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INTRAVENOUS INJECTION OF INSULIN FOR MEASURING INSULIN SENSITIVITY IN HORSES: EFFECTS OF EPINEPHRINE, FEEDING REGIMEN, AND SUPPLEMENTATION WITH CINNAMON OR FISH OIL

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Interdepartmental Program in
the School of Animal Sciences

by
Lisa RosaLee Earl
B.A., Louisiana State University, 2008
B.S., Louisiana State University, 2009
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ABSTRACT

Seven experiments were performed to assess the use of glucose responses to insulin injections as a means of estimating insulin sensitivity in horses; to compare the insulin sensitivities of normal horses vs. those displaying hyperleptinemia; and to put this method into practical application. Experiment 3.1 examined dose-responses in mares of potentially different insulin sensitivities. Recombinant human insulin was injected at doses of 8, 20, 50, and 125 mU/kg BW, as needed, to estimate the dose of insulin causing a 50% decrease in glucose concentrations (ED50). Five mares each of low leptin concentrations (LL) and low BCS, LL and high BCS, and high leptin concentrations and high BCS, were studied. The ED50 was similar for LL mares, regardless of BCS, and was lower ($P < 0.01$) than for mares with high leptin concentrations. It was concluded that a dose of 50 mU/kg BW of recombinant human insulin could be used safely to start the dose-response curve; lower or higher doses could then be used to estimate ED50. Experiment 3.2 assessed the repeatability of the estimates for ED50 obtained in Exp. 3.1. Estimates obtained were highly correlated (R2 = 0.822) with those obtained in Exp. 3.1, with an average within-mare CV of 8.9%. The next five experiments studied the effects of 1) prior administration of epinephrine, 2) overnight feed deprivation versus hay or pasture consumption, 3) 10-d acclimatization to hay in a dry lot versus pasture grazing, 4) cinnamon extract supplementation, and 5) fish oil supplementation on insulin sensitivity. Epinephrine stimulated blood glucose ($P < 0.05$) and prevented the insulin-induced decrease in blood glucose in both sensitive and insensitive mares. Overnight feed deprivation decreased ($P < 0.06$) insulin sensitivity relative to overnight ad libitum access to hay, and both regimens resulted in reduced insulin sensitivity relative to overnight pasture availability. Ten days of hay consumption in a dry lot reduced ($P < 0.05$) insulin sensitivity in insensitive mares relative to pasture grazing.

Supplementation with cinnamon extract or fish oil had no effect on insulin sensitivity of mares with known low insulin sensitivity under the conditions of these experiments.

CHAPTER 1

INTRODUCTION

Horses rarely have a total loss of insulin production, as seen in humans with type 1 (juvenile onset) diabetes, but obese horses commonly exhibit insulin resistance, which can contribute to other health risks, such as laminitis, pituitary adenoma, hyperlipidemia, osteochondritis dissecans, reproductive inefficiency, and a diminished ability to properly exercise (Kronfeld et al., 2005; Frank et al., 2006). Identification of horses with insulin resistance is important due to its link with these problems (Treiber et al., 2006). Identification can help producers identify which horses to monitor more closely for signs of laminitis as well as make changes in management practices for insulin resistant horses that may be predisposed to other conditions. Identification can also be beneficial to researchers, as these horses may skew data in some experiments involving insulin, leptin, or thyroid hormones (Gentry et al., 2002; Cartmill et al., 2003).

Insulin resistance is often aggravated in the horse, as many horses are increasingly being viewed as companion animals or pets. Due to this, they are often pampered and live a lifestyle with little exercise and diets high in carbohydrates, leading to increased insulin resistance (Frank et al., 2010). Thus, there is an increased need for horse producers and veterinarians to have an easy, reliable method of measuring insulin resistance. Current methods of measuring insulin resistance have been adapted from human medicine, and are complicated and often unreliable when used to measure insulin sensitivity in horses.

The research described herein was designed to develop a more simplistic and effective means of measuring insulin sensitivity in horses through the intravenous administration of insulin, and to put this method into practical application to determine its suitability for farm use

by veterinarians and producers. The first objective was to assess the insulin sensitivities of horses with high and low plasma leptin concentrations by evaluating glucose response curves to the intravenous administration of insulin to animals across a wide range of sensitivities and to refine this method. The second objective was to compare the insulin sensitivities of normal horses vs. those displaying hyperleptinemia. Finally, the third objective was to put this method into practical application by determining whether various factors would affect insulin sensitivity, such as 1) epinephrine administration (mimicking stress), 2) prior feeding regimen (feed deprivation, hay, and pasture), and 3) two common feed supplements (cinnamon extract and fish oil) reported to affect insulin sensitivity in other species.

