glucose oxidation, mitochondrial oxidative phosphorylation, and insulin signaling.[117]

Mitochondrial biogenesis is the process of mitochondrial replication, which is a complex process requiring the coordination of assembly of proteins that are nuclear and mitochondrial-encoded.[118] Mitochondrial biogenesis is stimulated by acute increases in ROS. Mitochondrial biogenesis is regulated primarily by the peroxisome proliferator activated receptor coactivators, PGC-1α and PGC-1β. PGC-1β is an important regulator during the basal state, while PGC-1α can be induced by a number of signals, including increased ROS production.[119,120] Downstream of the PGC-1 co-activators is the estrogen related receptor α (ERRα), nuclear respiratory factor (Nrf) 1 and 2 and mitochondrial transcription factor A (TFAM). Estrogen related receptor α is a critical downstream regulator of PGC-1α-induced mitochondrial biogenesis.[121] The nuclear respiratory factors regulate transcription of nuclear oxidative phosphorylation genes. Type II diabetes is associated with a reduction in skeletal muscle gene expression of regulators of mitochondrial biogenesis and oxidative phosphorylation, including PGC-1α, PGC-1β and Nrf1,[122,123] although these changes may in part be associated with a sedentary lifestyle.[124]

Increased ROS production alone may be insufficient to cause oxidative damage, as cells can upregulate antioxidant defense capacity in response to increased ROS. However, failure of antioxidant defenses to upregulate in response to increased ROS, overwhelming ROS production in the face of normal antioxidant defenses, or decreased antioxidant capacity may all result in oxidative stress. Within the mitochondria, primary antioxidant enzymes include the superoxide dismutase

(SOD), manganese SOD (MnSOD), the peroxiredoxins, and glutathione peroxidase (GPX).[125] Antioxidant defenses within the cytosol neutralize ROS that escape the mitochondria or derive from other sources. Key cytosolic antioxidants include the glutathione system, copper, zinc-SOD and catalase. The SODs catalyze the conversion of superoxide to the less reactive H_2O_2. The glutathione system is responsible for recycling glutathione from oxidized to reduced states, and consists of glutathione synthase (GSS), glutathione reductase (GRS), and glutathione peroxidase (GPX).[126] Glutathione synthase is an enzyme important in the creation of glutathione. Glutathione reductase converts oxidized glutathione to reduced glutathione, using NADPH as a cofactor. Glutathione peroxidase oxidizes glutathione in order to reduce H_2O_2 within the cell. Normally, the GRS is efficient at maintaining large store of reduced glutathione. Catalase is an antioxidant enzyme found in peroxisomes, lysosomes, and mitochondria that converts H_2O_2 into water. In people and rats, obesity and insulin resistance are associated with both upregulation and depletion of antioxidant capacity.[127,128] Differences in results may be attributed to severity or chronicity of ROS insult.[129]

Once antioxidant defenses are overwhelmed, oxidative damage can occur to DNA, lipids, or proteins. ROS can interact with nuclear or mitochondrial DNA, causing damage including modification of bases, breaks or cross linking of DNA, and damage to repair mechanisms.[130] Mitochondrial DNA is more sensitive to ROS-induced damage than nuclear DNA due to poor repair mechanisms and absence of protective histones.[131] Of the lipid molecules, the polyunsaturated fatty acids (PUFAs) are the most sensitive to free radicals.[132] Formation of lipid peroxides may alter membrane stability and function of membrane-associated proteins.[133] Proteins are also susceptible to oxidation, by attack of amino acid residues.[134]

ROS directly impact insulin signaling through activation of stress kinases, including JNK and IKKβ (Figure 2).[135] In IR people, ROS activates JNK, which phosphorylates a serine residue on the IRS1 protein, impairing downstream signaling.[135] ROS can also modulate insulin signaling through inhibition of several tyrosine phosphatases, including the protein tyrosine phosphatase 1B (PTP1B)

and phosphatase and tensin homologue (PTEN).[136] PTP1B dephosphorylates the tyrosine residue on the IRS1, while PTEN mediates the conversion of PIP_3 to PIP_2. Inhibition of these phosphatases results in enhanced downstream signaling.[136] Thus, it appears the quantity and location of ROS production are important in determining whether insulin signaling is impaired or enhanced.[136]

The source of ROS resulting in oxidative stress in obesity and insulin resistance remains a matter of debate. Although initial studies implicated mitochondria as the source of increased cellular ROS, recent studies have found evidence that mitochondrial dysfunction is the consequence of oxidative stress.[137] Whether mitochondrial dysfunction causes oxidative damage or merely perpetuates the pro-oxidant state, it is clear that impaired mitochondrial function occurs in association with obesity and insulin resistance in people.[138]

Evaluation of the role of oxidative stress in equine obesity and IR remains limited to studies of systemic oxidative stress. No relationship between systemic markers of oxidative damage or antioxidant capacity and obesity or IR has been identified.[139,140]

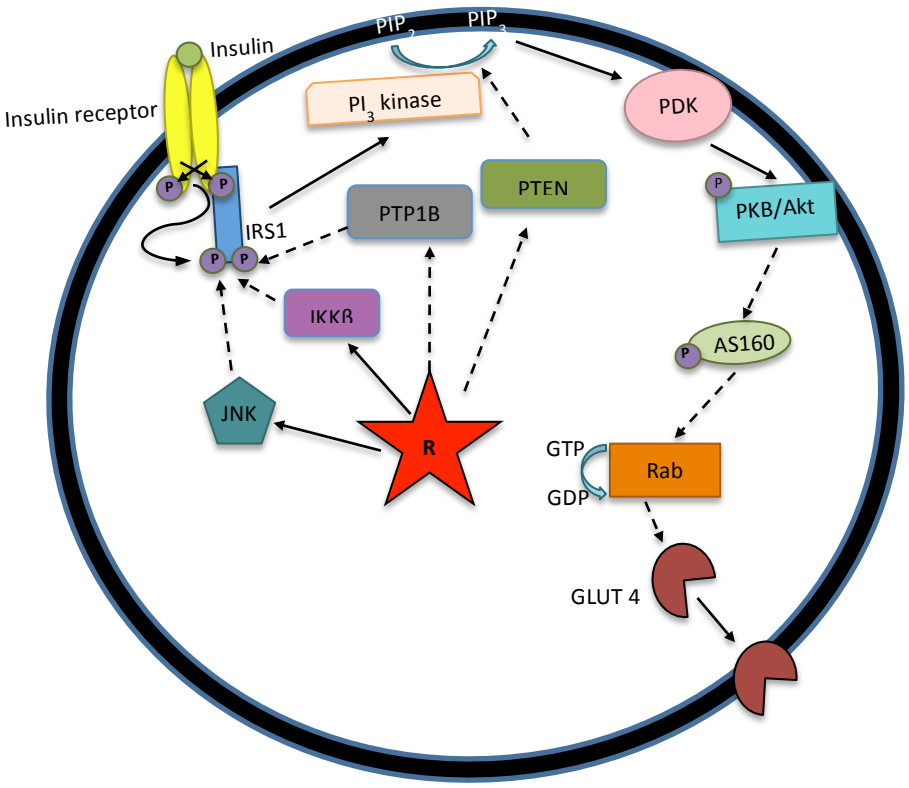

Figure 2. Interaction of ROS with normal insulin signaling pathway. Reactive oxygen species activate the stress kinases, c-Jun N-terminal kinase (JNK) and inhibitor of nuclear factor kappa B kinase β (IKKβ) through inhibitory phosphorylation of IRS1, impairing insulin signaling. ROS also enhance insulin signaling through inhibition of the phosphatases, phosphatase and tensin homologue (PTEN) and protein tyrosine phosphatase 1B (PTP1B). See Figure 1 for remainder of key.

Measurement of mitochondrial function and oxidative stress

Mitochondrial function may be directly assessed through measurement of individual complex activity, ATP synthesis, or by respirometry, either within cells or within isolated mitochondria.[141] Direct measurement of mitochondrial function is technically challenging, thus, surrogate markers of function, including evaluation of mitochondrial biogenesis and mitochondrial dynamics, are commonly employed.[141]

Quantification of oxidative stress within a biological system can be challenging, due to rapid metabolism *in vivo* and influence of *ex vivo* factors on stability of markers of oxidative stress. Oxidative stress may be quantified by direct measurement of free radical formation, or indirectly through assessment of antioxidant capacity or markers of oxidative damage. Due to the challenges in obtaining results representative of cellular oxidant status with any single method, it is prudent to measure multiple markers in order to determine cellular oxidant status.

Free radicals may be directly measured by electron spin resonance spectroscopy, spin trapping, or use of fluorescent or chemiluminescent probes.[142,143] Assays of free radical formation are expensive and technically difficult to perform. Therefore, measurement of antioxidant capacity and oxidative damage are often undertaken to evaluate the effect of increased ROS. Antioxidant capacity may be assessed through measurement total antioxidant capacity or by measurement of individual antioxidant content or enzymatic activity.[134]

Markers of oxidative damage include oxidized DNA,[144] lipids,[132,134] or proteins.[145,146] Markers of lipid peroxidation can be difficult to quantify, as they are predisposed to *ex vivo* degradation or formation.[132,134] Markers of protein oxidation include tyrosine oxidation products and carbonylated proteins.[146] Oxidized proteins are typically more stable than oxidized lipids, and perhaps are a better indicator of chronic oxidative stress.[145] However, in states characterized by intracellular lipid

accumulation, such as obesity-associated IR, measurement of lipid peroxides may be more indicative of cellular oxidative damage.[147]

Mechanisms of obesity-associated IR: Inflammation

A pro-inflammatory state exists in people with obesity-associated insulin resistance.[148] Systemic inflammation is strongly associated with obesity and regional (visceral) adiposity[149-151] and a risk of development of type II diabetes.[152] The chronic pro-inflammatory state identified within circulation of obese people is characterized by increased circulating concentrations of TNFα, IL1β, IL6 and the acute phase proteins, serum amyloid A (SAA) and C reactive protein (CRP).[149,151,153,154]

It has been proposed that these inflammatory signaling molecules act in an autocrine, paracrine, or endocrine fashion to promote additional pro-inflammatory cytokine production and impair insulin signaling in insulin sensitive tissues.[155] TNFα can bind to cellular receptors and activate intracellular stress kinases, including c-Jun-N terminal kinase (JNK) and inhibitor of nuclear factor kappa B kinase β (IKKβ). These stress kinases activate transcription of additional inflammatory cytokines in insulin-sensitive tissues. Furthermore, stress kinases impair insulin signaling by causing serine (inhibitory) phosphorylation of the IRS1 protein and inhibitory phosphorylation of the AS160 (Figures 2 and 3).[156]

The sources of circulating inflammatory cytokines remains under investigation, but both adipose and skeletal muscle are characterized by a pro-inflammatory state in obesity. Increased gene expression and protein secretion of TNFα was first identified in adipose tissue of obese mice.[157] These findings were later confirmed in adipose explants of obese people.[158] Although initial evidence suggested that adipocytes were the primary source of inflammatory cytokines in white adipose tissue (WAT),[157] more recent data suggests that it is primarily the non-adipocyte fraction (stromal-vascular cells and macrophages) that creates an inflammatory state within WAT.[159] The interplay between adipocytes and macrophages is important, as adipocytes secrete MCP-1 which promotes macrophage

infiltration of adipose tissue.[160,161] Macrophages secrete inflammatory cytokines and stimulate inflammatory cytokine expression by adipocytes. In WAT, cytokine expression varies with fat depot location. Visceral adipose is generally considered to be more pro-inflammatory than subcutaneous adipose, a difference which appears to be attributable to the vascular fraction.[162,163] Increased concentrations of TNF-α have also been demonstrated in skeletal muscle of obese, IR people, although whether this is a primary event or subsequent to systemic or adipose inflammation is unclear.[164,165]

In horses, systemic inflammation has not been strongly associated with obesity or insulin resistance when accounting for age.[19,139,140,166-169] Investigations of tissue inflammation in horses have been limited to comparisons between overconditioned IR and IS horses, without stratification by obesity.[77,170] Pro-inflammatory gene expression, including expression of MCP-1, was not different between IR and IS horses in visceral or subcutaneous adipose depots.[77] Evaluation of TNFα protein content yielded variable results based on the tissue examined; TNFα was increased in visceral adipose, but not nuchal adipose or skeletal muscle of IR compared to IS horses.[170] These early findings suggest that in horses, WAT inflammation varies by location, and visceral adipose inflammation may be associated with IR status.

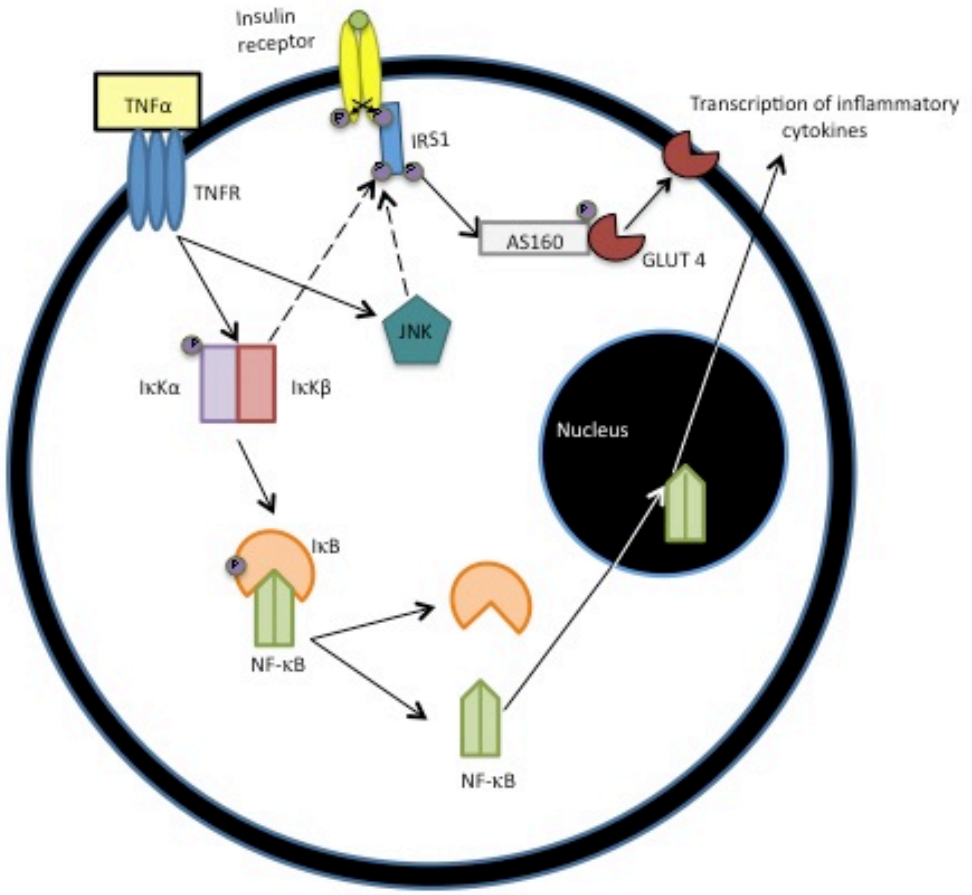

Figure 3. Inflammatory cytokines activate pathways to increase inflammatory cytokine transcription and impair insulin signaling. TNFα = tumor necrosis factor α. TNFR = Tumor necrosis factor receptor. IκK = inhibitor of nuclear factor kappa B kinase. NF-κB = nuclear factor kappa B. See Figures 1 and 2 for remainder of key.

Mechanisms of obesity-associated IR: lipotoxicity

Obese humans have impaired free fatty acid (FFA) metabolism manifested by increased circulating FFA concentrations, increased lipolysis, and reduced suppression of FFA turnover by insulin.[171,172] High circulating concentrations of FFAs trigger intramyocellular accumulation of fatty acids, primarily as di-, or triacylglycerol, long-chain fatty acyl-CoA, or ceramides. Cytosolic accumulation of free fatty acids has several effects on glucose metabolism. Di- and triacylglycerol activate protein kinase C θ, which causes serine (inhibitory) phosphorylation of IRS1.[173,174] Increased cellular FFAs may impair glucose oxidation by inhibiting pyruvate dehydrogenase.[175] Accumulation of fatty acid metabolites inhibits import of long chain fatty acids into the mitochondria[176] and impairs electron transport chain activity.[177] Plasma free fatty acids downregulate the expression of oxidative phosphorylation genes.[178] Although intramyocellular lipid accumulation is recognized to be associated with insulin resistance in human obesity, endurance athletes have increased intracellular lipid accumulation compared to non-obese, sedentary adults yet are highly insulin sensitive.[179] This finding is referred to as the "athlete's paradox."[180] Although the reason for the differences in consequences between lipid accumulation in obese people and accumulation in athletes is not known, it may be associated with type of lipid[179] or degree of lipid peroxidation.[181]

Measurement of serum insulin concentrations in the horse

Accuracy in measuring serum insulin concentrations is important for assessing insulin sensitivity in equids. Equine insulin (molecular weight, 5748 Da) has two amino acid substitutions which differ from human insulin (molecular weight, 5808).[182] One of these substitutions, amino acid 30 (threonine in humans, alanine in horses), exists in a location that may affect three dimensional structure of insulin, thus potentially impacting equine insulin binding in a human assay.[183] Multiple assays have been employed to assess equine serum insulin concentrations, including the

radioimmunoassay (RIA)[a] and the ELISA[b,184] The RIA is a competitive antigen binding assay using radioactive (I^{125}) insulin. Both the RIA[185] and the ELISA[186] have been validated in the horse. However, both the RIA and the ELISA substantially and consistently underestimate insulin concentration when compared to liquid chromatography-mass spectrometry.[184] Comparison between the RIA and the ELISA revealed poor agreement at high concentrations of insulin.[183]

The RIA is the most commonly used assay for measurement of equine insulin. However, it is an inconvenient assay, requiring overnight incubation. Furthermore, it requires special equipment and handling of radioactive isotopes. An assay which requires less technical expertise and improved accuracy in detection of equine insulin is desirable.

The Immulite[c] measures serum insulin by a chemiluminescent immunometric sandwich assay (CIA), using a mouse monoclonal capture antibody and a mouse monoclonal and sheep polyclonal detecting antibody.[187] This technique offers several advantages over the RIA in that it is performed quickly, requires little technical expertise, and does not require use of a radioactive isotope. The CIA has been recently used in horses to evaluate response to therapy.[188] However, to the author's knowledge, this method has not been validated in the horse.

Summary and Hypotheses

There is increasing recognition of equine obesity, insulin resistance or hyperinsulinemia, and equine metabolic syndrome (EMS). The mechanisms leading to the development of obesity-associated IR in horses have not been determined. Accurate assessment of insulin dysregulation and identification of mechanisms leading to IR are paramount for the development of preventative measures and treatments.

In order to improve understanding of the mechanisms leading to EMS, we developed the following central hypothesis: ***Impaired insulin signaling within skeletal muscle causes equine insulin resistance.***

To test this hypothesis, an accurate method for measurement of equine serum insulin is required. Radioimmunoassay (RIA) is a commonly employed method of measuring equine insulin within a research setting. However, it is an inconvenient assay requiring overnight incubation and handling of radioactive isotopes. Therefore, we sought to evaluate a more convenient, automated assay: the chemiluminescent immunometric assay (CIA). ***We hypothesized that the CIA offers improved detection of equine insulin compared to the RIA.*** To address this hypothesis, the following specific aims were constructed:

Specific Aim 1: To validate the chemiluminescent immunometric assay. For validation of the assay, linearity, recovery, and inter-assay precision were determined.

Specific Aim 2: To compare the CIA to the RIA. To assess this aim, insulin was measured by both methods in 40 equine serum samples.

Next, three specific hypotheses were developed to evaluate the role of skeletal muscle insulin resistance in EMS.

Hypothesis 1: The hyperinsulinemic euglycemic clamp yields comparable results to the oral sugar test and the insulin response to dexamethasone test.

Specific Aim: To determine the relationship between insulin sensitivity indices obtained by the HEC, OST, and IRDT. To achieve this aim, the insulin sensitivity index obtained by the HEC was compared to the insulin and glucose response of the OST and the insulin response of the IRDT.

Hypothesis 2: Skeletal muscle oxidative stress causes equine insulin resistance.

Specific Aim: To evaluate the role of skeletal muscle oxidative stress in IR. To assess this aim, markers of mitochondrial function, antioxidant capacity, and oxidative damage within skeletal muscle were quantified in insulin sensitive and insulin resistant horses.

Hypothesis 3: Skeletal muscle inflammation causes equine insulin resistance.

Specific Aim 3: To characterize skeletal muscle and systemic inflammatory state of insulin resistant and insulin sensitive horses. For evaluation of this specific aim, markers of systemic and skeletal muscle inflammation were measured.

Footnotes

[a]Coat-A-Count, Siemens, Tarrytown, NY

[b]Mercodia, Uppsala, Sweden

[c]Immulite 1000, Siemens, Tarrytown, NY

CHAPTER II

REVIEW OF IMMMUNOASSAY VALIDATION FOR THE VETERINARY CLINICIAN

INTRODUCTION

In order to accurately identify disease, it is critical that testing protocols and laboratory assays be appropriately validated. Furthermore, it is important to standardize across assay methodologies in order to allow for comparison between laboratories or studies. The purpose of this paper is to review laboratory assay standardization and cross-validation of methodologies, using an example of cross-validation of two commercially available methodologies, a chemiluminescent immunometric assay[a] (CIA) and a radioimmunoassay[b] (RIA) for determining equine insulin concentrations. Recent guidelines from the American College of Veterinary Clinical Pathology[189] were used as a reference during assay validation.

REVIEW OF ASSAY VALIDATION

Assay validation should include evaluation of accuracy, linearity, analytical range, precision, lower limit of detection, functional sensitivity, and effects of commonly encountered substances.[189] Accuracy is the closeness of agreement between the measured value and actual concentration of the analyte.[189] Precision refers to the reproducibility of the method. Development of an immunoassay requires determination of the working range, accuracy, intra- and inter-assay precision, interference, and recovery.[190] If possible, the new method should be compared to a

reference method to evaluate systematic error (or bias) of the new method.[191] For an assay to be meaningful, there must be a consistent, proportional relationship between analyte concentration and signal.[192] This relationship is defined as linearity, and is an important component to determination of accuracy and analytical range. To determine linearity, a standard curve must be developed with at least six standards in an appropriate matrix.[193] Standards should have concentrations spanning the expected working range.[193] Furthermore, sample linearity must be assessed from a high concentration to a low concentration. This will also allow for estimation of the lower limit of quantification in biological samples,[193] and will determine whether dilution is required for samples to enter the working range. Linearity should be assessed in a series of 5 dilutions using aliquots of varying concentrations of a sample with low concentration of analyte (low pooled) and high concentration (high pooled) of analyte.[189] Linearity is determined to exist if mean accuracy is 70-130% compared to undiluted sample and CV ≤ 25%.[193] For sample dilutions, a solution should be used that approximates real sample matrix.[189]

Recovery is a measurement of selectivity of the assay in the presence of endogenous matrix components.[193] It is a reflection of accuracy of the method. Recovery should be assessed in a sample of high concentration, or, if necessary, a sample that has been spiked with a standard with high concentration of analyte.[189] If a standard solution is to be added to the sample to evaluate recovery, dilution of the sample by the standard should not exceed 10% so as not to substantially alter sample matrix.[189] Recovery studies should cover the expected range of analyte values in patient samples.[189] For immunoassays, recovery is considered adequate if accuracy is 70-130% compared to unspiked sample and CV ≤25%.[193]

Specificity is another important component of accuracy. For antibodies, specificity should be tested by evaluating three samples spiked with a protein that belongs to the same family or has similar structure to the target biomarker. Specificity should be assessed at a physiologic concentration and at a supraphysiologic (100-fold) concentration.[193]

Precision is a measurement of random error that is determined by measurement of coefficient of variation (CV).[194] Coefficient of variation (CV) is calculated using the following formula:[195]

$$\% \text{ CV} = (\text{standard deviation/mean}) * 100$$

Precision should be defined in two ways, by measurement of the same sample within a single run of the assay (intra-assay CV) and by measurement of the same sample on separate runs (inter-assay CV). Precision should be evaluated by measurement of 20 replicates of at least 3 samples spanning the expected analytical range (i.e., low, medium, and high concentration).[189] Ideally, inter-assay CV should be performed at least 24 hours apart and should be assessed over 20 days.[190] Inter-assay variation should be performed on aliquots to eliminate the effects of repeated freeze/thaw cycles.[189] Intra- and inter-assay CVs should be <25%.[193]

Limit of detection and functional sensitivity should be established by using a series of low spiked samples and a blank sample.[189] Lower limit of detection is considered to be the mean of the blank plus 2 or 3 standard deviations of the blank,[189] and determines when analytical noise associated with the blank interferes with reliable detection of the analyte.[192] Functional sensitivity is the mean of the lowest spiked sample that has a coefficient of variation of ≤20%.[189] This measurement is used to determine imprecision at low analyte levels.[196] The coefficient of variation typically increases as analyte concentrations approach the lower limit of detection, and a concentration with a CV of 20% is considered to be at the limit of useful measurement.[189]

The effect of commonly encountered interfering substances should also be considered.[189] Common interfering substances in serum or plasma assays include bilirubin, hemoglobin, and lipids.[197] Interfering substances may falsely increase or decrease test results. When analyzing the effect of interfering substances, it is important to use concentrations that are within expected range of concentration in patient samples.[189] The volume of interfering substance added should be minimized in order to maintain the properties of the sample matrix.[189]

To compare the new method to a previously validated, established method, it is recommended that at least 40 samples with analyte concentrations covering the entire working range of the assay be measured in duplicate.[189] When diagnostic tests are to be used with samples with a low range ratio (i.e., expected maximum value and minimum value of analyte are close together), more than 40 samples may need to be included to achieve adequate statistical power.[194] Diagnostic test comparison should be conducted over several days or weeks. Creation of a difference plot (Bland-Altman) and calculation of total error should be performed using the following equation:[189]

$$TE = Bias_{meas} + 3 * CV$$

Bias can be determined from Bland-Altman analysis, and is a measure of systematic error.[189] Systematic error is the difference between the new method's measurement of the analyte and the true value, as determined by an established reference method.[194] Coefficient of variation is a measurement of random error. For this equation, CV is determined from the inter-assay repeatability experiment.[191] This calculated total error should be less than the established total allowable error for the initial test. Total allowable error is dependent upon biologic variation of the assay and rate of inappropriate categorization (e.g., normoinsulinemic horse being categorized as hyperinsulinemic).

Relationships between the two test methods should be further evaluated using correlation analysis. If correlation between the two methods is <0.975 for data with a narrow range or <0.99 for data with a broad range, data may be improved through increasing sample size or replicates.[194] Alternative statistical methods for regression should be employed if correlation is not improved with additional samples.[194] However, if the correlation coefficient is considerably lower, then the two tests are unlikely to be interchangeable.[198]

If correlation is ≥0.975 or ≥0.99, respectively, linear regression can be used to estimate bias.[194] Linear regression may also be used to determine whether constant or proportional systematic errors are present. Constant error is interpreted on the basis of intercept (constant error present if

intercept ≠0), and proportional error is interpreted by slope (a slope≠1 indicates presence of proportional error).[198] If no proportional error exists, data may be analyzed for agreement using a concordance coefficient.[199] If proportional error exists, alternative linear regression should be used. The clinical importance of proportional or constant error is reflected by determination of bias and total error;[200] however, significant systematic error of any type likely indicates that the methods should not be used interchangeably.

For clinical application of an assay, it is not only important to determine agreement with an established method but also diagnostic consequences of disagreement.[201] Disagreement with consequences as to clinical decision making (such as defining a horse as normoinsulinemic or hyperinsulinemic) may be defined as a discordance rate.[201] Discordance may be defined as a fraction of the total study group using the following equation:[202]

Unefficiency = (fp+fn)/(fp+fn+tp+tn)

In this equation, a false positive (fp) is defined as a difference between assays whereby the value from new assay is above and the reference assay is below the decision-making point for disease. A false negative (fn) exists when the value from the new assay is below and the reference assay above the cutoff for clinical decision making. True positives (tp) exist when measurements from both assays are above the cutoff value, and true negatives (tn) where both assays are below the cutoff value.

METHODS FOR MEASUREMENT OF EQUINE INSULIN

With increasing recognition of equine insulin resistance and its complications,[9] there is a demand for convenient, accurate tests for measurement of equine insulin. In addition to the RIA that has been validated for use in the horse,[203] several additional methods of measurement have been evaluated for equine use, including an additional RIA and four ELISAs.[184] An equine ELISA

validated in the horse, but the assay had poor concordance with the previously validated RIA.[184] Furthermore, the ELISA demonstrated poor concordance while the RIA had moderate concordance with liquid chromatography/mass spectrometry; both assays consistently underestimating plasma insulin concentrations.[184] These findings suggest that accurate measurement of equine insulin is not currently achieved using commercially available assays. Determination of agreement between insulin assays is an important issue to be addressed. Once agreement is evaluated, a method for standardization or harmonization of equine insulin assays will allow for consistent identification of insulin concentrations of horses in clinical and research settings. In people, initial recognition of poor agreement between insulin assays led to harmonization of assays and substantial improvement in method agreement.[204]

The chemiluminescent immunoassay is a convenient method of equine insulin measurement that has been used to assess response of insulin resistant horses to therapy.[56] However, to the authors' knowledge, this method has not yet been validated in the horse. Therefore, the purpose of this study was to validate the CIA and compare the CIA to the previously validated RIA.

ASSAY VALIDATION: MEASUREMENT OF EQUINE INSULIN USING THE CHEMILUMINESCENT ASSAY AND COMPARISON TO THE RADIOIMMUNOASSAY

Materials and Methods

Archived serum from 40 horses was used for measurement of serum insulin. Serum had been stored at -80°C until analysis, and all analysis was undertaken within 6 months of sample collection.

Linearity

Linearity of the CIA was determined using equine serum-based control solutions at four insulin concentrations from 0-320 μIU/mL. Dilutional parallelism was evaluated in a high

endogenous insulin equine serum (measured concentration using CIA was 111 μIU/ml) using both the RIA and CIA.

Recovery

A commercially available equine insulin standard[c] was used to determine recovery. Two different equine serum samples with low (<7 μIU/mL) serum insulin were spiked with the commercially available equine standard (12 ng/ml) and serially diluted with the low endogenous insulin serum to achieve concentrations of 75 μIU/mL, 37.5 μIU/mL, 18.8 μIU/mL, and 9.4 μIU/mL.

Intra-assay coefficient of variation

Intra-assay coefficient of variation was determined on the CIA with two equine serum-based control solutions, one at low and one at medium concentrations. As all samples were evaluated in duplicate with the RIA, intra-assay coefficient of variation was evaluated for all 40 serum samples.

Inter-assay coefficient of variation

Inter-assay coefficient was performed in three pooled samples, high (mean, 134.2 μIU/mL), medium (mean, 24.4 μIU/mL), low (2.3 μIU/mL) and on the provided medium (mean, 11.53 μIU/mL) and high (mean, 59.3 μIU/mL) equine serum-based control solutions. Six replicates of controls, four replicates of medium pooled serum, and five replicates of high and low serum were evaluated.

Comparison of methods

Two commercially available assays for measuring insulin were used, the RIA and CIA. The RIA has been previously validated[203] for use in horses. Forty serum samples with insulin concentrations that were expected to vary over a wide range were measured with RIA and CIA. Samples evaluated by RIA were measured in duplicate.

Treatment of discordant samples

Samples from 5/40 horses were found to be discordant between methods, using the previously recommended concentration of 20 μIU[45] as the level of clinical decision making. All five of these horses had higher insulin values on the CIA. Two of these horses underwent repeated sampling over a four-month period, during a fasted state or an oral glucose challenge. Six of eight samples from Horse 1, and 4/4 samples from Horse 2 were discordant. Discordant results occurred during fasting and following an oral glucose challenge. Two samples that were discordant between the RIA and CIA and were further evaluated subsequent to polyethylene glycol (PEG) treatment, as previously described for displacement of antibodies.[205,206] Samples were treated with an equal volume PEG 6000 solution (250 g/L PEG in 0.05 mmol/L phosphate buffer, pH 7.4, containing 0.5 g/L Triton X-100). The solution was vortexed and allowed to sit at room temperature for 20 minutes followed by a 15 minute centrifugation at 1200 g. The supernatant was decanted and used for analysis.

In addition, in order to determine if discordance was related to recognition of a substance other than insulin within the equine matrix, human recombinant insulin[d] was used to spike a low serum sample, and recovery was assessed.

Statistical Analysis

Linearity and dilutional parallelism were assessed by linear regression and determination of a best fit line. A Pearson's correlation coefficient was calculated for the 40 samples analyzed by both methods. Deming's regression was performed to evaluate the relationship between the RIA and CIA. A Bland-Altman difference plot was created and a paired t-test was used to calculate bias.

RESULTS

Linearity using equine-serum based control solutions was excellent (r^2=0.99, $p<0.001$) (Figure 4). Dilution of a high endogenous insulin (96 μIU/mL) sample indicated a strong relationship

between expected concentrations of the diluted sample and measured concentrations from both assays (RIA, r^2=0.99, p<0.001, CIA, r^2=0.92, p=0.009, Figure 5).

Recovery of the equine insulin standard using both the RIA (53.8±3.9%) and the CIA (8.4±2.1%) was poor (Figure 6). When evaluating precision of the CIA, intra-assay CV of the low (10.44 μIU/mL) and high (55.40 μIU/mL) equine-serum based control solutions was 2.5% and 3.1%, respectively, while inter-assay CV was 13.7% and 5.5%, respectively. The pooled equine serum samples had inter-assay CV of 30% (medium) and 4.1% (high). The low pooled sample was not included in analysis as 4/5 readings were below detection.

Additional determination of the validity of the CIA was performed by cross-referencing to the commonly used RIA. Insulin concentrations of forty samples ranged from <3.5-215.6 μIU/mL (median, 11.54) using the RIA and <2-224 μIU/mL (median, 11.9) using the CIA. Correlation analysis between the two assays revealed good correlation (r=0.58, p<0.001). Regression analysis yielded a best-fit line of y=1.81x -5.06 (Figure 7). There was no significant difference between the CIA and RIA measurements (p=0.81). However, a bias did exist, with the CIA an average of 12.7 μIU/mL higher than the RIA (95% limits of agreement, -70.9 to 96.9, Figure 8). The bias was due to proportional error (95% limits of agreement for slope, 1.04-2.58; 95% limits of agreement for y-intercept, -37.7 to 27.6). Total error was 63.1%. The bias was primarily associated with 5 discordant samples; removal of these samples from analysis yielded a bias of 1.5 μIU/mL (95% limits of agreement, -16.6 to 19.5). However, even with exclusion of these five samples, assay performance exhibited a total error of 51.9%.

Due to the discordance between the CIA and RIA, investigations were undertaken to try to determine possible reasons for method discordance. Discordance in measurement may be associated with substances that interfere in one assay but not another, including bilirubin, hemoglobin, lipids, antibodies, or other proteins. As visual inspection did not reveal the presence of interfering pigments

or lipids, the possibility of interference by antibodies (heterophilic or anti-insulin) was evaluated by treatment of discordant samples with polyethylene glycol (PEG). Polyethylene glycol allows for displacement of antibodies from solution, decreasing antibody interference with the assay.[206] Treatment with PEG did not significantly alter serum insulin concentrations of 2 discordant samples ($p=0.07$; Table 1). In addition to PEG treatment, serial dilution of an equine serum sample with added non-equine insulin (human recombinant insulin) was performed to determine if discordant results were a result of an intrinsic component of the equine matrix. Serial dilution of an equine serum spiked with recombinant human insulin yielded excellent recovery (91.2±4.7%, Figure 9) with the CIA.

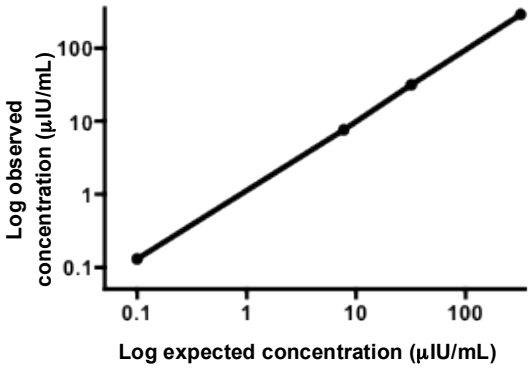

Figure 4. Linearity of the equine serum-based control solutions measured by CIA with best-fit line $y=0.90x +1.021$ ($r^2=0.99$, $p<0.001$).

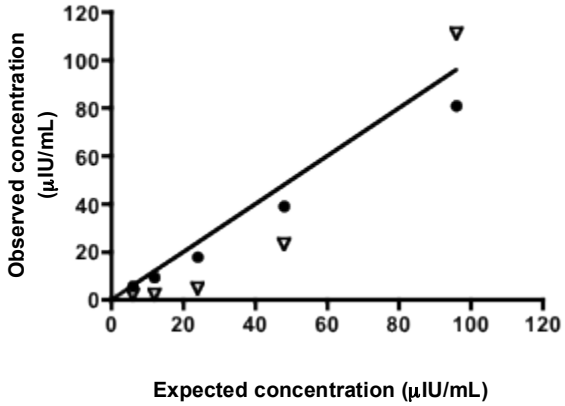

Figure 5. Dilution of a high endogenous insulin serum sample using a pooled low serum sample measured by RIA (filled circles) and CIA (open triangles). Line indicates expected dilution. Best fit line for the RIA: $y = 0.85x - 0.87$ ($r^2=0.99$, $p<0.001$). Best fit line for the CIA: $y = 0.90x + 1.021$. ($r^2=0.92$, $p=0.009$).

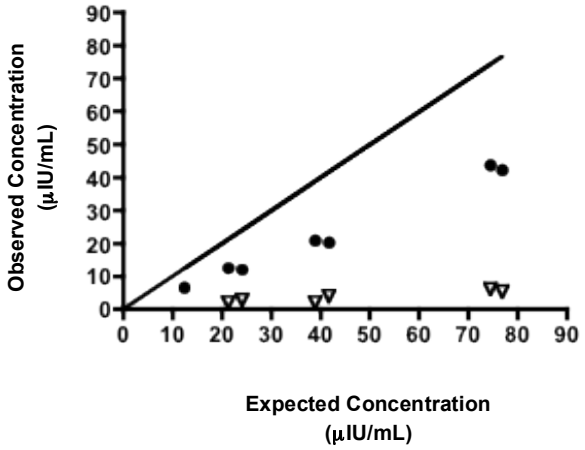

Figure 6. Recovery of equine insulin standard with the RIA (filled circles) and CIA (open triangles). Line indicates perfect recovery.