CHAPTER 2

REVIEW OF LITERATURE

Normal Insulin Action

Insulin is a protein hormone synthesized by cell clusters in the pancreas, referred to as the islets of Langerhans, in response to elevations in blood glucose and/or amino acid concentrations (Hadley and Levine, 2007). Insulin is needed by almost all cells (except the brain, retina, and testes) to transport glucose across cellular membranes, and its action is directed towards the metabolism of carbohydrates, fats, and proteins (Hadley and Levine, 2007). In this mechanism, glucose and insulin are directly proportional; as blood glucose levels increase, an increase in insulin is prompted. When glucose levels decrease, less insulin is released (Hadley and Levine, 2007).

In healthy fasted humans, blood glucose concentrations typically range between 80 to 90 mg/dL (Guyton and Hall, 2006). These limits are closely upheld in the fasted state. After consumption of a meal (in the first half hour), these levels rise to 120 to 140 mg/dL, but normally return to fasted values within 2 h after a meal. Horses maintain comparable blood glucose levels, with between-meal concentrations typically ranging from 60 to 90 mg/dL (Ralston, 2002). Horses absorb glucose differently depending on several factors, including diet, daily routine, exercise level (metabolic demands), and genetics. After a meal high in carbohydrates, disaccharides are hydrolyzed into monosaccharides in the duodenum by intestinal microvilli brush border enzymes (Guyton and Hall, 2006). Glucose is the main end result. After production, it moves into portal blood and is transported to vital tissues. When stimulated by glucose, the pancreas produces insulin. This impedes gluconeogenesis by the liver. The half-life

of insulin in humans is about 6 min and it circulates mostly unbound to serum proteins (Guyton and Hall, 2006).

Eighty percent of the body requires insulin as a moderator for glucose entry into the cell. The insulin receptor is composed of alpha and beta subunits, connected by disulfide bonds (Tritos and Mantzoros, 1998). Tyrosine kinase, the enzyme linked to the insulin receptor, is promptly autophosphorylated when insulin binds to the receptor's alpha subunit. This activates the tyrosine kinase, which in turn phosphorylates numerous enzymes within the cell as well as the insulin receptor substrate (**IRS**). These products drive the incorporation of glucose transport (**GLUT**) proteins to the cell membrane for glucose absorption from interstitial fluid. Any glucose that remains unused for energy is transformed to glycogen and warehoused in the liver and muscle cells until necessary. Any additional surplus carbohydrates are used to produce fats, which are stored in adipose tissue (Guyton and Hall, 2006). In a discussion by Storer et al. (2007), it was postulated that in wild horses, insulin production is relatively steady, as they graze for most of the day. In contrast, humans and meal-fed horses rely on free fatty acids (**FFA**) and volatile fatty acids (**VFA**) for energy between meals, as most tissues (excepting nervous tissue) are only somewhat porous to glucose (Guyton and Hall, 2006).

Insulin Resistance

Insulin resistance occurs when some aspect(s) of the insulin receptor-intracellular cascade stop responding normally to insulin and the body as a whole becomes less sensitive to insulin (Treiber et al., 2006). It is most likely the outcome of both polygenic flaws and environmental influences (Qin et al., 2003). This resistance to normal amounts of insulin causes the pancreas to produce increasing amounts of insulin in order to keep blood glucose at a normal level, meaning

higher concentrations of insulin are required to keep glucose at the same level (Treiber et al., 2006).

Many bodily factors can compromise the standard physiologic reaction to glucose (Qin et al., 2003). In humans, over 50 mutations of the insulin receptor gene have been characterized (Tritos and Mantzoros, 1998). Mechanisms of these mutations can be grouped into three divisions involving receptors: receptor, pre-receptor, and post-receptor insulin resistance (Chang et al., 2004). When the number or avidity of insulin receptors declines, receptor type insulin resistance occurs. Pre-receptor insulin resistance, on the other hand, occurs when circulating antibodies are developed against insulin receptors, causing insulin to not bind to the target cells well (Chang et al., 2004). The third group, post-receptor type insulin resistance, is the most common type of insulin resistance. This type is caused by a signaling failure by intracellular effectors of insulin's actions (Chang et al., 2004). These receptor failures can cause serious problems in both humans and horses.

With insulin resistance, the body is forced to produce greater amounts of insulin to signal the target tissues to incorporate GLUT proteins in the face of hyperglycemia (Frank et al., 2006). Sustained hyperglycemia from insulin resistance may ultimately overpower the pancreas' ability to produce insulin (Chang et al., 2004). In humans, this can eventually lead to frank diabetes (a state where no insulin is produced at all), a condition called type I diabetes. Total insulin production loss is rarely seen in horses, but insulin resistance can still cause major problems for equines, such as laminitis, pituitary adenoma (Frank et al., 2006), hyperlipidemia, osteochondritis dissecans, reproductive inefficiency, and the ability to exercise properly (Kronfeld et al., 2005).

Contributing Factors to Insulin Resistance

Breed and lifestyle. Many factors can cause a predisposition to insulin resistance. There is often a genetic component to insulin resistance; certain breeds of horses (Morgans, Spanish Mustangs, European warmbloods, American Saddlebreds, Arabians, and ponies) often have an increased incidence of insulin resistance, most likely as a genetic adaptation to sparse vegetation (Johnson, 2002; Harris et al., 2006). Within an individual breed, some lines may be more predisposed than others, as insulin resistance is rarely a factor taken in account when choosing a sire for breeding.

Insulin resistance can also be aggravated by the increasing perception of horses as companion animals or pets, rather than work animals. Due to this, many horses receive little exercise and have improper nutrition, as well as often consuming diets too high in carbohydrates (Buff et al., 2005). Such a diet and lifestyle is commonly hard for even a healthy horse to overcome, as diet has been shown to affect insulin sensitivity. An insulin resistant horse is frequently unable to override the glycemic response of a diet high in sugars and starches because great vacillations in glucose and insulin after these meals give incorrect energy signaling (Harris et al., 2006). In Throughbred weanlings, insulin sensitivity was lower in those fed sugar and starches as opposed to those fed fats and fiber (Treiber et al., 2005). Similar results were seen in Throughbred geldings (Hoffman et al., 2003).

According to a discussion by Storer et al. (2007), wild horses adapted to an active lifestyle, evolving without any sources of concentrated carbohydrates in their diet and requiring movement throughout most of the day. In wild horses, insulin production is relatively steady, as they graze for most of the day. This roaming lifestyle directly contrasts to the lifestyle of most horses today, where the majority of their time is spent in a barn or small pasture consuming rich

grass or grain, which often leads to inadequate exercise and obesity (Buff et al., 2005). Even active horses may have a lifestyle predisposed to insulin resistance, as equine athletes are often fed sweet feed in order to improve performance (Kronfeld et al., 2005). Horses that may be susceptible to insulin resistance can often be distinguished from healthy horses by excessive weight gain or loss, muscle loss, lack of stamina, cresty necks, and abnormal fat pockets across the body (Johnson, 2002).

Fat intake and obesity. Obesity occurs when there is a disparity between energy intake and expenditure (Geor and Harris, 2009). Obese horses are horses with a body condition score (**BCS**) of 7 or more (Henneke et al., 1983). These are the horses most at risk for developing metabolic problems. The National Animal Heath Monitoring System of the Department of Agriculture reported (through owner surveys) that 5.5% of the horse population was obese in 1995. Of horses not reported as obese, many were probably still heavier than ideal. This value is most likely much lower than actuality due to errors in owner measuring (Geor, 2008). While not all insulin resistant horses are overweight and not all overweight horses are insulin resistant, there is a general correlation between obesity and insulin resistance. In one experiment, 35% of the high BCS mares were found to be hyperleptinemic (Waller et al., 2006), which is a trait associated with insulin resistance.

Harry Himsworth (1935) was the first to test the effects of dietary fat on insulin action. Subjects on a liquid diet were fed 13 to 80% fat. Himsworth found that administration of insulin depressed the oral glucose tolerance curve in some diabetic patients (insulin sensitive), but not others (insulin insensitive). He found a reduced depression of blood glucose in the higher fat content diets (Himsworth and McNair-Scott, 1935).