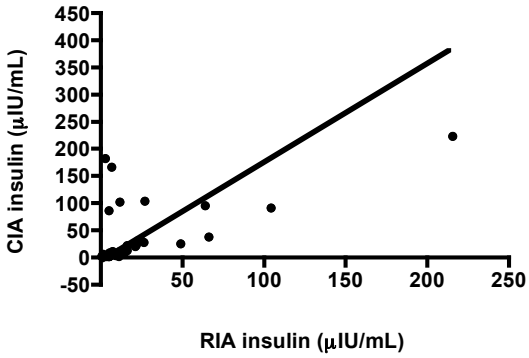

Figure 7. Scatterplot of RIA compared to CIA results. Deming's regression analysis indicates a best-fit line of y= 1.81x - 5.06.

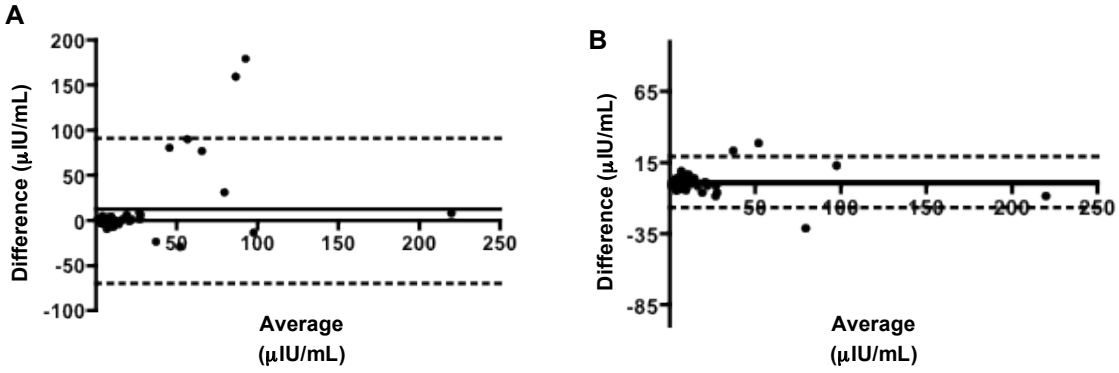

Figure 8. a) Bland-Altman plot of average compared to difference CIA minus RIA. b) Bland-Altman plot of average compared to difference CIA minus RIA with discordant results (n=5) removed.

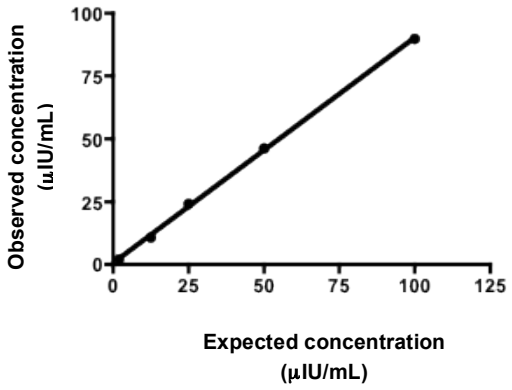

Figure 9. Recovery of human insulin in equine serum matrix using the CIA. Best-fit line of y=0.90x +0.54 (r^2=0.99, p<0.001).

Table 1. Serum insulin concentrations (μIU/mL) before and after polyethylene glycol treatment.

	CIA	**CIA PEG**	**RIA**	**RIA PEG**
Horse 1	120	159.6	5.8	6.8
Horse 2	158	151.2	10.6	12

DISCUSSION

Equine insulin is commonly evaluated by RIA in research studies. However, this assay consistently underestimates equine insulin concentrations, when compared to LC-MS.[184] Although mammalian insulins demonstrate substantial homology, equine insulin does differ from porcine, human, and bovine insulin by 1-3 amino acids.[182] These substitutions may affect insulin binding due to different secondary, tertiary, or quaternary protein structure.[207,208] Therefore, depending upon the amino acid sequence recognized by antibodies in different immunoassays, commercially available insulin assays may vary in detection of equine insulin. This was recently confirmed in an evaluation of six different commercially available insulin assays in comparison to LC/MS.[184] An accurate, quick method for measuring serum insulin in the horse remains to be identified. The CIA performs well in people, with 95% of samples falling within 32% total error[204] allowed for insulin assays.[209] However, despite being in use in clinical equine practice,[188] the CIA has not yet been validated for use in horses.

Recovery was poor in both assays, but much worse (average <10%) when using the CIA. Poor recovery of equine insulin using commercially available assays has been previously reported,[184] and may be a product of decreased binding of assay antibodies to equine insulin compared to human insulin. The observation that the linearity of the CIA was excellent when using provided equine-serum based control solutions, but deteriorated when using a high endogenous insulin equine serum, would support this explanation. It is also possible that the equine insulin standard had an actual concentration that was lower than the labeled concentration, leading to a lower than expected recovery. A role of the equine insulin standard in poor recovery is supported by the observation of lower than expected concentrations of the standard in both assays. Inter-assay CIA CVs were more variable using pooled equine serum samples, compared to the provided control solutions. Poor recovery and high inter-assay variability on pooled serum samples suggest that the CIA would not be an acceptable method of insulin measurement in the horse.

Additional evaluation of the CIA included comparison to the commonly used RIA. Poor agreement was found between the CIA and RIA assays for measuring equine insulin. For assays measuring human insulin, it is recommended that total error not cause more than 12% error in appropriate categorization (i.e., within reference range or outside of reference range) for a given sample.[209] Acceptable measurement bias and total acceptable error are in part determined by biological variation of the analyte. For human insulin assays, within-individual variation has been reported to be 21.1%, and within-group variation for healthy individuals has been reported to be 58%.[210] Recommendations for measurement of insulin concentrations in people include a bias < 15.5%, imprecision < 10.6%, and total analytical error < 32% for a single result at concentrations within the reference interval.[209] While this allowable error may seem substantial, within the reference interval, considerable variation may exist without impacting test interpretation. For example, a horse with an initial insulin measurement of 10 may have a subsequent insulin measurement of 13.2 or 6.8 μIU/mL and still be considered insulin sensitive. Although bias of the CIA was within acceptable ranges, total error using inter-assay precision exceeded recommendations for insulin immunoassays.

Much of the bias and wide limit of agreement observed between assays was attributable to five samples. When these five horses were removed from analysis, average bias improved to 1.5 μIU/mL. Due to the dramatic improvement of agreement with removal of samples from these horses, additional analysis was undertaken to determine possible reasons for the discordant results. Multiple samplings from two of these horses yielded repeatable results, with ten samples being discordant and two samples being concordant by the two methods. In all discordant samples, insulin concentrations were substantially higher on the CIA. As common interfering substances (lipids, hemolysis, bilirubin) have been previously documented to falsely lower CIA results in people,[187] and visual inspection of the samples did not indicate lipemia, hyperbilirubinemia, or hemolysis as a likely source of interference, the possibility of an effect by heterophilic or anti-animal antibodies was explored. Heterophilic antibodies are weak polyspecific antibodies that are either natural idiotypic antibodies or

autoantibodies that interfere with antibody binding in immunoassays by a non-competitive mechanism.[211] Anti-animal antibodies are typically high affinity antibodies against another species following exposure to antigens from that species.[211] Interestingly, two-site immunometric assays appear to be more prone to interference by heterophilic antibodies than antigen binding assays.[211] Although heterophilic and anti-animal antibodies are not commonly reported in animals, exposure of horses to other animal-derived antigens (e.g., rodents) seems plausible.

Multiple methods are available to eliminate the effects of heterophilic or anti-animal antibodies, including polyethylene glycol (PEG) treatment,[211,212] heat treatment,[211,212] or use of blocking serum.[211] Insulin is heat-sensitive,[213] and determining appropriate concentrations of blocking serum can be challenging.[213] Therefore, in this study, we chose to evaluate the effect of PEG treatment on insulin concentrations. Polyethylene glycol is a synthetic polymer that may be used to precipitate proteins from solution.[206] In human serum, it has been used to precipitate immunoglobulins and immunoglobulin-antigen complexes to improve detection of hormones and other analytes.[214] Polyethylene glycol treatment decreased serum insulin concentrations by approximately 40% in both the RIA and CIA. Furthermore, assay results remained discordant following treatment, suggesting that changes in insulin concentrations were an effect of dilution, rather than an effect of decreased interfering antibody. Demonstration of consistent recovery of a human recombinant insulin within a pooled equine serum sample indicates that the CIA recognizes insulin within the equine matrix. Discordant samples may be associated with other matrix components other than interfering antibody or may be due to binding to a substance with similar structure to insulin. Additional matrix components that have been reported to interfere with immunoassays include complement or lysozyme.[211]

In summary, the CIA does not appear to be an acceptable method of measuring equine insulin due to poor recovery of an equine insulin standard and high inter-assay coefficient of variation. This study highlights the importance of validating and cross-referencing assays prior to implementation

into clinical use. Several aspects of initial assay validation indicated that the CIA would not yield acceptable results. Cross-referencing to the RIA allowed for identification of a bias, with the CIA tending to measure higher concentrations than the RIA. Furthermore, cross-referencing allowed for identification of discordant samples. Reasons for the discordant results are unknown, but appear unlikely to be due to interference by antibodies.

Footnotes

[a]Immulite 1000, Siemens, Tarrytown, NY

[b]Coat-A-Count, Siemens, Tarrytown, NY

[c]Shibayagi Company, Ishihara, Japan

[d]Novolin R, Novo Nordisk Pharmaceuticals, Princeton, NJ

CHAPTER III

COMPARISON OF THREE METHODS FOR EVALUATION OF EQUINE INSULIN RESISTANCE

ABSTRACT

Multiple dynamic field tests are used for assessment of equine insulin resistance. However, the relationship between markers of glucose homeostasis and insulin disposal obtained by different testing protocols is unknown. We hypothesized that two recently developed field tests for evaluation of equine IR, the insulin-response to dexamethasone test (IRDT) and oral sugar test (OST), would yield comparable results to the hyperinsulinemic euglycemic clamp (HEC). Fifteen light breed horses with body condition scores of 3/9 to 8/9 were used in this study. Eight horses underwent an OST, HEC, and IRDT over a four-week period. The OST was performed under two different housing conditions, pasture and stall. Eight horses underwent an OST on pasture three times over a 14-16 week period during the summer and fall. The HEC did not correlate with either the OST or IRDT, but the OST correlated with the IRDT. OST was not significantly different when performed in the pasture compared to in a stall, but did change significantly over time on pasture. These results suggest that the OST and IRDT results are not primarily determined by tissue insulin sensitivity among insulin sensitive horses.

INTRODUCTION

Hyperinsulinemia is a recognized risk factor for the development of laminitis.[35] In horses presenting to a first opinion hospital for evaluation of laminitis, endocrine disorders were the most frequently identified cause of laminar disease, with 67% of horses presenting with hyperinsulinemia.[32] Despite the clinical importance of hyperinsulinemia, there is not an established standard for evaluation of glucose homeostasis and insulin disposal in the horse. Insulin dysregulation has been assessed by fasting hyperinsulinemia or proxies,[65] and dynamic glucose or insulin tolerance testing.[76,78,79] In people, the gold standard for evaluation of tissue IR is the hyperinsulinemic euglycemic clamp (HEC).[67] The HEC has been adapted for use in equids, including ponies,[68] Warmbloods,[68] Belgian horses,[70] Standardbreds,[71] and Quarter Horses.[69,73,215] The HEC is ideal for assessing tissue insulin sensitivity, as it eliminates the impact of pancreatic insulin secretion or enteral glucose absorption on glucose homeostasis. However, the HEC technique requires the use of infusion pumps and frequent monitoring and thus is not amenable to field testing of client horses or epidemiological studies. Furthermore, the specificity of the HEC for IR within insulin-sensitive tissues may be a disadvantage in cases where enteral glucose absorption or pancreatic response to glucose may be altered.

Several tests have recently been developed or adapted for use to improve ease of detection of equine insulin dysregulation by practitioners. The oral sugar test (OST) is an oral glucose challenge test that was developed for use in the field.[49] The simplicity of this test makes it an attractive possibility for ambulatory practice. However, as the response to an oral glucose challenge is influenced by enteral glucose absorption, pancreatic insulin secretion, and tissue insulin sensitivity, results may differ from the HEC.

Initial evaluation of the usefulness of the OST in detecting alterations of insulin regulation and glucose homeostasis was performed in a controlled environment (stalls). Despite being in a

controlled environment, substantial day-to-day variation in insulin response was observed in a small population of horses undergoing repeated measurements (area under the curve for insulin coefficient of variation=45%).[94] It is uncertain how this test performs when horses are on pasture, which may introduce greater variability due to amount of grass consumed and non-structural carbohydrate (NSC) content of pasture. Finally, in horses normally kept on pasture, altering housing may induce stress and influence test results. Thus, it is important to understand how environment impacts test results.

The dexamethasone suppression test (DST) has historically been used to identify pituitary pars intermedia dysfunction.[81] Recently, the response to dexamethasone was evaluated as a dynamic test of insulin sensitivity. Laminitic, IR ponies were found to have higher insulin concentration post-dexamethasone compared to control ponies.[82] Although these initial findings are promising, these test results have not yet been replicated in horses or IR non-laminitic ponies.

The purpose of this study was to compare three different methods of insulin sensitivity testing in Quarter Horses: HEC, OST, and the insulin response to dexamethasone test (IRDT) (Experiment 1). We hypothesized that the HEC, OST and IRDT would correlate well. A second objective was to evaluate the effect of season on the OST in horses maintained on pasture (Experiment 2).

MATERIALS AND METHODS

Experiment 1: Comparison of Hyperinsulinemic Euglycemic Clamp, Oral Sugar Test, and Insulin Response to Dexamethasone Test

Horses

Eight Quarter Horses, aged 7-14 years and weighing 518-645 kg were used in this study. There were 7 geldings and 1 mare. Body condition score ranged from 4.5-7 (median, 5.8). Horses were free of clinical signs of systemic disease including pituitary pars intermedia dysfunction. Two

horses (BCS 4.5 and 5) had navicular disease. Horses were housed on pasture with free choice grass hay.

Morphometrics

Height, length, heart girth, abdominal girth,[24] and neck circumference[28] were measured as previously described. Body mass index was calculated as previously described.[22]

Effect of Environment on OST

To evaluate the effect of environment on OST results, OST was performed as previously described.[49] All OSTs were performed during a 4 week period between mid-October and mid-November and initiated between 11 am-12 pm to minimize potential seasonal and diurnal variation in glucose and insulin response due to changes in pasture non-structural carbohydrate (NSC) content. The order of the OSTs was randomized, with 4 horses undergoing an OST on pasture first and 4 horses undergoing an OST in a stall first. OST pasture and OST stall for each horse was performed 24 hours apart. Horses undergoing OST stall were fasted for 12 hours (no hay) prior to initiation of the OST. Horses were weighed using an electronic scale on the day of testing. Blood samples were collected at baseline and 75 minutes after the administration of Karo[a] syrup (0.15 ml/kg, PO). Immediate analysis of blood glucose was performed by hand-held glucometer[b] that has been previously validated in the horse[216] and was independently validated by the investigators. Additional blood samples were placed on ice until centrifuged. Serum was stored at -80°C for analysis of insulin concentration by radioimmunoassay.[c,203] Horses were considered to be insulin sensitive if fasting (T0i) and T75i were <60 μIU/mL.[49]

Dynamic Insulin Sensitivity Testing

Over a four-week period, all horses underwent an OST while stalled (OST stall), HEC, and IRDT. The HEC was performed 36-48 hours after the last OST. All horses were given at least a seven-day washout period between the IRDT and any other dynamic test.

Hyperinsulinemic Euglycemic Clamp

The HEC was performed as previously described. [71] Following a 24-48 hour stall acclimation period, intravenous catheters were placed. Intravenous catheters were placed at least 12 hours prior to initiation of the clamp. Horses were weighed the morning of the clamp. Insulin was prepared in 0.9% saline (100 mU/mL using 2 mL of homologous serum). Baseline blood samples were taken prior to initiation of the clamp for immediate analysis of blood glucose using a hand-held glucometer.[b] Whole blood was saved on ice for evaluation of insulin concentration. A constant rate infusion of insulin (3 mU/kg/min) was then initiated with infusion of 50% dextrose used to maintain euglycemia.[69-71,215] Blood glucose was monitored every ten minutes. Target blood glucose concentration was 99 mg/dL. Serum was saved for subsequent analysis of insulin concentrations by chemiluminescent immunoassay.[d] The first 90 minutes were used as an equilibration period. Once a steady-state of euglycemia was achieved, serum glucose and insulin concentrations from a 30 minute period were used to calculate the amount of glucose metabolized (M) and insulin concentration (I).

Insulin response to dexamethasone test

Analysis of insulin response to dexamethasone administration was performed as described.[82] A blood sample was obtained at 4 pm for assessment of serum insulin concentration. Dexamethasone (0.04 mg/kg) was injected intramuscularly and a second blood sample obtained 19 hours after injection.

Statistical analysis

Correlation coefficients were calculated to evaluate the relationship between BCS, morphometrics, and results of dynamic tests. The blood glucose (T75g) and insulin (T75i) concentrations 75 minutes after initiation of the OST for the OST pasture and OST stall were compared by a paired t-test to determine repeatability. As the OST results in the pasture did not differ from that in the stall, only the results of the OST stall were used for additional comparison to the HEC, and IRDT. For comparisons of insulin sensitivity status, the M:I ratio (HEC), T0 and T75 insulin and glucose concentrations (OST) and 19-hr post dexamethasone insulin concentrations (IRDT) were compared using a Spearman rank coefficient of correlation.

Experiment 2: Seasonal variation of the OST

Horses

Horses used in this study were aged 8-23 years. Body condition score ranged from 3-8 (median, 5). There were two Arabian mares, five Quarter Horses (two geldings and three mares), and one Thoroughbred gelding. Horses were housed on pasture with free choice grass hay. The three Quarter Horse mares were supplemented with 2.5 pounds of grain once daily. Horses underwent OSTs (as described above) three times, once between June 8 - July 10 (OST Summer) and twice in fall (OST September and OST October). Blood glucose and serum insulin concentrations were measured at baseline and 75 minutes after the administration of Karo syrup as described above.

Statistics

Analysis of variance for repeated measures was used to evaluate the effect of time (month) on fasting insulin concentration, T75i and T75g. Bonferroni's *post-hoc* correction was applied when significance was $p \leq 0.05$.

RESULTS

Experiment 1

Based on OST results, all horses were considered to be insulin sensitive.[49] When evaluating the relationship between OST stall and OST pasture, there was no significant difference between OST T75g ($p=0.32$), although there was a trend toward a difference in OST T75i (mean difference, 2.7 μIU/mL; $p=0.08$; Figure 10). Due to the similarities between the two tests, the OST stall was used for additional comparisons between insulin sensitivity methods. There was poor correlation between the HEC and other tests (Figure 11). A moderate correlation existed between the IRDT and OST T75i ($r=0.54$), although this relationship failed to reach significance ($p=0.16$) (Figure 3).

Simple correlation analysis revealed a strong correlation between girth and other assessments of obesity or body mass (BCS, weight, body mass index, and neck circumference; Table 2). No index of obesity correlated with indices of insulin sensitivity.