There are two primary theories linking obesity to insulin resistance. The first involves the buildup of lipids in cells in tissues that are sensitive to insulin (particularly skeletal muscles). This is called lipotoxicity, and involves fat cells producing toxins that interfere with insulin action at the target cell. A horse's natural diet is low in fat, but extra glucose is often converted into fat by de novo lipogenesis. These fats are then either used for metabolic demands or stored within cells as triglycerides. When adipose tissues no longer have storage area, the fats are repartitioned to nonadipose tissues, such as skeletal muscle, liver, and pancreatic tissues. These tissues increase β-oxidation in an effort to utilize fats, but in this process, as the lipids amass, normal cell functions can be altered, including insulin signaling (Frank et al., 2010).

The second theory involves insulin signaling pathways that are down-regulated by adipokines and cytokines manufactured in fat tissue (Frank et al., 2010). As fat cells enlarge, the concentration of insulin receptors on their exterior diminishes (O'Dea, 1992). To compensate for the decreased receptor number, the body increases its insulin production. As a result, hyperinsulinemia may cause insulin receptors in other tissues to be down-regulated (O'Dea, 1992). Thus, high insulin levels lead to fat, and fat leads to improper insulin action, leading to more insulin, creating a malicious progression of disease.

Insulin resistance can cause compensated hyperinsulinemia, a syndrome of severe insulin resistance, which occurs from an augmented insulin production in order to compensate for the ongoing insulin resistance and decreased insulin clearance (Tritos and Mantzoros, 1998). This may upset the "switching" apparatus of lipid breakdown in humans and in horses and produce dyslipidemia. Dyslipidemia is characterized by augmented circulating very low density lipoproteins (**VLDL**), elevated triglyceride concentrations, and low concentrations of high density lipoprotein (**HDL**) cholesterol (Carr and Brunzell, 2004; Alberti et al., 2006).

An exact mechanism for this process is unknown, but obesity is thought to have an impact on insulin resistance and dyslipidemia. Insulin sensitivity has been reported to be less in obese geldings compared to non-obese geldings (Hoffman et al., 2003). One cytokine produced by adipose tissue is tumor necrosis factor-alpha (**TNF-alpha**). It stimulates the release of nonesterified fatty acids (**NEFA**) and may facilitate the gene repression for assembly of glucose and NEFA (Vick et al., 2008). Large amounts of TNF-alpha and NEFA may impair insulin signaling, particularly in muscle tissue, because as NEFA oxidation is fulfilling energy requirements, less glucose uptake is needed (Randle et al., 1963). Extreme NEFA contact may directly impact the islets of Langerhans. In an experiment by Boden and Laakso (2004), elevation of NEFA for 2 to 4 d (experimentally-induced) triggered increased insulin secretion in humans (Boden and Laakso, 2004). Adipose tissue products may also be harmful to the liver, as the products are secreted straight into portal blood. (Mlinar et al., 2007).

Clinically, insulin resistant horses as compared to healthy horses have 86% higher NEFA, 104% higher VLDL, and 29% higher HDL-cholesterol concentrations in blood. The NEFA concentrations in blood are considered fat metabolism and mobilization markers (Frank et al., 2006). In healthy obese humans with normal β cells, FFA stimulate insulin secretion and induce insulin resistance. This process is thought to have developed as a beneficial adaptation to situations like pregnancy or starvation (Boden and Laakso, 2004). An adaptation for survival in conditions of unreliable food supply could explain why some breeds have a higher incidence of insulin resistance, as these breeds lived in harsher environments (Treiber et al., 2006). During pregnancy, mares have higher insulin levels after an increase in glucose due to an enhanced β cell response to glucose, because glucose is redirected to nourish the fetus, not taken up by

maternal tissues. Around the time of parturition, insulin concentrations fall (Fowdean et al., 1984).

Because insulin resistance was originally an adaptation, in many active people, the body is able to compensate for the insulin resistance created by FFA (Boden and Laakso, 2004). So not all obese insulin-resistant humans develop disease, but in many sedentary people and those genetically predisposed to disease, FFA are unable to compensate for the insulin resistance they create. Concentrations of NEFA rise as result of fat tissues satisfying their total storage ability for fat, which lessens the inhibitory properties of insulin on lipase (Frank et al., 2006). Due to this, fat must be stored in other tissues (like liver and muscle cells), which can cause tissue insulin resistance from yields of fat use, upsetting insulin signaling conduits (Boden and Laakso, 2004). Additional indicators of NEFA uptake in the human liver are rises in VLDL and triglycerides (Carr and Brunzell, 2004; Frank et al., 2006).

In humans, hypertriglyceridemia often leads to high blood pressure, and as such, is commonly linked with metabolic syndrome, obesity, and insulin resistance. Although seldom measured in equines, pony breeds considered insulin resistant with a prior history of laminitis showed raised insulin, blood pressure, VLDL, and triglyceride concentrations during times when high-fructans (high-glycemic, non-structural carbohydrates linked to insulin resistance in the horse) were available for grazing (Bailey et al., 2008). Forhead (1994) reported comparable findings in hypertriglyceridemic donkeys.

Equine Metabolic Syndrome

The previously described risk factors for insulin resistance have been clustered into one disorder, equine metabolic syndrome (**EMS**). Metabolic syndrome in humans is a disorder comprising a string of medical abnormalities judged as threats for type II diabetes and coronary

artery disease (Alberti et al., 2006). In 1988, Gerald Reaven described a compilation of abnormalities thought to cause the progress of cardiovascular disease, kidney disease, and diabetes mellitus. He called this collection of disorders “syndrome X”, a disorder consisting of: decreased HDL cholesterol, elevated VLDL triglyceride, hyperglycemia, hypertension, and insulin resistance (Reisin and Alpert, 2005). Presently, in human medicine, this condition is more commonly referred to as the metabolic syndrome, with added constituents: dyslipidemia and obesity. By 2020, this syndrome is expected to impact 40% of the world's population (Alberti et al., 2006).

In 2002, it was suggested that some of the same disorders, obesity, insulin resistance, and laminitis, were also constituents of a syndrome in equines (Johnson, 2002). Thus, the term EMS was accepted to describe this condition due to its resemblance to the human disorder. Established components of EMS include obesity, insulin resistance, regional fat deposits, and laminitis unconnected to other causes like grain overload or colic. Other related perturbations may include hypertriglyceridemia or dyslipidemia, hyperleptinemia, arterial hypertension, and altered reproductive cycling (Frank et al., 2010).

As opposed to the human syndrome, horses are not typically diagnosed with diabetes mellitus. They are instead diagnosed with insulin resistance and hyperinsulinemia (Geor and Frank, 2009), as the horse is able to produce sufficient amounts of insulin and does not have beta-cell failure as occurs in humans (Johnson, 2002). The horse also often presents with laminitis rather than cardiovascular disease (Johnson et al., 2004). In an experiment by Asplin et al. (2007), horses infused with insulin to produce prolonged hyperinsulinemia were found to have signs of Obel grade 2 laminits (Obel, 1948) in all feet within 72 h.

Hyperleptinemic-Hyperinsulinic Syndrome in Horses

Adipose cells (including adipocytes, preadipocytes, and macrophages) secrete various biologically active molecules. Jointly, these are called adipokines (Hutley and Prins, 2005). Adipokines have a variety of functions, such as regulating energy metabolism, reproductive status, and immune function, as well as regulating cardiovascular functions. Obese individuals often have abnormal adipokine production. White adipose tissue helps with insulation of the body and provides mechanical support and storage areas for extra energy (Radin et al., 2009).

One peptide hormone secreted by adipocytes, leptin, has been studied extensively in horses due to its link to insulin and insulin sensitivity. Leptin's purpose, as defined mainly from rodent and human research, is to constrain food intake at the central nervous system, one of its main target tissues (Hadley and Levine, 2007). When low doses of leptin are infused into the ventricles of the brain, food intake and body weight are reduced in sheep (Morrison et al., 2001) as well as pigs (Barb et al., 1998). Conversely, failure to produce leptin or any leptin receptor defect results in massive obesity (Radin et al., 2009). In addition to controlling feed consumption, leptin is thought to supply the brain with a hormonal signal of the body's nutritional and energy state (Houseknecht et al., 1998). In contrast to these known effects of leptin, most overweight human subjects have normal or high serum leptin levels, and thus the obese state in humans is most likely not a leptin deficiency, but a consequence of resistance to leptin's actions (Hadley and Levine, 2007).