Experiment 2

Season had a significant impact on the OST T75g ($p=0.02$), with the difference attributable to the Summer to September interval ($p<0.05$, Figure 12). Peak OST insulin (T75i) exhibited a trend toward significance ($p=0.06$) with season, but fasting insulin did not change ($p=0.17$, Figure 13).

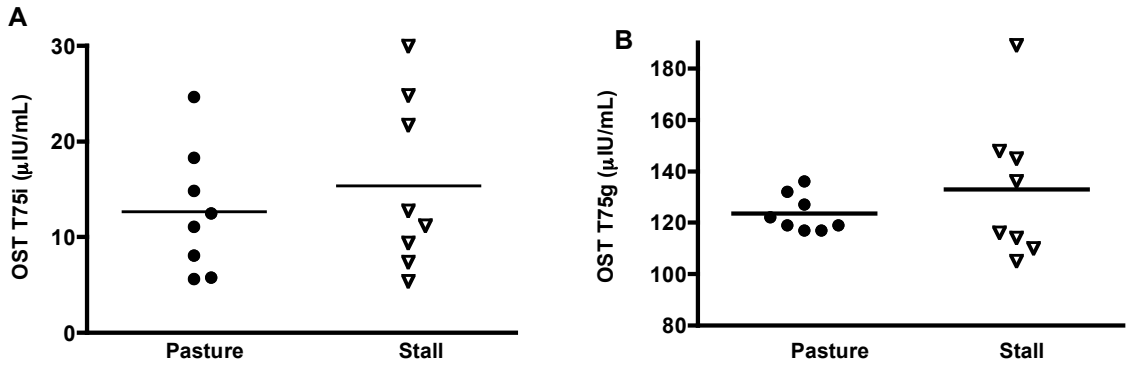

Figure 10. Comparison between OST pasture and OST stall: A) OST T75i, B) OST T75 glucose concentration (OST T75g).

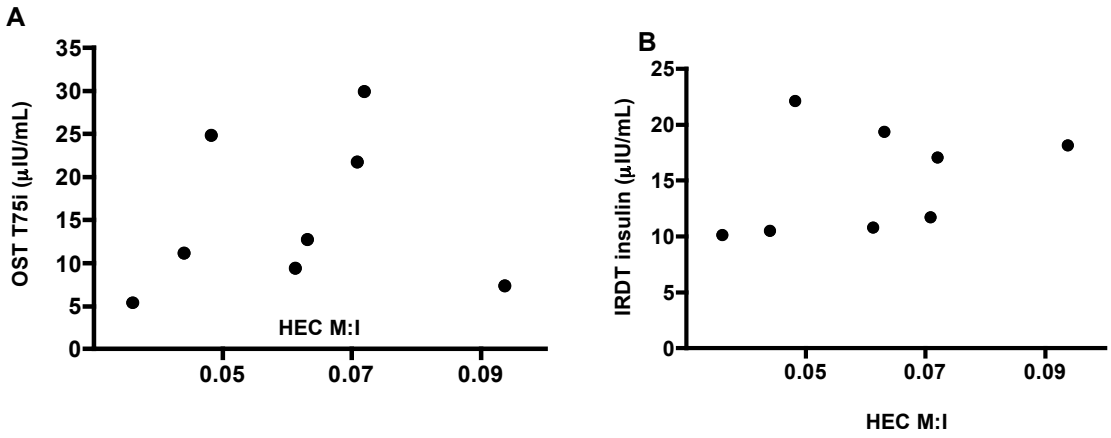

Figure 11. Scatterplot depicting the relationship between dynamic insulin sensitivity test results. a) HEC and OST T75 insulin concentration (OST T75i); r=0.31, p=0.46, and b) HEC and IRDT insulin (IRDTi); r=0.50, p=0.22. M:I = mmol glucose/kg•min^{-1}/pmol insulin/L*100.

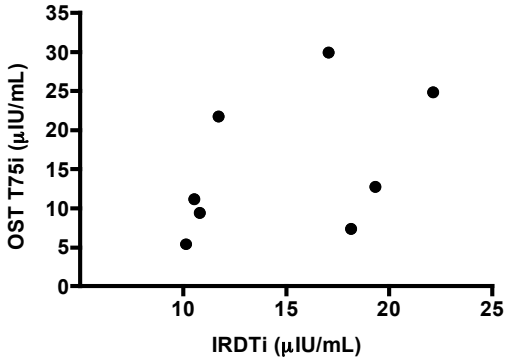

Figure 12. Scatterplot demonstrating relationship between OST T75i and IRDTi (r=0.55, p=0.16).

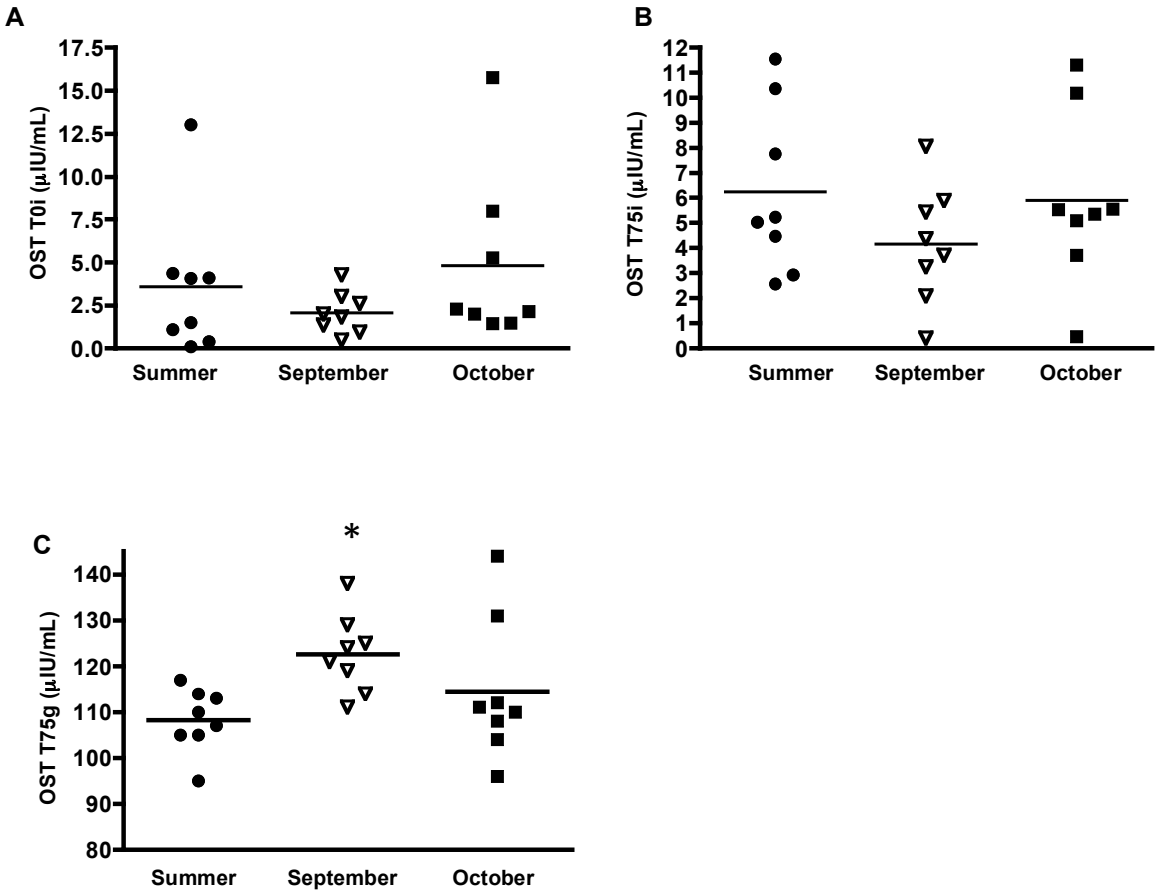

Figure 13. Differences between OST results across season. a) OST T0i, b) OST T75i, c) OST T75g.

*p≤0.05 compared to Summer.

Table 2. Coefficients of correlation for indices of obesity and insulin sensitivity for the HEC, IRDT, and stall OST. BMI = body mass index. M = rate of glucose metabolism. M:I = rate of glucose metabolism: serum insulin concentration. OST T75i = oral sugar test (OST) serum insulin concentration at 75 minutes. OST T0i = fasting serum insulin prior to the OST. OST T75g = OST blood glucose concentration at 75 minutes. IRDTi = serum insulin concentration 19 hours after dexamethasone. *p≤0.05. **p≤0.01.

	BCS	**Weight**	**BMI**	**Neck**	**Abdominal girth**	**Girth**	**M:I**	**OST T0i**	**OST T75i**	**OST T75g**
Weight	0.61									
BMI	0.75*	0.88**								
Neck	0.51	0.52	0.57							
Abdominal girth	0.52	0.74*	0.74*	0.29						
Girth	0.82**	0.88**	0.86**	0.71*	0.53					
M:I	0.20	-0.17	-0.33	-0.40	0.05	-0.10				
OST T0i	0.49	-0.05	0.24	0.17	0.21	0.25	0.26			
OST T75i	0.58 (p=0.07)	0.45	0.52	0.24	0.64 (p=0.09)	0.55	0.31	0.79*		
OST T75g	0.14	0.17	-0.05	0.24	-0.36	0.42	0.19	0.10	0.19	
IRDTi	0.40	0.17	0.12	-0.19	0.10	0.30	0.50	0.38	0.55	0.50

DISCUSSION

Insulin resistance is defined as decreased responsiveness of tissues to insulin.[14] Diagnosis of IR can be challenging due to the dynamic nature of glucose homeostasis. Glucose tolerance is often used as a surrogate marker of insulin resistance. In people, multiple tests have been developed for evaluation of glucose tolerance and tissue insulin sensitivity in research and clinical settings, including IV glucose and insulin tolerance tests, oral glucose tolerance tests, and proxies. The HEC is considered to be the gold standard of insulin sensitivity testing.[14] The HEC prevents pancreatic insulin secretion in response to glucose by providing an exogenous source of insulin. In people, approximately 85% of infused glucose is taken up by skeletal muscle, with lesser uptake occurring in splanchnic bed, insulin-insensitive tissues, and adipose.[15] Unfortunately, the HEC is impractical for widespread clinical use in human medicine due to technical challenges and expense.

The IVGTT was the first method used for evaluation of insulin and glucose dynamics in horses.[72] Intravenous dynamic glucose tolerance test results are reflective of both tissue insulin sensitivity and pancreatic beta cell response. In order to improve identification of tissue IR, an insulin-modified frequently sampled IV glucose tolerance test (FSIGTT) was subsequently developed.[76] The minimal model analysis used to interpret the FSIGTT can characterize pancreatic response as well as tissue insulin sensitivity.[217] Despite this improvement in diagnosis of tissue insulin sensitivity, the frequent sampling required makes this test challenging to use in the field. A combined glucose insulin tolerance (CGIT) test was created as a more practical field test for evaluation of tissue insulin sensitivity following simultaneous administration of glucose and insulin. Less frequent sampling is performed during the CGIT compared with the FSIGTT, but the test still necessitates placement of an IV catheter, making it less convenient as a field test. An oral glucose tolerance test (OGTT) was adapted for use in the horse[85] which does not require IV catheter placement. However, nasogastric intubation must be performed in order to ensure administration of

oral dextrose solution. Nasogastric intubation is a procedure that horses may be stressful for some horses. Stress hormones can impact glucose dynamics,[218] potentially resulting in testing inaccuracy.

An easy, accurate test for diagnosis of equine insulin sensitivity in a clinical setting remains to be identified. In people, the oral glucose tolerance test is considered a reasonable surrogate dynamic test of insulin sensitivity in human clinical medicine, as it correlates well with the HEC.[219] In horses, the relative roles of the entero-insular axis and tissue insulin sensitivity in maintenance of glucose homeostasis following administration of oral glucose or intramuscular dexamethasone are unknown. In this study, two recently developed field tests were compared to the HEC in order to determine how the new tests compared with the established method of diagnosis of insulin sensitivity.

The results of the current study revealed no relationship between the HEC and either the IRDT or OST. A moderate correlation was observed between the IRDT and OST. The lack of association between the HEC and other tests may be due to the differences in relative roles of tissue insulin sensitivity, pancreatic insulin secretion, and enteral absorption in each test. The HEC measures tissue insulin sensitivity, isolated from the influence of pancreatic response.[67] Glucose homeostasis following dexamethasone administrations is influenced by tissue insulin sensitivity and pancreatic insulin response.[84,220,221] Interestingly, glucocorticoids have also been demonstrated to enhance enteral uptake of glucose in rats,[222] but not in people.[223] The OST is influenced by GI absorption, incretin hormones, pancreatic insulin secretion, and tissue insulin sensitivity.[89,90,92] The absence of relationship between the HEC and other dynamic tests observed here suggests that among insulin sensitive horses, tissue insulin sensitivity is not the key predictor of serum insulin concentrations following an oral glucose challenge or administration of dexamethasone.

The OST was previously found to correlate well with an IVGTT in a group of 18 obese (BCS≥7, n=10) and non-obese (BCS≤6, n=8) horses.[94] The correlation found in that study may have been the result of a more diverse population as 4/10 obese horses had evidence of severe IR,

including fasting hyperinsulinemia.[94] In contrast, the horses included in the current study were insulin-sensitive and normoinsulinemic, although two of the selected horses had a history of an abnormal OST within the previous six months. Alternatively, it may be that the IVGTT is better correlated to the OST as it reflects both pancreatic response and insulin sensitivity, while the HEC is indicative of only tissue insulin sensitivity. In our study, there was a stronger association between the OST and IRDT than between the OST and HEC or IRDT and HEC. It is attractive to speculate that an exaggerated pancreatic response to an IV or oral glucose challenge may be an important component of insulin dysregulation in the horse.

In addition to alterations in glucose dynamics caused by disease state, glucose homeostasis can also be impacted by changes in housing environment through stress of a new environment or changes in feed composition. Stress hormones such as catecholamines inhibit pancreatic secretion of insulin and impair tissue sensitivity.[218] Housing changes may also influence the results of testing due to differences in NSC content consumed in hay compared with pasture. Increases in dietary NSC content result in a rapid and dramatic increase in circulating insulin concentration,[82] and long term feeding of a high starch diet can impair tissue insulin sensitivity.[76] In this study, there was a trend towards a difference in T75i from OST pasture to OST stall. However, the difference between insulin concentrations in both tests was small (mean, 2.7 μIU/mL) indicating that an acute change in housing environment has minimal influence on OST results.

With any oral glucose challenge test, it is likely that season will have an effect due to differences in NSC content. In order to further characterize differences that occurred with the OST across time, the OST was evaluated in 8 horses on pasture at three time points during the summer and fall. In the horses of this study, there were no significant differences detected in fasting plasma insulin over time. However, there was an effect of time on T75g, with horses having a higher glucose response in Summer than in September. A similar, although non-significant, change in T75i was observed over time. These results indicate that alterations in season can impact results of the OST.

In contrast, there was no seasonal impact on results of a CGIT.[224] In that study, the horses were kept on a dry lot with free choice grass hay. The differences between that study and the OST results reported here may be due to seasonal changes in pasture composition.[225]

In conclusion, this study highlights the differences between dynamic testing methodologies in a population of Quarter Horses of varying BCS. Care must be taken when attempting to extrapolate these findings to other breeds. Further studies are needed to identify the relative contribution of pancreatic secretion, tissue insulin sensitivity, and enteral glucose absorption to abnormalities of glucose homeostasis in the horse.

Footnotes

[a]Karo Light syrup, ACH Food Companies, Inc., Memphis, TN

[b]AlphaTRAK, Abbott Laboratories, Abbott Park, IL

[c]Siemens Coat-A-Count, Tarrytown, NY

[d]Siemens Immulite 1000, Tarrytown, NY

CHAPTER IV

ROLE OF OXIDATIVE STRESS IN EQUINE OBESITY-ASSOCIATED INSULIN RESISTANCE

ABSTRACT

Obesity in other species is associated with oxidative stress within insulin-sensitive tissues and systemic circulation. We hypothesized that obesity-associated mitochondrial dysfunction causes oxidative stress, which leads to skeletal muscle insulin resistance in horses. Thirty-five light breed horses with body condition scores of 3/9 to 9/9 were included in the study. Biomarkers of oxidative stress were evaluated in skeletal muscle biopsies and plasma. Relationships between serum insulin concentration, body condition score (obesity) and markers of mitochondrial function, antioxidant capacity, and oxidative damage were evaluated. Markers of oxidative stress were compared between insulin resistant (IR) and insulin sensitive (IS) horses. Of the markers evaluated, obesity and gene expression of mitofusin 2 (Mfn2) predicted serum insulin. Regulators of mitochondrial dynamics were upregulated with obesity and IR. While there was no evidence that oxidative damage was associated with IR status, lipid peroxidation and antioxidant capacity did increase with obesity. Obesity is associated with increased skeletal muscle oxidative stress; however, upregulation of antioxidant capacity and alterations in mitochondrial dynamics appear to protect against oxidative stress-induced IR.

INTRODUCTION

Obesity is an increasingly common condition of all domestic species, including horses. Owner surveys and prospective observational studies in the United States and Europe indicate an equine obesity prevalence of 45-55%.[2-4] In people, obesity is associated with the development of insulin resistance (IR) and type II diabetes. Similarly, in horses, obesity is associated with IR[19] and fasting hyperinsulinemia.[20] In the obese state, fasting hyperinsulinemia may be an indication of pancreatic beta cell compensation for peripheral tissue insulin resistance, although it is recognized that hyperinsulinemia can also occur independent of IR and even induce IR.[16] In people, beta cells are unable to maintain a sustained state of compensation and type II diabetes ensues. In contrast, horses appear to be able to maintain a state of compensated insulin resistance, as type II diabetes is rarely recognized.[226]

In people, obesity-associated insulin resistance is attributed to intracellular lipid accumulation,[227] inflammation,[158] and/or oxidative stress.[147] Increased oxidative stress is a consistent finding in obesity, and is identified both within insulin-sensitive tissues[228,229] and systemic circulation.[229]

Oxidative stress is defined as a disruption in the balance between exposure of the cell to reactive oxygen species (ROS) and its antioxidant capacity resulting in a pro-oxidant state. This pro-oxidant state allows for structural modifications to lipids, proteins and DNA that can alter cellular function. These changes may be reversible or permanent depending upon the severity and duration of oxidative insult to the cell. Either increased ROS production or decreased antioxidant capacity can result in oxidative stress. Increased ROS production may be due to increased production by mitochondria or by enzyme systems including the nicotinamide adenine dinucleotide phosphate oxidase and xanthine oxidase. In people with type II diabetes, skeletal muscle has been demonstrated to have low mitochondrial content,[110,230] decreased respiratory capacity[231] and alterations in

mitochondrial dynamics.[112,232] Impaired mitochondrial function may in part be due to decreased expression of transcription factors that regulate mitochondrial biogenesis and mitochondrial oxidative phosphorylation.[122,123]

Despite the prevalence of equine obesity, mechanisms of obesity-associated insulin resistance remain to be identified. Systemic oxidative stress has not been identified.[140,233] However, oxidative stress in tissues likely precedes the development of systemic oxidative stress,[229] and the role of oxidative stress in insulin-sensitive tissues has not been evaluated.

We hypothesized that equine obesity is associated with mitochondrial dysfunction and oxidative stress, which leads to insulin resistance (Figure 14). To test this hypothesis, relationships between serum insulin concentration, body condition (obesity) and markers of mitochondrial function and oxidative stress were assessed.