Leptin receptors can be found in peripheral tissues as well as the central nervous system. Receptors are particularly prevalent in the hypothalamus and brainstem. These receptors control satiety, energy expenditure, and neuroendocrine function (Buff et al., 2002, 2005). Produced by the adipocytes, leptin is secreted in proportion to body mass index or BCS in many animals,

including the horse (Prolo et al., 1998; Buff et al., 2002; Wild and Byrne, 2006), humans (Prolo et al., 1998), and ruminants (Chilliard et al., 2000). Conversely, the lack of leptin or leptin receptors (as seen in mice, rats, and humans) causes obesity (Hadley and Levine, 2007). In mares, a 24-h period of feed deprivation resulted in reduced leptin concentrations (Fitzgerald and McManus, 2000). In a longer term feed restriction, after inducing a low BCS through a heavily restricted diet, Gentry et al. (2002) found mares had lowered circulating leptin concentrations and a longer seasonal anovulatory period. Although low BCS mares had lowest leptin concentrations, well-fed mares showed a wide variation in leptin concentrations, with some exhibiting excessively high concentrations relative to other obese mares. This could mean that in mares of high BCS, factors other than BCS may influence leptin secretion (Gentry et al., 2002).

Leptin has been shown to play a role in fatty acid metabolism in both humans and rats. In humans, leptin encourages fatty acid oxidation within muscle and impedes hepatic triglyceride buildup by triggering phosphoinositol-3-kinase activity. In rats, leptin diverts lipids from non-adipose tissue (Hutley and Prins, 2005). This prevents lipotoxicity, a condition that occurs when lipids are deposited into nonadipocytes, such as the liver, muscle, pancreas and kidneys. It is essential to prevent this condition (lipotoxicity), as it results in altered glucose metabolism, fat metabolism, and impaired organ function (Radin et al., 2009).

A consistent low leptin (<5 ng/mL) or high leptin (7 to 20 ng/mL) separation of horses was described over a 2-yr period by Gentry et al. (2002) and Cartmill et al. (2003). In these experiments, leptin and insulin concentrations were highly correlated, similar to results previously reported for rats (Sivitz et al., 1998) and pigs (Ramsay and White, 2002). In one experiment, 35% of the high BCS mares were found to be both hyperleptinemic and hyperinsulinemic (Waller et al., 2006). Hyperinsulinemia, a syndrome associated with

compensated insulin resistance, occurs from an augmented insulin production in order to compensate for the outlying insulin resistance and decreased insulin clearance (Tritos and Mantzoros, 1998). In rats, hyperinsulinemia increases leptin concentrations within 3 to 5 h (Cusin et al., 1995); in contrast, leptin secretion is decreased when insulin concentrations are low (Sivitz et al., 1998). In horses, it has been reported that that hyperinsulinemic-hyperleptinemic horses have an increased and extended insulin response to glucose infusion (Cartmill et al., 2003).

In an experiment by Huff et al. (2008), the hyperleptinemic state was further evaluated in post-foaling broodmares by measuring resting leptin concentrations in blood samples taken 2 wk apart. Results were compared in a frequency diagram, which showed a normal distribution of mares across leptin concentrations varying from 0 to 6 ng/mL; hyperleptinemic mares were defined as those with mean leptin concentrations in excess of 10 ng/mL. Given that leptin is believed to have a role in insulin signaling/sensitivity in the horse, and hyperleptiniemia-hyperinsulinemia has been reported clinically in insulin resistance horses of high BCS (Frank et al., 2006), it is possible that links between high-leptin, high-insulin horses can be evaluated. Still, further research is needed to reveal relationships between the hyperinsulinemic-hyperleptinemic condition and insulin resistance in the horse.

Current Methods of Measuring Insulin Resistance

Oral glucose tolerance test. An oral glucose challenge in horses consists of administering glucose (usually as 1g/kg BW as a 20% dextrose solution) through intubation of the nasogastric cavity (Firshman et al., 2007). This typically follows a 12- to 16-h period of food deprivation, and blood samples are taken every half hour to hour following infusion.

Concentrations of glucose are expected to return to the original baseline by 4 to 5 h post-administration of dextrose (Ralston, 2002; Hoffman et al., 2003).

This method is done infrequently in an equine clinical setting, as it cannot substitute for evaluation of glucose tolerance by measuring blood glucose levels. Despite this, oral challenges can give important data, as they allow a researcher to measure how well an animal may absorb glucose through the intestine and the impact of this intestinal absorption on insulin sensitivity, measurements not typically taken into account in intravenous glucose tolerance tests. In this test, the response curves for both insulin and glucose are typically elongated as compared to intravenous methods, and often have a lengthened crest as well (Ralston, 2002).

In an experiment by Hoffman et al. (2003), an oral glucose tolerance test was used to show how reproductive status and nutritional status impacted glucose metabolism. They determined horses in late gestation had highest insulin concentrations, and that horses in early lactation have the lowest insulin concentrations. They also found that the response to glucose via the nasogastric tube was more sluggish in those fed fats and fiber than those fed starches and sugars (Hoffman et al., 2003).