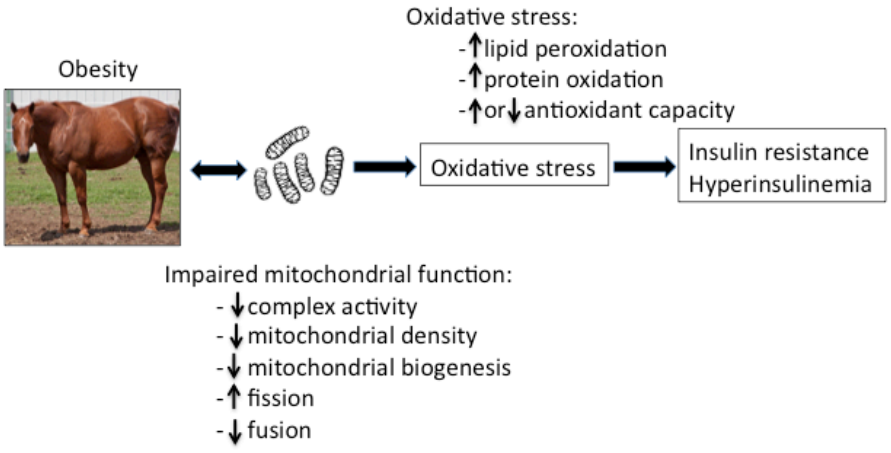

Figure 14. Schematic of the proposed relationship between obesity, mitochondrial function, oxidative stress and insulin resistance. Photo courtesy of Kim Hill.

MATERIALS AND METHODS

Sample population

Blood samples and skeletal muscle biopsies were collected from 35 horses. Breeds represented included Quarter Horse (n=20), Thoroughbred (n=4), Paint (n=3), Paso Fino (2), Appaloosa (1), Missouri Foxtrotter (1), Azteca (1), Arabian (1), Tennessee Walking Horse (1), and Morgan (1). Samples were collected from horses in the fall (August-October, n=5) and non-fall (November-July, n=30). There were 16 mares and 19 geldings. Age ranged from 2-27 (mean 14 +/- 6 years). Body condition score was assessed in all animals, while dynamic insulin sensitivity testing was undertaken in a subset of 19 horses, and consisted of either a frequently sampled IV glucose tolerance test (FSIGTT, n=6),[76] hyperinsulinemic euglycemic clamp (HEC, n=3),[68] or an oral sugar test (OST, n=10).[49] Horses were considered insulin resistant (IR) on the basis of an insulin >60 μIU/ml at 0 or 75 minutes (OST), an insulin sensitivity index <1.0 (FSIGTT), or a glucose infusion rate < 0.015 mmol/kg/min (HEC). Semi-membranosus muscle biopsies were collected ante mortem (n=15) or immediately following euthanasia (n=20). Samples were obtained in accordance with the Institutional Animal Care and Use Committee at Oklahoma State University.

Hormone analysis

Serum insulin concentration was measured by radioimmunoassay.[a] Plasma ACTH concentration was determined by chemiluminescent assay.[b] Both assays were previously validated for use in horses.[203,234]

Muscle homogenates

Muscle samples were diluted in PBS and homogenates prepared using a tissue homogenizer.[c] Homogenates were centrifuged at 1000 g for 10 minutes. Supernatant protein concentration was quantified using a commercially available assay.[d]

Carbonylated proteins

Carbonylated proteins were derivatized in a sample of skeletal muscle homogenate as previously described,[235] with slight modifications. A commercially available oxidized protein[e] was used as a positive control. Samples were diluted in TBS and 2.5 μg of protein applied to a PVDF membrane via a slot blot apparatus. The membrane was stained with Ponceau S to evaluate protein loading. The membrane was then blocked in 5% milk/TBS for one hour, washed three times in TBS/0.05% Tween, and incubated with anti-DNPH primary antibody[f] (1:13000) overnight at 4°C. Following washing with 5% milk/TBS/1% Tween, the membrane was incubated in secondary antibody[g] (1:10000) at room temperature for one hour. Detection was carried out by chemiluminescence.[h] Bio-Rad software[d] was used for quantitative analysis of the slot blot, accounting for any difference in protein loading with Image J software.[i]

Thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS) were evaluated in plasma and muscle homogenate supernatant via a commercially available kit.[j] Plasma was analyzed as detailed by the manufacturer. Muscle supernatants were diluted to a protein concentration of 2 mg/ml in PBS, and samples were analyzed according to manufacturer's directions.

Gene expression

Total RNA was extracted from approximately 30 mg tissue, using TRIzol extraction.[k] For quantitative polymerase chain reaction (PCR), total RNA was treated with DNAse[l] for 30 minutes at 37°C to remove potential residual DNA, and then cDNA was transcribed according to the

manufacturer's directions.[m] Equine-specific primers were designed with Primer3[n] from published equine sequence data[o] and used to amplify peroxisome proliferator activated receptor gamma coactivator 1α (PGC1α), PGC1β, estrogen related receptor α (ERRα), manganese superoxide dismutase (MnSOD), glutathione peroxidase (GPX), glutathione synthase (GSS), glutathione redutase (GRS), catalase, peroxiredoxin (PRX), nuclear respiratory factor (Nrf) 1, Mfn2, and dynamin-related protein (Drp) 1 using β-actin as a housekeeping gene. Quantitative PCR was performed in triplicate in a thermocycler.[m] Reaction volume was 25 µL, consisting of 10 µL of 1/20 dilution of cDNA, 12.5 µL of a SYBR Green master mix[m] and 10 mM of forward and reverse primers.

The relative expression (RE) of each gene was calculated using the formula:

$$RE = 2^{-(Ct\ gene\ of\ interest - Ct\ \beta actin)}$$

Mitochondrial DNA content

DNA was extracted from approximately 15 mg of tissue using a commercially available kit.[p] Equine-specific primers were designed with Primer3[n] using published equine sequence data[o] and used to amplify the mitochondrial-encoded genes, nicotinamide adenine dinucleotide dehydrogenase (NADH-dh) and cytochrome c oxidase subunit 2 (COX2) using β-actin as a nuclear reference gene.

Antioxidant assays

Total SOD (TotSOD) activity was measured in muscle homogenate supernatants using a commercially available kit[q] and MnSOD activity was determined by inhibition of Cu,Zn-SOD with 2.8 mmol sodium cyanide. For assessment of total glutathione, 50 µL of supernatant was added to a reaction mixture containing 0.32 mM 5,5'-dithiobis (2-nitrobenzoic acid), 0.32 mM NADPH, 1.3 U/ml glutathione reductase as previously described.[236] Glutathione peroxidase activity was assessed

in 10 μL supernatant as previously described,[236] using serial dilutions of bovine glutathione peroxidase[e] as an assay standard.

Statistical analysis

Forward stepwise regression was performed to determine the impact of BCS, age, breed, gender, plasma ACTH concentration and markers of oxidative stress on serum insulin concentration. Hormone concentrations and markers of oxidative stress were $\log_{10}$ transformed prior to analysis to improve variance for the model. Markers of oxidative stress consisted of markers of oxidative damage (carbonylated proteins, skeletal muscle TBARS, plasma TBARS), markers of antioxidant capacity (GSH concentration, MnSOD and TotSOD activity, and gene expression of antioxidant enzymes), and markers of mitochondrial function (mitochondrial density and gene expression of PGC1α, PGC1β, ERRα, Drp1, Mfn2, Nrf1). As both markers of mitochondrial density were strongly correlated ($r=0.84$, $p<0.001$), only COX2 was used in statistical analysis. Because dynamic insulin sensitivity testing allows for better evaluation of tissue insulin sensitivity than fasting insulin concentrations, additional analysis was performed comparing markers of oxidative stress between IR (n=11) and insulin sensitive (IS, n=8) horses using a Mann-Whitney U test.

In order to determine if obesity was associated with mitochondrial dysfunction or oxidative stress, Spearman coefficient of correlation was used to evaluate the relationship between obesity, mitochondrial function, and markers of oxidative stress. As obesity may not have a substantial impact on oxidative stress until a state of obesity (BCS ≥7) develops, a Spearman's rank correlation coefficient was also calculated using data only from horses with a BCS ≥ 7/9.

Because large numbers of samples had GPX activity below detection, GPX activity was dichotomized (detectable versus undetectable) and the frequency of detectable enzymatic activity in obese horses was compared to that in non-obese horses using chi square analysis.

RESULTS

Body condition score ranged from 3/9 to 9/9 (median, 5.5). Thirteen horses were considered to be obese (BCS ≥7). Serum insulin concentrations ranged from <3.5 μIU/ml to 405 μIU/mL (median = 8.3 μIU/mL). Eight horses tested IR on the basis of dynamic IR testing, and all of these horses were hyperinsulinemic (serum insulin concentrations >70 μIU/mL). Plasma ACTH concentrations ranged from 12-166 pg/mL (median, 35.4 pg/mL). ACTH was included in the forward stepwise regression model but was not retained, indicating limited influence of ACTH on serum insulin concentration.

The final linear regression model contained two independent variables that accounted for approximately 67% (adjusted r^2=0.67) of the variance in serum insulin concentration. Body condition score was the strongest predictor, accounting for 59% of the variance in the model ($p<0.001$) while expression of Mfn2, the regulator of mitochondrial fusion, accounted for 8% of the variance ($p=0.012$; Figure 15). When comparing markers of oxidative stress between IR and IS horses, gene expression of regulators of mitochondrial dynamics (Drp-1, mitochondrial fission, $p=0.007$; Mfn-2, $p=0.004$), and the transcriptional regulator of oxidative phosphorylation genes, Nrf1 ($p=0.006$) were significantly increased in IR horses (Figure 16). Antioxidant capacity was also altered with IR, with an increase in GSH ($p=0.05$) and a decrease in GSS expression ($p=0.01$; Table 3). No evidence of skeletal muscle ($p>0.05$) or systemic oxidative damage was associated with IR status (Figure 17).

When examining the relationship between BCS and markers of oxidative damage, carbonylated protein concentration ($r=-0.48$, $p=0.005$) was negatively correlated with BCS while skeletal muscle TBARS concentration was positively correlated ($r=0.39$, $p=0.02$; Figure 18). Neither of these markers of oxidative damage remained significantly associated with obesity when only obese horses were examined. Of the markers of antioxidant capacity, only TotSOD activity ($r=0.51$, $p=0.002$) was correlated with BCS, while among obese horses, both TotSOD ($r=0.63$, $p=0.02$) and

GSH (r=0.58, p=0.04) were correlated with increasing obesity (Table 4). There was no significant difference in detection of GPX activity between obese and non-obese horses (p=0.64).

Upregulation of gene expression of Drp1 (r=0.45, p=0.007), a regulator of mitochondrial fission but not Mfn2 (r=0.23, p=0.11), a regulator of mitochondrial fusion was found with increasing BCS. Among obese horses both Drp1 (r=0.61, p=0.03) and Mfn2 (r=0.64, p=0.02) were correlated with increasing obesity. Transcriptional activators of mitochondrial biogenesis were upregulated with increasing BCS (ERRα, r=0.50, p=0.002; PGC1α, r=0.40, p=0.02), but there was no relationship between BCS and mitochondrial content or between gene expression of regulators of mitochondrial biogenesis and mitochondrial content (Table 4).

Evaluation of the relationship between markers of oxidative damage, antioxidant capacity, and mitochondrial function, revealed that Drp1 and Mfn2 were strongly correlated with each other (r=0.83, p<0.001) and also correlated with other mitochondrial and antioxidant genes (Table 4). Mitofusin 2, Drp1, the antioxidant enzyme MnSOD, and the regulator of transcription of oxidative phosphorylation genes, Nrf1, were all negatively correlated with the marker of systemic lipid peroxidation, TBARS, while Mfn2 was positively correlated with skeletal muscle TBARS. Antioxidant gene expression was generally well correlated (Table 4).

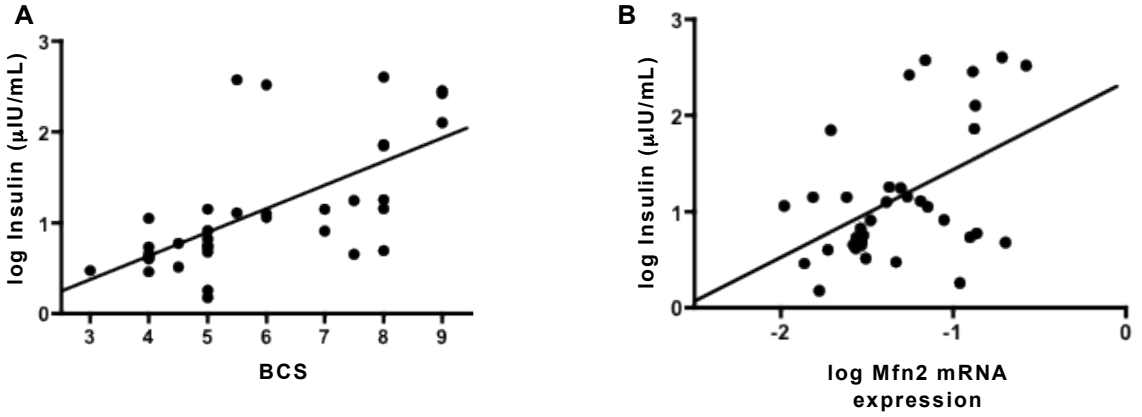

Figure 15. Final linear regression model of serum insulin concentration. Scatterplot of relationship between a) BCS and serum insulin concentrations ($r=0.71$, $p<0.001$) and b) skeletal muscle Mfn-2 expression and serum insulin concentration ($r=0.43$, $p=0.01$). Final regression equation for prediction of serum insulin: 0.676 (BCS) + 0.310 (Mfn2) + 0.485 (adjusted $r^2=0.67$, $p<0.001$).

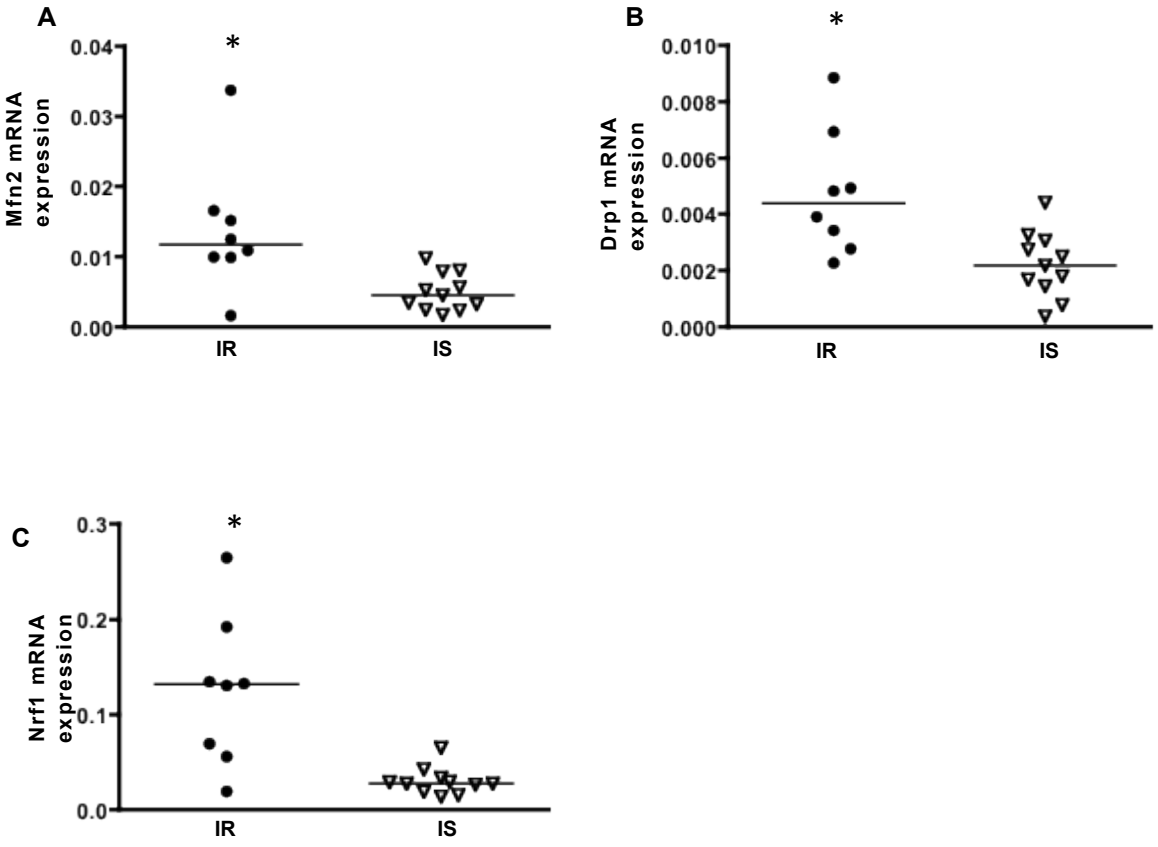

Figure 16. Scatterplot of gene expression of mitochondrial dynamics and mitochondrial function between IR and IS horses. Line indicates median, * p≤0.05. a) Mfn2, b) Drp1, c) Nrf1.

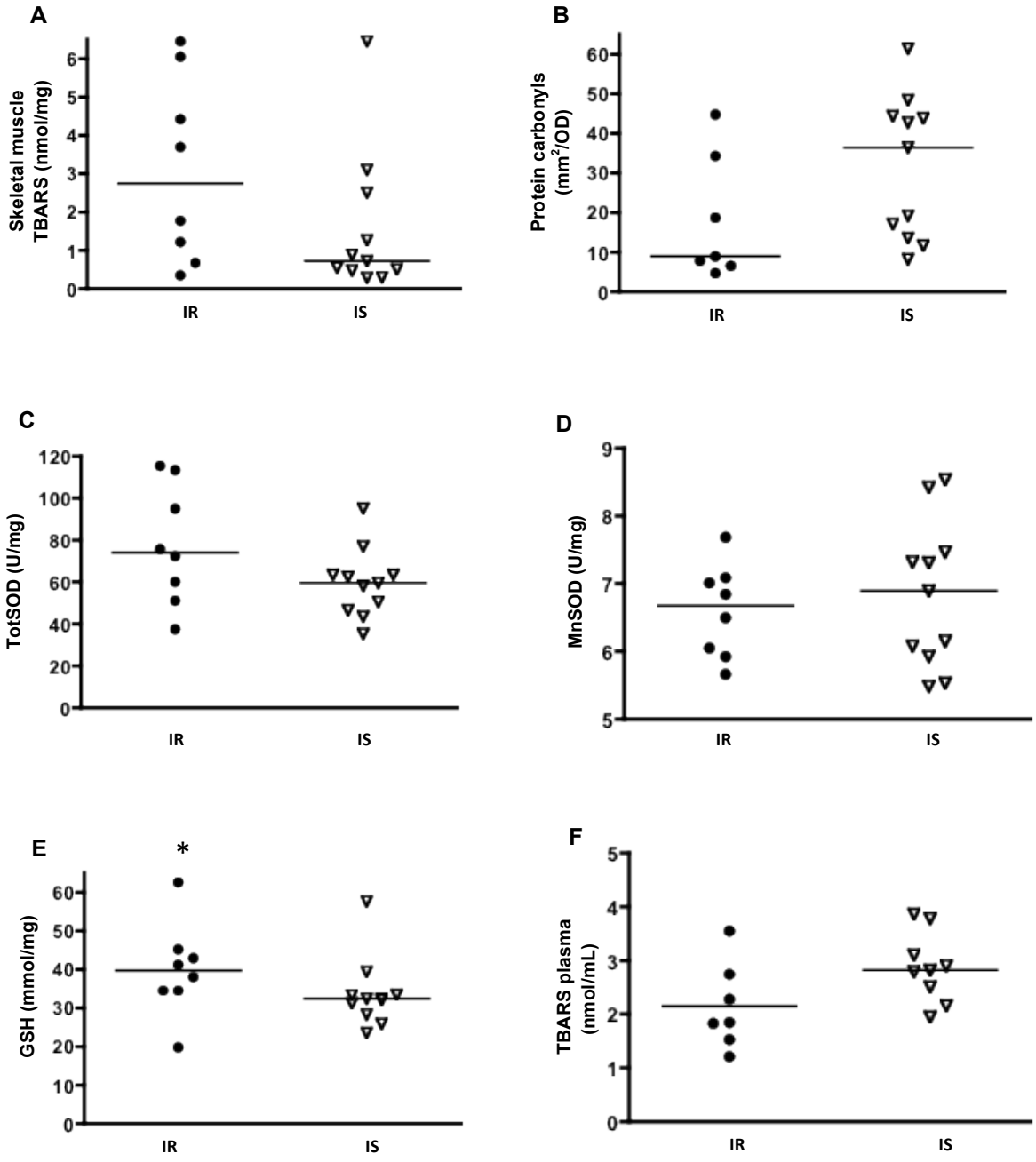

Figure 17. Scatterplot of markers of oxidative damage and antioxidant capacity in skeletal muscle between IR and IS horses. Line indicates median, *p≤0.05. a) skeletal muscle TBARS, b) carbonylated proteins, c) TotSOD activity, d) MnSOD activity, e) GSH, and f) plasma TBARS.