Intravenous glucose tolerance test. Most commonly known as a glucose tolerance test, first described by Mehring and Tyznik (1970), the intravenous glucose tolerance test is performed after an overnight (10 to 12 h) of food deprivation. A baseline blood sample is taken and followed with a large bolus dose of dextrose (Ralston, 2002). Blood samples are also taken at 0, 5, 15, 30, 60, and 90 min post injection, and then hourly for 5 to 6 h after the injection (Firshman et al., 2007). Following the dose of dextrose, glucose values normally peak within 15 min of the injection. Insulin levels also peak around 30 min post-dextrose injection. Within 1 h of treatment, both insulin and glucose levels return to normal in a typical non-resistant horse

(Ralston, 2002). Glucose intolerance occurs when there is a prolonged, or excessively high, response to the injection; this would mean a delay in the return to baseline of greater than 2 h (Firshman et al., 2007). As this method was adapted to horses from a human model, despite being considered as the "gold standard" for many years, it is also often considered unpredictable and not well defined in horses. Thus, other methods are more commonly used in horses today.

The intravenous glucose tolerance test was first conducted in horses by Mehring and Tyznik (1970) to study the rate of glucose utilization. They found the horse to be intermediate in glucose utilization when compared to previously studied species (humans, monkeys, rabbits, lambs, calves, and sheep) and found ruminants to be less sensitive than non-ruminants.

Frequently sampled intravenous glucose tolerance test and minimal model assessment. The frequently sampled intravenous glucose tolerance test (**FSIGT**) followed by minimal model analysis (Bergman et al., 1987) has been used extensively in human medicine and has been applied to cattle, (Stanley et al., 2002), sheep (Williams et al., 2002), pigs (Behme, 1996), and horses (Hoffman et al., 2003). This assessment uses computer software and mathematical equations to extrapolate the changes in glucose and insulin over time after an intravenous load of glucose (Hoffman et al., 2003).

There are two main advantages to this test. It can evaluate the sensitivity of pancreatic beta cells to glucose, as well as calculate both insulin-dependent and insulin-independent glucose utilization (Treiber et al., 2005). Despite these advantages and its contemporary well-known use, there are limits to this test. First, the test is complex and necessitates costly software, which is a restrictive factor to clinicians, who may use the test only infrequently. Second, both glucose and insulin data are required. Although glucose concentrations can be easily determined with hand-held glucometers, insulin measurement requires more complicated laboratory analysis. Many

insulin resistant horses do not have a large insulin response, even with very high doses of insulin (Treiber et al., 2005). During the initial 20 min of this test, glucose concentrations may sometimes be greater than 2.0 g/L, exceeding a horse's renal threshold, which can cause urinary glucose spilling. This is not accounted for in the minimal model method, so would be a confounding factor (Menzies-Gow et al., 2009). Despite these disadvantages, the FSIGT and minimal modeling is commonly used by some equine researchers.

Protocol for this test requires horses to be restricted to stalls the night before the test. Reference point samples are attained to find fixed insulin and glucose values. After this, a bolus of dextrose is infused intravenously. For the following 180 min, numerous blood samples are drawn. In a modified version of the test, a single dose of insulin is given 20 min after the glucose infusion in order to create a distinct second-phase insulin response. This modification supposedly increases accuracy (Hoffman et al., 2003).

After the test, samples are evaluated by computerized algorithms to provide three main variables: the insulin sensitivity index (**Si**), glucose effectiveness (**Sg**), and the acute insulin response to glucose (**AIRg**). The Si is an approximation of net fractional glucose clearance rate per unit change of insulin, or more commonly, insulin sensitivity. The Sg indicates net fractional glucose clearance rate in the absence of insulin (i.e., non-insulin dependent glucose clearance). And thirdly, AIRg is the incremental area under the insulin curve (this calculation is found in the first 20 min of sampling post-admin) and provides an index of beta cell response to the infused glucose. Once calculations are obtained, they are evaluated together (Treiber et al., 2005). In addition, the incremental areas under the concentration versus time curves (**AUC**) for glucose (**AUCg**) and insulin (**AUCi**) are calculated. Plasma half-life time for glucose clearance (**T1/2g**) is also found through non-linear regression analysis (Hoffman et al., 2003).