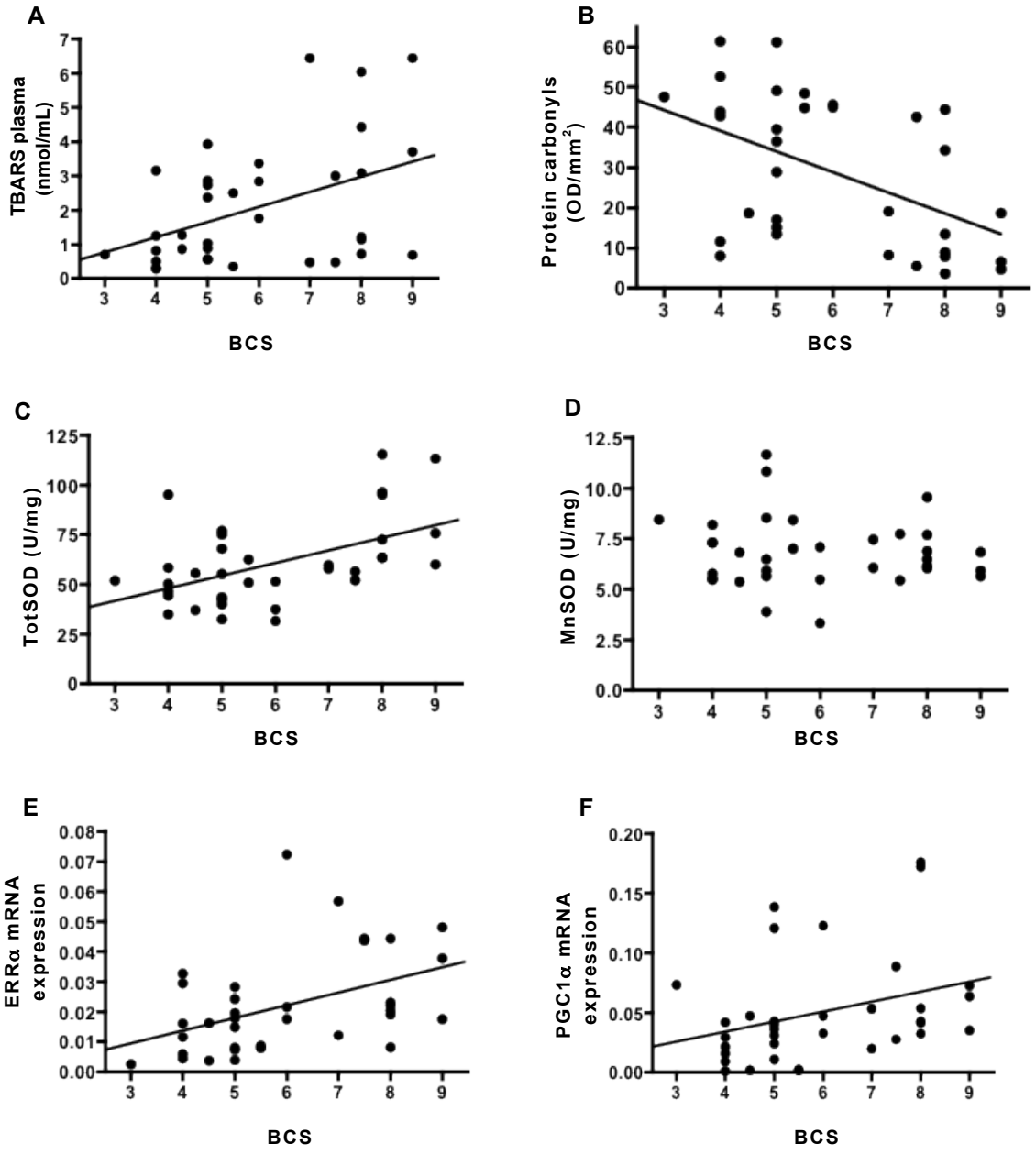

Figure 18. Scatterplot of relationship between BCS and skeletal muscle and selected markers of skeletal muscle oxidative stress. BCS and skeletal muscle a)TBARS (r=0.43, p=0.01), b) carbonylated proteins (r=-0.50, p=0.004), c) TotSOD activity (r=0.52, p=0.002), d) MnSOD activity (r=0.27, p=0.1), e) ERRα mRNA expression (r=0.45, p=0.001), and f) PGC1α mRNA expression (r=0.40, p=0.02).

Table 3. Mitochondrial DNA content and gene expression of markers of oxidative stress in insulin resistant (IR) and insulin sensitive (IS) horses. Data reported as relative expression (RE), median (interquartile range).

Gene expression (RE) or DNA copy number	IR (n=11)	IS (n=8)	P-value
PGC1α (RE)	0.030 (0.0090-0.12)	0.042 (0.034-0.056)	0.64
PGC1β (RE)	0.030 (0.0017-0.0056)	0.0047 (0.0036-0.0097)	0.43
ERRα (RE)	0.022 (0.0088-0.028)	0.028 (0.013-0.046)	0.39
COX2 (DNA copy number)	693 (577-1178)	1067 (678-1232)	0.54
GSS (RE)	0.0025 (0.0017- 0.0041)	0.0060 (0.0045-0.0099)	0.01
GRS (RE)	0.00070 (0.00035-0.0019)	0.0022 (0.0011-0.0033)	0.06
GPX (RE)	0.070 (0.044-0.12)	0.11 (0.07-0.18)	0.20
MnSOD (RE)	0.71 (0.49-0.89)	0.89 (0.56-1.14)	0.30
Catalase (RE)	0.0044 (0.0020-0.0075)	0.0066 (0.00090-0.018)	0.71
PRX (RE)	0.16 (0.083-0.267)	0.38 (0.20-0.44)	0.09

Figure 4. Spearman's coefficient of correlation for obesity, markers of mitochondrial function, oxidative damage, and antioxidant capacity. RE = relative expression. *p<0.05, **p<0.01.

	BCS	TBARS plasma	TBARS muscle	GSH	Protein carbonyls	MnSOD activity	TotSOD activity	PGC1α (RE)	ERRα (RE)	COX2 (RE)	Nrf1 (RE)	MnSOD (RE)	Mfn2 (RE)	Drp1 (RE)	PGC1β (RE)	Cat (RE)	PRX (RE)	GSS (RE)	GRS (RE)
TBARS plasma	-0.22																		
TBARS muscle	0.39*	-0.16																	
GSH	0.19	-0.08	-0.15																
Protein carbonyls	-0.48	0.23	-0.18	-0.31															
MnSOD activity	-0.03	-0.01	0.15	-0.14	0.24														
TotSOD activity	0.51*	-0.12	0.22	0.48*	-0.37*	0.32													
PGC1α (RE)	0.40*	-0.23	0.22	0.05	0.10	0.27	0.21												
ERRα (RE)	0.50*	-0.15	-0.07	0.08	-0.27	0.01	0.10	0.39*											
COX2 (RE)	0.07	0.37*	-0.18	-0.18	0.14	0.06	-0.19	-0.09	0.05										
Nrf1 (RE)	-0.08	-0.48	0.15	0.16	-0.14	-0.35*	-0.20	-0.08	0.00	-0.31									
MnSOD (RE)	0.27	-0.34*	-0.01	0.18	-0.25	0.21	0.24	0.36*	0.42*	-0.24	0.27								
Mfn2 (RE)	0.23	-0.36*	0.35*	0.06	-0.07	0.29	0.09	0.27	0.26	-0.17	0.49**	0.53**							
Drp1 (RE)	0.45*	-0.35*	0.28	0.03	-0.22	0.16	0.09	0.26	0.50**	-0.17	0.36*	0.57**	0.83**						
PGC1β (RE)	-0.06	-0.12	-0.25	0.04	0.08	-0.05	-0.05	-0.19	0.00	-0.13	0.28	0.39*	0.46*	0.28					
Cat (RE)	0.11	0.02	0.05	-0.26	0.24	0.05	-0.11	0.18	0.31*	0.01	0.10	0.11	0.30	0.19	0.47**				
PRX (RE)	-0.01	-0.29	-0.04	-0.09	-0.15	-0.07	-0.16	-0.16	0.24	0.02	0.45	0.26	0.59**	0.47**	0.47**	0.28			
GSS (RE)	0.13	-0.23	-0.01	0.08	-0.23	-0.16	-0.03	-0.13	0.32	0.00	0.54**	0.15	0.61**	0.41*	0.52**	0.44**	0.83**		
GRS (RE)	0.01	-0.23	0.07	-0.04	-0.14	-0.04	-0.13	-0.14	0.41*	-0.01	0.45**	0.17	0.62**	0.48**	0.40*	0.38*	0.86**	0.87**	
GPX (RE)	0.32	-0.14	0.07	-0.17	0.10	0.11	-0.07	0.14	0.50**	0.24	0.04	0.16	0.36*	0.34*	0.19	0.38**	0.46*	0.51**	0.52*

DISCUSSION

Obesity-associated insulin resistance is a risk factor for the development of cardiovascular disease in people and laminitis in horses. In other species, obesity has been associated with mitochondrial dysfunction, oxidative stress and insulin resistance.[109,137,229,237] In obese states, an increase in ROS production may initially be compensated for by an upregulation in antioxidant capacity.[127-129] Oxidative damage occurs due to insufficient antioxidant response and eventual depletion of antioxidant capacity. Normally, a majority of cellular ROS are produced by the mitochondrial electron transport chain, primarily complex I (NADH-dehydrogenase) and complex III (cytochrome bc), as a byproduct of oxidative phosphorylation.[238] Excessive substrate delivery to the mitochondria or impaired mitochondrial function can substantially increase ROS production.

Increased mitochondrial ROS production may affect the mitochondria in multiple ways. ROS can cause mtDNA mutations due to proximity of ROS production. ROS also stimulate mitochondrial biogenesis,[239] although this adaptation does not appear to persist in states of chronic ROS exposure. Mitochondrial ROS production is also associated with alterations in mitochondrial dynamics, including increased fission[115] and decreased fusion. Initially, increased ROS production may be neutralized by an upregulation in antioxidant defenses. Within the mitochondria, the primary antioxidant enzymes include MnSOD, PRX, and GPX. Cytosolic free radicals may be scavenged by additional antioxidants, including the glutathione system and the antioxidant enzymes Cu, Zn-SOD and catalase. Insufficient upregulation of antioxidants can result in a cellular pro-oxidant state and oxidative damage.[129]

In addition to causing oxidative damage to the cell, ROS can also activate several stress kinases, including inhibitor of nuclear factor B kinase-β (IKKβ) and c-Jun-N-terminal kinase (JNK). In insulin-sensitive tissues, including skeletal muscle, both IKK and JNK can cause inhibitory (serine) phosphorylation of the insulin substrate protein 1, decreasing downstream signaling. The end result is

decreased translocation of the glucose transporter 4 protein to the cellular membrane and decreased insulin-dependent glucose transport into the cell. Fasting hyperinsulinemia is often considered a surrogate marker of IR in obesity, as high serum insulin concentrations are considered an appropriate compensatory response of pancreatic beta cells to IR. However, because IR may precede the development of hyperinsulinemia, dynamic testing is an important component of evaluating tissue insulin sensitivity. In people, the HEC is considered the gold standard for evaluation of insulin sensitivity.[67] The HEC has been demonstrated to correlate well with oral glucose tolerance testing,[219] and both tests are widely used. In contrast to people, where methodology is well-established, dynamic insulin sensitivity testing in horses is an evolving process with several new tests being developed over the past several years. Over the course of sample collection for the present study, recommendations for testing insulin sensitivity varied. In addition, it was not feasible to do the more resource intensive tests in all animals. Therefore, dynamic testing was performed in a subset of 19 horses using a variety of methods. Additionally, fasting serum insulin concentrations were used as an index of insulin sensitivity across the entire population.

In people, skeletal muscle IR has been attributed to impaired mitochondrial function. Obesity is associated with decreased oxidative phosphorylation within skeletal muscle, which leads to increased mitochondrial ROS production, oxidative stress, and impaired insulin signaling.[240] Impaired insulin-dependent glucose transport has been identified in the skeletal muscle of IR horses, but the mechanism causing the defect has not been elucidated.[105] Therefore, in this study, we chose to evaluate several markers of mitochondrial function, including mitochondrial biogenesis, content, and dynamics to determine if mitochondrial function was impaired in horses with obesity-associated IR. Because impaired mitochondrial function is associated with increased ROS production, measurements of oxidative damage and antioxidant capacity were undertaken.

Mitochondrial dynamics describes the morphological changes mitochondria can undergo in response to cellular environment and intracellular signals. The mitofusins, Mfn1 and Mfn2, are

membrane proteins involved in mitochondrial fusion. Fusion is the process of mitochondrial union, which allows for transfer of mtDNA and rescue of membrane potential. Mitochondria may also fragment in order to allow for removal of dysfunctional parts of the mitochondria, a process known as fission. Fission is mediated primarily by two proteins, Drp1 and fission 1 (Fis1). Excessive fission or inadequate fusion causes the formation of small, globoid mitochondria, while the converse results in formation of long reticulated networks. The maintenance of a normal mitochondrial network is important for mitochondrial function, and downregulation of Mfn2[113] or Fis1[114] results in impaired oxidative phosphorylation. In the horses of this study, the only oxidative stress marker that was predictive of plasma insulin concentration was expression of Mfn2. Mfn2 and Drp1 expression were also increased in IR horses and with increasing obesity among obese horses.

In addition to its key role in mitochondrial morphology, Mfn2 functions as a regulator of metabolism. Mitofusin 2 has been demonstrated to be important in mediating glucose oxidation, insulin signaling, and mitochondrial oxidative phosphorylation in skeletal muscle and liver, and overexpression of Mfn2 improves glucose oxidation and mitochondrial oxidative phosphorylation.[117]

Gene expression of Mfn2 in skeletal muscle is decreased in human obesity[113] and type II diabetes.[116] An increase in mitochondrial-associated fission protein, Fis-1, and mitochondrial fragmentation have been observed in high fat fed and genetically obese rodents.[112] Taken together, these findings indicate a shift in mitochondrial dynamics towards fission in the obese, IR state. In contrast, in this study, regulators of both fission and fusion were increased with IR, while only the regulator of fusion, Mfn2, was predictive of serum insulin concentrations. This suggests that Mfn2 may be upregulated as a protective mechanism, either in response to a defect in glucose metabolism or in response to increased fission. In a normal physiological state, mitochondrial dynamics are auto-regulated and increased fission activates signaling mechanisms that lead to increased fusion. Increased ROS production is associated with increased mitochondrial fission in cultured myoblasts during conditions of excessive glucose[115] or saturated fatty acids,[112] and inhibition of fission

normalizes ROS level.[241] Activation of Mfn2 is protective against ROS-induced damage to the mitochondrial matrix.[111]

Mitochondrial dynamics proteins also play an important role in mitochondrial biogenesis. Mitochondrial biogenesis is the process of mitochondrial division, which is a complex process that involves replication of mitochondrial DNA (mtDNA) and incorporation of proteins and lipids into the mitochondrial membrane.[118] The peroxisome proliferator-activated receptor gamma coactivators are considered to be the master regulators of mitochondrial biogenesis. PGC1β is constitutively active, and appears to be the most important regulator under normal physiologic conditions, while PGC1α is inducible. Estrogen related receptor α (ERRα) is a transcriptional factor that is downstream of PGC1α and PGC1β. ERRα activates transcription of regulators of oxidative phosphorylation genes, including Nrf1. Mitochondrial biogenesis may be assessed by gene expression or protein content of regulators of mitochondrial biogenesis, or through evaluation of mitochondrial content. Mitochondrial content may be evaluated by electron microscopy or indirectly, through measurement of mitochondrial DNA content.[242] Each mitochondria has between 2-10 genome copies.[243] Therefore, comparison of mitochondrial DNA copy number to nuclear DNA copy number can generate an approximation of mitochondrial content.

In this study, Nrf1 was increased with IR, but not correlated with increasing BCS. Interestingly, mitochondrial biogenesis was upregulated with obesity, as demonstrated by increased transcription of PGC1α and ERRα. This upregulation in transcriptional activators of biogenesis in BCS was not paralleled by an increase in Nrf1 transcription or mitochondrial copy number. In contrast, in people with type II diabetes, there is a coordinated reduction of genes involved in mitochondrial biogenesis, including decreased mitochondrial PGC1α, PGC1β, ERRα, and Nrf-1[122,123] and decreased mitochondrial content.[109,110] The findings reported here may indicate impaired signaling between ERRα and transcription of oxidative phosphorylation genes that occurs with

obesity. Although PGC1α and PGC1β are considered the master regulators of biogenesis, a functional ERRα is critical for downstream signaling.[121] The absence of response of mitochondrial DNA replication in light of the upregulation of regulators of biogenesis with increasing BCS warrants additional investigation. In the IR state, the increased expression of Nrf1, a regulator of oxidative phosphorylation gene transcription, may be a physiological response to increased substrate demand by enhancing cellular respiratory function.

Lipid peroxidation increased while protein oxidation decreased with increasing BCS, although this relationship was not maintained when examining obese horses only. The discordant findings between TBARS (increased) and protein carbonyls (decreased) across BCS may be reflective of upregulated proteasomal-mediated degradation of oxidized proteins.[244] Increased TBARS concentration may be a function of increased intramyocellular lipid content and increased availability of lipid for peroxidation by intracellular ROS.[127,181] The increase in antioxidant capacity paralleling obesity suggests compensation for an increase in cellular ROS exposure occurring with BCS.

In conclusion, although several lines of evidence support the hypothesis that increased ROS is associated with equine obesity and obesity was a strong predictor of IR, there was no relationship between markers of oxidative stress and IR. It is of interest that Mfn2, a regulator of mitochondrial fusion, a process that protects the mitochondrial against ROS damage, was upregulated in horses with IR, suggesting alterations in mitochondrial dynamics may be protective against increased ROS associated with obesity. Further studies are needed to confirm the role of mitochondrial dynamics in obesity and IR in horse.

Footnotes

[a]Coat-A-Count, Siemens, Tarrytown, NY

[b]Immulite 1000, Siemens, Tarrytown, NY

[c]Fisher Scientific, Pittsburg, Pennsylvania

[d]Bio-Rad, Hercules, CA

[e]Cell Biolabs, Inc, San Diego, CA

[f]Sigma-Aldrich, St. Louis, MO

[g]Goat-anti-mouse IgE, Southern Biotech, Birmingham, AL

[h]GE Healthcare, Piscataway, NJ

[i]http://rsb.info.nih.gov/ij

[j]Zeptometrix Corporation, Buffalo, NY

[k]Invitrogen, Eugene, OR

[l]Ambion, Inc, Crawley, TX

[m]Life Technologies, Carlsbad, CA

[n]www.primer3.sourceforge.net

[o]www.ncbi.nlm.nih.gov/nuccore

[p]Qiagen, Valencia, CA

[q]Enzo Life Sciences, Inc, Farmingdale, NM

CHAPTER V

ABSENCE OF INFLAMMATION IN EQUINE OBESITY-ASSOCIATED INSULIN RESISTANCE

ABSTRACT

Local (skeletal muscle and adipose) and systemic inflammation are implicated in the development of obesity-associated insulin resistance (IR) in people. In horses, obesity is not strongly or consistently associated with systemic inflammation. The role of skeletal muscle inflammation in the development of equine obesity-associated IR remains to be determined. We hypothesized that skeletal muscle inflammation causes obesity-associated IR in horses. Thirty-five light breed horses with body condition scores of 3/9 to 9/9 were included in the study. Inflammatory biomarkers were evaluated in skeletal muscle biopsies and plasma. Relationships among local and systemic inflammatory markers, obesity, and serum insulin concentration were evaluated. Inflammatory biomarkers were compared between insulin resistant (IR) and insulin sensitive (IS) horses. None of the inflammatory biomarkers increased with serum insulin concentration. In contrast, skeletal muscle TNFα and plasma serum amyloid A concentrations were negatively correlated with obesity and lower in IR compared to IS horses. We conclude that skeletal muscle inflammation is not a primary mechanism for development of equine obesity-associated IR. Systemic inflammation was not increased with obesity or IR status.

INTRODUCTION

Obesity in people is associated with a low-grade, chronic inflammatory state.[245] Obesity is primarily due to accumulation of white adipose tissue (WAT). Historically, WAT was recognized for its role as a primary site of energy storage. More recently, it has been identified as an important endocrine organ that secretes proteins, known as adipokines, which are involved in regulation of metabolism, coagulation, and inflammation. Key inflammatory adipokines secreted by WAT include the cytokines IL-6 and tumor necrosis factor α (TNFα) and the acute phase reactants, serum amyloid A (SAA) and c-reactive protein (CRP).[153,246-248]

High concentrations of circulating inflammatory cytokines have been implicated in the development of type II diabetes.[152] Circulating inflammatory cytokines, primarily TNFα, perpetuate the inflammatory state by activating the intracellular stress kinases, inhibitor of nuclear factor kappa B (NFκB) kinase β (IKKβ), Jun N terminal kinase (JNK) and mitogen activated protein kinase 4.[102] IKKβ causes translocation of NFκB, allowing for transcription of inflammatory cytokines and perpetuation of the pro-inflammatory state. In insulin-sensitive tissues, including skeletal muscle, JNK interacts directly with the insulin signaling pathway by causing inhibitory phosphorylation of the insulin receptor substrate 1 protein. A pro-inflammatory state has been identified within skeletal muscle of people with type II diabetes.[249,250] Thus, it has been proposed that inflammation within skeletal muscle may in part be responsible for obesity-associated insulin resistance.

In contrast to studies in people and in mice, research into obesity in horses has not demonstrated a consistent association between systemic inflammation and obesity. Initial studies found that obesity in horses was correlated with systemic inflammation,[19,166] but these findings were confounded by failure to control for age in the obese population surveyed. In contrast, in ponies with a history of laminitis, circulating TNF-α concentrations were not correlated with obesity or IR, but did correlate with age.[139] Age has been independently associated with a pro-inflammatory state in

horses.[167] In Thoroughbred geldings fed to promote weight gain, there was no correlation between BCS and serum TNFα.[168]

The role of systemic inflammation in equine obesity-associated insulin resistance is similarly unclear. Circulating pro-inflammatory cytokine concentrations do not appear to be a key component of obesity associated IR. A previous study of hyperinsulinemic obese horses demonstrated a trend toward decreased circulating TNFα and decreased mononuclear inflammatory cytokine expression when compared with lean controls.[140] IL1β, IL6, and TNFα plasma concentrations were not correlated with obesity or plasma insulin concentrations in another study.[169] There have been conflicting findings with respect to the acute phase response. No change in CRP concentrations was found in hyperinsulinemic obese horses compared to controls.[140] In contrast, SAA did correlate with insulin concentrations and weakly with BCS.[169]

Despite multiple investigations into the relationship between systemic inflammation, obesity and insulin resistance in horses, knowledge of tissue inflammation is limited. Inflammatory cytokine gene expression in adipose tissue of IR horses was not significantly different than that of IS controls.[77] Furthermore, TNFα protein content was increased in visceral adipose but not skeletal muscle or subcutaneous adipose of IR horses compared to IS controls.[170] Notably, in both of these studies, horses were stratified solely on the basis of dynamic insulin sensitivity testing and were similar with respect to BCS.

We hypothesized that obesity-associated skeletal muscle inflammation leads to the development of IR in horses. To test this hypothesis, relationships between serum insulin concentration, obesity (BCS), and markers of local and systemic inflammation were explored.

MATERIALS AND METHODS

Sample population

Blood samples and skeletal muscle biopsies were collected from 35 horses. Breeds represented included Quarter Horse (n=20), Thoroughbred (n=4), Paint (n=3), Paso Fino (2), Appaloosa (1), Missouri Foxtrotter (1), Azteca (1), Arabian (1), Tennessee Walking Horse (1), Morgan (1). There were 16 mares and 19 geldings. Age ranged from 2-27 (mean 14 ± 6 years). Body condition score (BCS) was assessed in all animals, while dynamic insulin sensitivity testing was undertaken in a subset of 19 horses, and consisted of either a frequently sampled IV glucose tolerance test (FSIGTT, n=6),[76] hyperinsulinemic euglycemic clamp (HEC, n=3),[68] or an oral sugar test (OST, n=10).[49] Horses were considered insulin resistant (IR) on the basis of an insulin >60 μIU/ml at 0 or 75 minutes (OST), an insulin sensitivity index <1.0 (FSIGTT), and a glucose infusion rate < 0.015 mmol/kg/min (HEC). Semi-membranosus muscle biopsies were collected ante mortem (n=15) or immediately following euthanasia (n=20). Samples were obtained in accordance with the Institutional Animal Care and Use Committee at Oklahoma State University.

Hormone analysis

Serum insulin[a] and plasma α-melanocyte stimulating hormone[b] (α-MSH) concentrations were measured by radioimmunoassay. Assays were previously validated for use in horses.[203,251]

Muscle TNFα

Muscle samples were diluted in PBS and homogenates prepared using a tissue homogenizer.[c] Homogenates were centrifuged at 1000 g for 10 minutes. Supernatant protein concentration was quantified using a commercially available assay.[d] Skeletal muscle TNFα was evaluated by ELISA.[e] Sample dilutional parallelism was assessed in a spiked sample with high TNFα concentrations (r^2=0.95, p=0.005). Percent recovery was determined by spiking a pooled low homogenate sample with reconstituted standard, at concentrations ranging from 62.5-1000 pg/mL. Recovery (mean ± standard deviation) was 79.7 ± 13%.

Gene expression

Total RNA was extracted from approximately 30 mg skeletal muscle using TRIzol extraction.[f] For quantitative polymerase chain reaction (PCR), total RNA was treated with DNAse[g] for 30 minutes at 37°C to remove potential residual DNA, and then cDNA was transcribed according to the manufacturer's directions.[h] Equine-specific primers were designed with Primer3[i] using published equine sequence data[j] and used to amplify TNFα, IL1 and IL6 mRNA using β-actin and GAPDH as housekeeping genes. β-actin and GAPDH were determined to have the best stability as housekeeping genes following analysis with a commercially available software program.[l] Quantitative PCR was performed in triplicate in a thermocycler.[h] Reaction volume was 25 µL, consisting of 10 µL of 1/20 dilution of cDNA, 12.5 µL of a SYBR Green master mix[h] and 10 mM of forward and reverse primers.

The geometric mean of both housekeeping genes was used to create a normalization factor. This normalization factor was applied to each gene to determine relative expression (RE).

Systemic inflammatory biomarkers

Serum amyloid A[m] and TNF-α[e] were measured in plasma using commercially available ELISAs as previously described.[19,252]

Statistical analysis

Forward stepwise regression was performed to determine the impact of BCS, age, breed, gender, and markers of inflammation on serum insulin concentration. For the purpose of statistical analysis, breed was dichotomized into Quarter Horses (n=20) and other breeds (n=15). Hormone concentrations and inflammatory biomarkers were $\log_{10}$ transformed for the model. Because dynamic insulin sensitivity testing allows for better evaluation of tissue insulin sensitivity than fasting insulin concentrations, skeletal muscle and systemic markers of inflammation in IR (n=8) horses was

compared to that of IS (n=11) horses using a Mann-Whitney U test. Since the ratio of pro and anti-inflammatory cytokines is often considered a better indicator of inflammatory status than either cytokine alone, a ratio of TNFα:IL10 and IL6:IL10 gene expression was also evaluated in IR and IS horses.

In order to determine the role of obesity in the development of local and systemic inflammation, a Spearman rank correlation coefficient was calculated to evaluate the relationship of BCS and skeletal muscle and circulating inflammatory biomarkers.

RESULTS

Body condition score ranged from 3/9 to 9/9 (median, 5.5). Thirteen horses were considered to be obese (BCS ≥7). Serum insulin concentrations ranged from <3.5 μIU/ml to 405 μIU/mL (median, 8.3 μIU/mL). Eight of 19 horses were IR on the basis of dynamic testing. All of these horses were also hyperinsulinemic with serum insulin concentrations >70 μIU/mL. Plasma samples from IR horses were collected during the summer (June-July, n=7) and spring (March-May, n=1). Plasma samples from IS horses were collected during fall (Aug-Nov, n=1), winter (Dec-Feb, n=2), and spring (Mar-May, n=8). Plasma α-MSH concentrations ranged from 7.5-345 pg/ml (median, 42.5).

The final regression model for prediction of serum insulin concentration consisted of BCS, gender, and age (adjusted r^2=0.72, p<0.001; Table 5). Body condition score accounted for 42% of the variance of the serum insulin concentrations (p<0.001), while age accounted for 23% (p=0.001) and gender for 9% (p=0.02). When comparing skeletal muscle inflammatory biomarkers of IR to IS horses, TNFα protein content was decreased in IR horses (Figure 19). No significant differences in TNFα, IL6, IL10, TNFα:IL10, or IL6:IL10 gene expression were detected between groups (Figure 19). Simple correlation analysis between BCS and skeletal muscle inflammatory biomarkers revealed

a moderate negative association between obesity and skeletal muscle TNFα (r=-0.40, p=0.04), but no association with IL6 or IL10 gene expression (Table 6). There was a trend towards a relationship between skeletal muscle and plasma TNFα (r=0.33, p=0.06), but no other associations between systemic inflammation and skeletal muscle inflammation were found.

The relationship between systemic inflammation, IR status, and obesity was also evaluated. Serum amyloid A (r=-0.38, p=0.05) and plasma TNFα (r=-0.40, r=0.02) were negatively correlated with BCS and SAA was significantly decreased in IR horses compared to IS horses (p=0.007, Figure 20). Plasma TNFα was not associated with IR status. Alpha MSH was positively correlated with obesity (r=0.65, p<0.001) and significantly increased (p=0.05) in horses with IR.

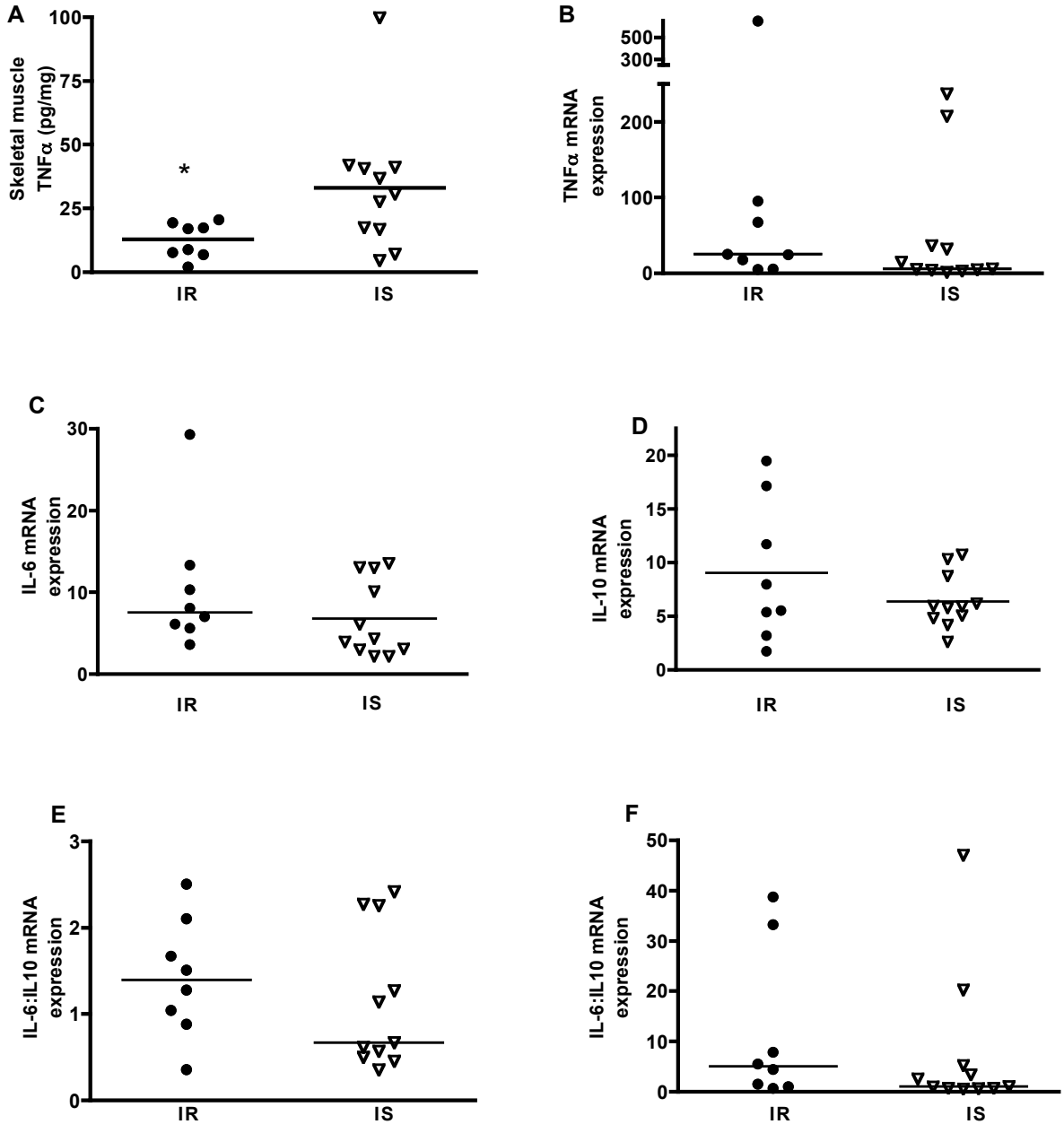

Figure 19. Skeletal muscle inflammatory markers in IR and IS horses. a) TNFα protein content b) TNFα gene expression, c) IL6 gene expression, and d) IL10 gene expression, e) IL6:IL10 gene expression, f) TNFα:IL10 gene expression. *p≤0.05.

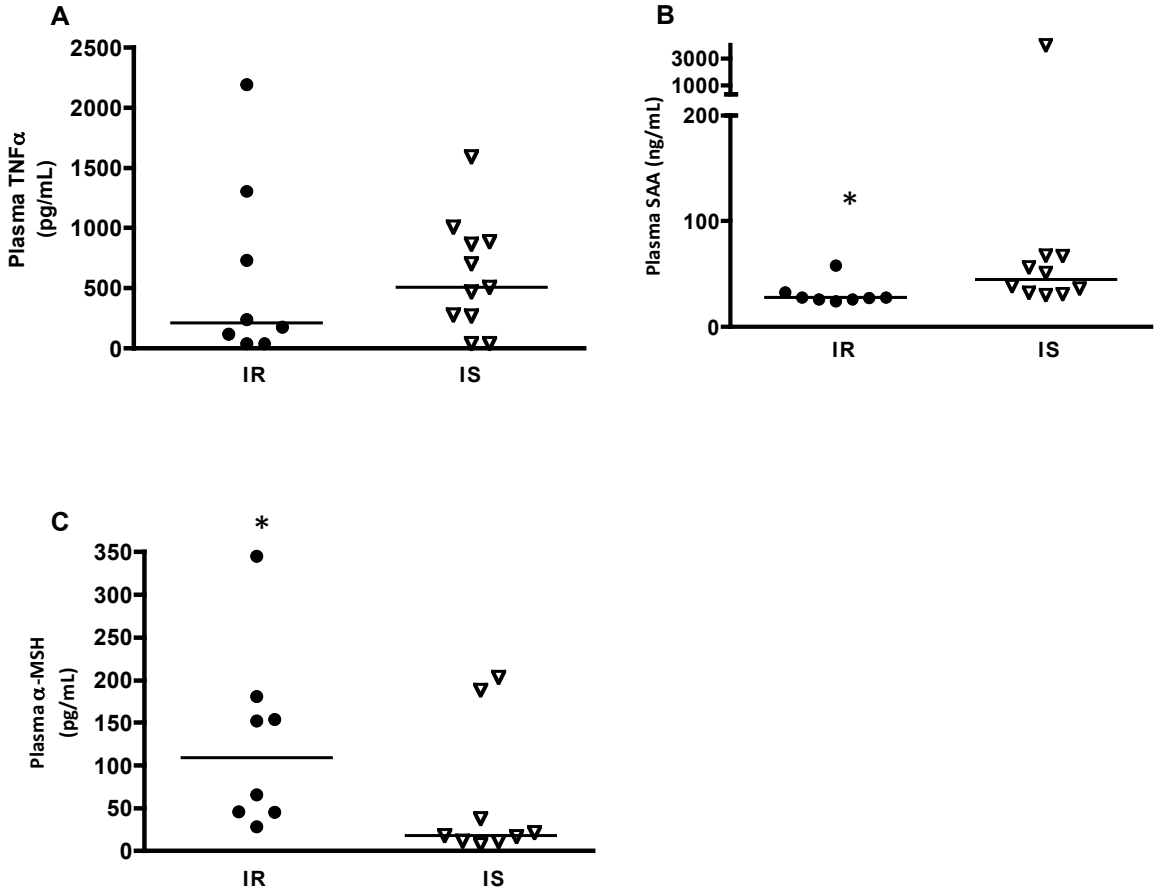

Figure 20. Comparison between systemic markers of inflammation in IR and IS horses: a) TNFα, b) Serum amyloid A, c) α-MSH. * $p \leq 0.05$

Table 5. Predictors of serum insulin concentration using linear regression modeling. Final regression equation: -1.251 + 0.76(BCS) + 0.433(age) -0.287(gender).

Step	**Variable**	**r^2**	**adjusted r^2**	**P**
1	BCS	0.44	0.42	<0.001
2	Age	0.67	0.64	0.001
3	Gender	0.75	0.71	0.021

Table 6. Relationship between body condition score and inflammatory biomarkers. *p≤0.05, **p≤0.01

	BCS	**Plasma α-MSH**	**Plasma TNFα**	**Plasma SAA**	**IL6 (RE)**	**IL10 (RE)**	**TNFα (RE)**	**IL6:IL10 (RE)**	**TNFα:IL10 (RE)**
Plasma α-MSH	0.65**								
Plasma TNFα	-0.40*	-0.16							
Plasma SAA	-0.38*	-0.18	0.00						
IL6 (RE)	0.11	0.06	0.23	-0.12					
IL10 (RE)	0.16	0.01	0.36*	-0.07	0.64**				
TNFα (RE)	0.23	0.06	-0.14	0.02	0.26	0.58 **			
IL6:IL10 (RE)	0.11	0.25	-0.24	-0.09	--	--	-0.05		
TNFα:IL10 (RE)	0.06	0.22	-0.29	0.09	0.006	--	--	--	
TNFα muscle	-0.40*	-0.22	0.33 (p=0.06)	0.29	-0.15	-0.15	-0.49*	-0.15	-0.46**

DISCUSSION

Inflammation is considered to be a central component of obesity-associated insulin resistance in people, with systemic and local (adipose and skeletal muscle) inflammation reported. In horses, the relationship between obesity and inflammation, or inflammation and insulin resistance, remains less clear. To date, most equine studies have focused on systemic inflammation. The limited investigations of the relationship between local inflammation and IR have used horses of similar BCS in an attempt to evaluate IR without the obesity confounder.[77,170] However, mechanisms of IR in obese and non-obese states are not always similar.[253] Given the prevalence of obesity-associated IR in horses, it is important to investigate mechanisms of IR while including the obesity-IR interaction.

Skeletal muscle is the most important site of insulin-mediated glucose disposal in the post-prandial state, with an estimated 67% of glucose uptake occurring within this tissue in people.[11] Whole body IR is typically reflective of IR within skeletal muscle. In horses, IR can be challenging to identify. Multiple dynamic insulin tolerance tests have been developed for use in the horse,[68,76,78,254] but few have been compared to the HEC,[255] which is considered the gold standard in diagnosis of human IR. In this study, multiple dynamic tests were employed for diagnosis of IR due to emergence of new, and potentially improved, methods for diagnosis of IR during the sample collection period. Because comparisons between dynamic tests are not standardized, and not all horses underwent dynamic testing, fasting serum insulin concentration was used as a surrogate marker for IR across the population. All horses considered to be IR on the basis of dynamic testing also had fasting hyperinsulinemia. Dynamic testing also confirmed insulin sensitivity in 11 horses with normal fasting insulin.

In other species, obesity-associated inflammation is mediated by a number of cytokines. TNFα is a key pro-inflammatory cytokine that initiates increased transcription of pro-inflammatory

cytokines, and activates stress kinases that interfere with insulin signaling. Interleukin 6 is an important mediator of the hepatic acute phase response that is secreted primarily from activated lymphocytes and macrophages.[256] It is also recognized as an important adipokine and myokine involved in insulin signaling.[257] Interleukin 10 is primarily an anti-inflammatory cytokine that counters the effect of IL6 and TNFα. Interleukin 10 also appears to counter-regulate the effects of IL6 and TNFα on insulin signaling. In mice, infusion of IL6 decreased skeletal muscle insulin signaling, an effect that was ameliorated by infusion of IL10.[258] In order to better understand the role of skeletal muscle inflammatory state in insulin resistance, gene expression of both pro-inflammatory (TNFα, IL6) and anti-inflammatory (IL10) cytokines was evaluated. Tumor necrosis factor α was also evaluated at the protein level, both within skeletal muscle and systemic circulation.

In the current study, signalment factors (obesity, age, and gender) were the only significant predictors of serum insulin concentrations, with mares having higher insulin concentrations. The findings reported here suggest that in horses, signalment is more important than inflammation in altering insulin sensitivity. Insulin sensitivity has been previously demonstrated to decline with age in horses.[19] In people, the decline in insulin sensitivity observed with age is thought to be due to a combination of obesity and a decline in physical activity.[259]

Insulin resistance and type II diabetes are associated with increased skeletal muscle TNFα concentrations in people.[164,165] In contrast, in our horses, skeletal muscle TNFα was negatively associated with obesity and IR status. Gene transcription of pro- or anti-inflammatory cytokines was not altered with IR status or obesity. These findings indicate that skeletal muscle inflammation is not an important contributor to equine obesity-associated IR.

In agreement with a previous study from our laboratory, the current data showed a decrease in systemic inflammation in IR horses compared to IS horses and with obesity.[140] In contrast, in human obesity, both circulating acute phase proteins and TNFα are increased.[153,246] Although acute

phase proteins are typically produced by hepatocytes, in states of obesity, adipose tissue is recognized as an important site of production and secretion.[153,247,248] In horses, SAA appears to be a better indicator of acute inflammation than other acute phase proteins as SAA normally circulates at low concentrations but has a more rapid, profound (several hundred fold) increase in response to an inflammatory stimulus.[252] Previously, SAA was reported to have a moderate correlation with insulin concentration and a weak correlation with BCS in horses.[169] In contrast, no relationship between SAA and BCS was found in a subsequent study of laminitis prone ponies but a significant impact of season on SAA concentration was reported, with concentrations higher in winter than summer.[260] Seasonal differences in sample collection between IR and IS horses may be responsible for discrepancies between our study and previous reports; Suagee et al collected samples during June and July,[169] while in this study, samples were collected throughout the year.

The relationship between α-melanocyte stimulating hormone (α-MSH), IR status and obesity is intriguing. Alpha melanocyte stimulating hormone, a pro-opiomelanocortin peptide produce by the pars intermedia of the pituitary, is a potent anti-inflammatory hormone.[261,262] In this study, there was no relationship between α-MSH and other pro- or anti-inflammatory mediators, suggesting that inflammation was not the main stimulus for α-MSH secretion in this population. However, it may be that high circulating α-MSH represses obesity-associated inflammation. In addition to its anti-inflammatory effect, α-MSH is also an important regulator of satiety.[263,264] The parallel increase in α-MSH and BCS in the horses of this study suggest α-MSH may contribute to obesity in horses through its role in central regulation of appetite.

In summary, despite what has been reported in other species, we were unable to show a relationship between skeletal muscle or systemic inflammation and obesity-associated IR. Our findings indicated that obesity was the strongest predictor of insulin resistance, with no significant

positive influence of either systemic or local pro-inflammatory biomarkers. The mechanism of equine obesity-associated IR remains to be discovered.

Footnotes

[a]Coat-a-Count, Siemens, Tarrytown, NY

[b]Eurodiagnostica, Malmö, Sweden

[c]Fisher Scientific, Pittsburg, PA

[d]Bio-Rad, Hercules, CA

[e]Pierce, Rockford, IL

[f]Invitrogen, Eugene, OR

[g]Ambion, Inc, Crawley, TX

[h]Applied Biosystems

[i]www.primer3.sourceforge.net

[j]Life Technologies, Carlsbad, CA

[k]www.ncbi.nlm.nih.gov/nuccore

[l]geNorm, Biogazelle, Zwijnaarde, Belgium

[m]TriDelta, Maynooth, County Kildare, Ireland

CHAPTER VI

DISCUSSION

Equine metabolic syndrome was a term coined in 2002[10] to describe a condition in horses characterized by obesity, regional adiposity, laminitis, and insulin resistance or hyperinsulinemia.[45] The term equine metabolic syndrome was chosen due to similarities between EMS and human metabolic syndrome, which is cluster of risk factors for cardiovascular disease, including central obesity, fasting hyperglycemia, hypertension, and dyslipidemia.[46] Equine metabolic syndrome has been associated with adverse health consequences including infertility in mares and exercise intolerance. However, the primary health concern associated with EMS is the development of laminitis, a painful condition that in some cases necessitates humane euthanasia in affected horses. Recent data indicates that in horses presenting to a hospital for laminitis, endocrine disease is the most common cause of laminar disease.[32]

Despite being a recognized clinical syndrome for over ten years,[10] EMS remains a challenging disease to diagnose and treat, in large part because the mechanisms that lead to insulin resistance and hyperinsulinemia in horses are not understood. Altered insulin regulation and glucose homeostasis may occur by disruption anywhere along the pathway of insulin-

mediated glucose disposal, including enteral glucose absorption, pancreatic insulin secretion, hepatic gluconeogenesis, or insulin-mediated glucose uptake in skeletal muscle or adipose. In people, skeletal muscle insulin resistance has long been believed to be a central defect in the development of type II diabetes.[265] Therefore, we set out with the central hypothesis that insulin resistance within skeletal muscle was the primary defect in horses with EMS. In people, oxidative stress,[266] inflammation, [148] and lipotoxicity[267] are key mechanisms associated with skeletal muscle insulin resistance.

Emerging evidence at the time of initiation of the study indicated that mitochondrial dysfunction was a key mechanism of oxidative stress and insulin resistance within skeletal muscle of insulin resistant humans.[240] Increased systemic oxidative stress has not been previously been identified either in a population of previously laminitic ponies compared to non-laminitic counterparts[139] or in a population of hyperinsulinemic obese horses compared to lean, normoinsulinemic horses.[140] However, systemic oxidant status is not always reflective of tissue oxidant status. We hypothesized that obesity-associated mitochondrial dysfunction leads to oxidative stress and insulin resistance within skeletal muscle of obese horses. Because preliminary investigations into mitochondrial content and function failed to yield significant findings, a more global approach exploring oxidative stress within skeletal muscle was undertaken, including analysis of antioxidant enzyme activities, antioxidant concentrations, and markers of lipid peroxidation and protein oxidation within skeletal muscle. Using serum insulin concentration as a surrogate marker of insulin resistance, linear regression modeling did not identify any marker of oxidative damage to be predictive of IR. However, Mfn2, a regulator of mitochondrial fusion, was predictive of IR. Furthermore, expression of Mfn2 and fission (Drp1) were increased in IR horses. This evidence suggests that mitochondrial dynamics are altered in association with IR. In people and rodents, mitochondrial dynamics are altered with obesity and type II diabetes; however, this involves an overall shift in mitochondrial dynamics towards fission.[112,116] In contrast, in horses, there appears to be a shift toward fusion.

Importantly, analysis of mitochondrial dynamics was confined to gene expression analysis. Further investigation into the role of mitochondrial dynamics in EMS is warranted, including measurement of fission and fusion protein content and direct evaluation of changes in morphology using fluorescent labeling techniques.[268]

As mitochondrial dysfunction and oxidative stress did not appear to be key mechanisms of equine skeletal muscle insulin resistance, a second hypothesis was constructed. Due to the strong association between obesity, IR, and local[157,158,164,165] and systemic[149,151] inflammation in people, we hypothesized that inflammation within skeletal muscle of horses causes equine insulin resistance. Gene expression of pro- or anti-inflammatory cytokines within skeletal muscle and TNFα protein content of skeletal muscle revealed no significant differences between IR and IS horses. As both skeletal muscle[164] and adipose tissue[157,158] can secrete inflammatory cytokines in states of obesity, systemic inflammation was also assessed. There was no evidence of a positive association between systemic or skeletal muscle inflammation and obesity, IR, or hyperinsulinemia in the horses of this study. The findings relating to skeletal muscle inflammation should be interpreted with caution for several reasons. First, only one protein (TNFα) was evaluated. TNFα was chosen as previous studies have documented an increase in skeletal muscle of people with type II diabetes.[164,165] However, in this study, TNFα was negatively associated with IR and obesity. It may be that other pro- or anti- inflammatory cytokines are more important in characterizing the inflammatory state of equine obesity and IR. There were no significant differences in pro- or anti- inflammatory gene expression between IR and IS horses, but cytokine gene expression may not always be reflective of protein content due to post-transcriptional regulation.[269] However, when coupled with analysis of systemic inflammatory markers in this and other[19,140,166,270] studies, it appears unlikely that inflammation is the principle mechanism of equine IR.

When evaluating the results found here, it is important to take into account that the horses of the obese, IR group did not all have EMS, as defined by the 2010 ACVIM consensus statement.[45] In that statement, laminitis was defined as a necessary characteristic for inclusion as an EMS-affected

horse. In this study, only 4/8 IR, hyperinsulinemic horses had a history of clinical laminitis. Obesity-associated IR may have a different mechanism in horses suffering from laminitis than in horses that are not affected by laminitis. Alternatively, laminitis may merely be an indication of chronicity or severity of obesity-associated IR and hyperinsulinemia. Regardless, it is important to recognize that findings in obese, hyperinsulinemic non-laminitic equids may not be applicable to those with laminitis.

The wide variation in signalment represented in this study should also be considered when drawing conclusions. Ten breeds were represented, with age of included horses ranging from 2-27 years. In linear regression modeling, breed was not retained for prediction of serum insulin when evaluating either inflammation or oxidative stress. However, for the purposes of the model, breed was dichotomized into Quarter Horse and other breed. The other breeds represented included both historically EMS-predisposed as well as EMS-resistant breeds. The diversity of breeds included coupled with the large proportion of Quarter Horse-type breeds represented in the population limit our ability to interpret the influence of breed on our findings. It may be that some breeds which were not well-represented in this study (i.e., breeds other than the Quarter Horse) have a primary defect in skeletal muscle insulin signaling that this study was could not identify. Differences in lipid profiles, insulin sensitivity and glucose tolerance have been previously been demonstrated between breeds.[68,70,79,271] Although findings from early studies were limited by a small number of animals[68,70,79] and a failure to control for obesity,[68,70] anecdotal reports of increased incidence of EMS in certain breeds[10,45,49] supports the idea that alterations in metabolism may precede the development of both obesity and IR in some breeds. However, a recent study did not demonstrate differences in fasting insulin concentrations or lipid profiles between an EMS-predisposed breed (Morgan horses) and an apparently EMS-resistant breed (Thoroughbred horses).[272] Furthermore, in a recent epidemiological study of hyperinsulinemia, obesity and age but not breed were associated with an

increased risk of hyperinsulinemia.[20] The relationship of breed with development of EMS deserves further study.

Signalment factors other than breed may also be important in assessment of IR and EMS. Age has been demonstrated to be negatively associated with insulin sensitivity in a population of mares.[19] Both age and gender (female > male) were associated with serum insulin concentration in the evaluation of inflammation in the present study. However, there was no significant difference in age or gender between IR and IS horses. Future studies evaluating mechanisms of equine insulin dysregulation or EMS should include a larger population of horses in order to allow for impact of signalment factors, including breed, to be adequately assessed.

In people, obesity and type II diabetes are typically considered to be chronic disease states. In the horses of the current study, records on subjective or objective assessments of obesity were not available. Therefore, the relationship between duration of excessive adiposity and development of skeletal muscle oxidative stress or inflammation cannot be accurately assessed. However, several horses were members of a research herd and had been identified as "easy keepers" for several years, with a propensity towards obesity. Other horses were very obese with markedly elevated fasting serum insulin concentrations, perhaps suggesting chronicity within the IR population studied. Differences in duration of obesity may have impacted findings. In a mouse model of dietary-induced obesity, adipose inflammatory cytokine expression varied across time.[159] In mice with either diet or genetically-induced obesity, markers of oxidative stress can vary over time.[127,229] Therefore, is possible that inflammation or increased ROS production is an early event in the pathogenesis of equine obesity-associated IR that is not detected at later stages.

As two key mechanisms of skeletal muscle insulin resistance did not appear to be associated with equine IR or hyperinsulinemia, the possibility of an alternative primary site of insulin dysregulation was explored by use of dynamic insulin sensitivity testing. Multiple tests have been

recently developed[49,78,94] or modified[80] for diagnosis of IR in the field, but none have been compared to the gold standard of tissue insulin sensitivity, the hyperinsulinemic euglycemic clamp (HEC). In this experiment, the HEC was compared to an insulin-response to dexamethasone test (IRDT) and the recently developed oral sugar test (OST).[49,94] Eight horses were chosen for this study. In an attempt to look at the relationship between tests across a broad spectrum of insulin sensitivity, three obese horses were included, two of which were previously documented to be IR based on results of an OST. At the time of the experiment, all horses had a normal response to an oral glucose challenge based on previously recommended cut-off values,[49] although there was a reasonable range of glucose metabolism indices and glucose metabolism to serum insulin concentration ratios. Unfortunately, the true status of tissue insulin sensitivity in this group of horses is not known, as breed-specific reference ranges for the HEC have not yet been established. Despite these limitations, the absence of correlation between the HEC and the OST or IRDT, suggests that in horses, tissue insulin sensitivity is not the primary determinant of glucose and insulin disposition following an oral glucose challenge. Furthermore, the absence of correlation between the HEC and fasting serum insulin concentrations suggest that tissue insulin resistance may not be the primary mechanism driving fasting serum insulin concentrations. All of the horses of this study were Quarter Horses, so findings may not apply to other breeds.

Interestingly, while our obese horses appeared to be insulin sensitive, previous studies have indicated a state of insulin resistance among non-obese, normoinsulinemic, apparently healthy horses using dynamic test results.[77,105,170,273] Unfortunately, dynamic testing may be inherently stressful to a horse due to the use of restraint within a stall, catheter placement, and frequent sampling required. Stress hormones are known to promote a state of insulin resistance in people.[218] Therefore, it may be that some thin horses are inappropriately diagnosed as IR on the basis of dynamic testing under stressful conditions or that some dynamic tests are not useful for the diagnosis of insulin dysregulation in horses.

Taken together, the results from these experiments on mechanisms of impaired insulin signaling within skeletal muscle and dynamic insulin sensitivity and glucose tolerance testing suggest that in horses, skeletal muscle insulin resistance may not be the central mechanism of insulin dysregulation. Instead, hyperinsulinemia may precede the development of tissue insulin resistance. Hyperinsulinemia is closely linked to the presence of insulin resistance, and hyperinsulinemia may induce IR.[16,274] Interestingly, recently it has been suggested that hyperinsulinemia may be the initial defect in glucose homeostasis and insulin disposal in people.[16,275]

As the mechanism of insulin dysregulation in the horse remains undetermined, it is unclear whether there is an ideal method for measurement of glucose homeostasis and insulin sensitivity that can be applied across all breeds and types of horses. Until the relative contributions of enteral absorption, pancreatic insulin secretion, and tissue insulin sensitivity to equine glucose homeostasis and insulin disposal can be established, no dynamic test of glucose tolerance or insulin sensitivity can be considered the gold standard. Furthermore, results from dynamic tests should not be used interchangeably for diagnosis of equine insulin dysregulation.

In conclusion, evidence presented here suggests that although EMS shares some phenotypic characteristics of human metabolic syndrome, the pathogenesis of the EMS may not be similar. Identification of the primary site of obesity-associated insulin dysregulation is critical to improve understanding of the pathogenesis of EMS and to allow for development of effective treatment strategies.

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