Hyperinsulinemic-euglycemic clamp method. Long considered the gold standard for assessing insulin sensitivity, the hyperinsulinemic-euglycemic clamp method was first developed for use in human medicine. This method measures insulin sensitivity by measuring the amount (infusion rate) of glucose that is required to counteract the glucose-lowering effects of a constant, simultaneous infusion of insulin. Despite being the most accepted methods of measuring insulin sensitivity, the clamp technique and the FSIGT test are the most impractical, expensive, and time-consuming tests.

This procedure, described by Pratt et al (2005), calls for a period of overnight feed deprivation with only water available. For the test, baseline samples are collected, and then 2 mL of the horse's serum is combined with 5 mL recombinant insulin (100 U/mL) and 493 mL of 0.9% NaCl. The insulin solution is infused via one jugular vein to maintain a steady rate of prolonged hyperinsulinemia. This state must be maintained for the entire 180 min of the test. During this time, blood samples are drawn and tested with a glucometer every 5 min, and the amount of glucose infused, via the contralateral jugular vein, is adjusted until a constant euglycemia is achieved for at least 60 min. Blood samples are drawn every 15 min for insulin measurement as well (Pratt et al., 2005).

Based on the data from the last 60 min of the test, whole body glucose uptake (**M**) and insulin sensitivity index are calculated. Since glucose cannot be measured perfectly constantly during the clamp, no matter how careful or skilled the technician, an intricate correction factor must account for any margin of error (Pratt et al., 2005).

The clamp technique has been criticized for its nonphysiological nature, given that the glucose disposal rate relates to an unnaturally high plasma insulin concentration. The test is also very complicated and requires a highly trained person and great attention to detail. Session et al.

(2004) used this test to develop a method of inducing insulin resistance and to determine the effect of insulin resistance on the estrous cycle. They found that insulin resistant mares had a diminished ability of insulin to encourage the uptake of FFA.

Combined glucose and insulin tolerance tests. Many of the disadvantages described in the previous tests are limiting to clinicians and researchers seeking to measure insulin sensitivity. Thus, a more practical horse-specific model for measuring insulin sensitivity is needed. The combined glucose insulin tolerance test (**CGIT**) was developed in an attempt to meet this need. First described by Eiler et al. (2005), this procedure entails an overnight period of hay, trace minerals, and water only. The next morning, the subject horse is stalled and allowed free access to hay and water. An intravenous catheter is inserted, and a baseline blood sample is obtained. Dextrose is then infused intravenously (150 mg/kg BW), followed by a bolus injection of insulin. Blood samples are collected at 1, 5, 15, 25, 35, 45, 60, 75, 90, 105, 120, 135, and 150 min after infusion. In normal horses, glucose concentrations, elevated by the initial infusion, return to baseline levels by 45 min (Eiler et al., 2005; Frank et al., 2010); in insensitive horses, glucose concentrations are well above baseline at 45 min. Serum insulin can also be measured at time 0 and 45 min. If the 45-min measurement is greater than 100 mU/L, the horse is secreting more insulin than normal and/or clearing insulin at a slower than normal rate. Iatrogenic hypoglycemia may be seen (blood glucose levels <40 mg/dL), with sweating, muscle fasciculation, and weakness are often observed as symptoms (Frank et al., 2010).

Intravenous insulin injection. Intravenous injection of insulin has been studied in horses as well as mules (Silver et al., 1987; Alexander et al., 1997; Forhead and Dobson, 1997). Despite this, it has not often been used due to safety concerns, such as hypoglycemic shock, which can lead to death (Given et al., 1988). There is also the possibility of inducing laminitis

through prolonged insulin exposure (Asplin et al., 2007). Yet, experiments in donkeys have shown doses of insulin up to 0.4 U/kg BW have no serious side-effects (Forhead and Dobson, 1997). Likewise, in previous research conducted by Gentry et al. (1999), no significant side effects were noted in horses after a single intravenous dose of insulin of 0.1 U/kg BW.

Epinephrine, Glucocorticoids, and Insulin Resistance

In reaction to hypoglycemia, there is often a prompt secretion of epinephrine, norepinephrine, adrenocorticotropin (**ACTH**), glucagon, and growth hormone (**GH**; Morita et al., 2007). Under stressful states, epinephrine depresses insulin secretion (Deibert and Defronzo, 1980; Hadley and Levine, 2007) so that glucose is available to essential tissues in an emergency, rather than being converted and stored as glycogen or fat for use later. In addition, epinephrine and norepinephrine activate beta receptors of pancreatic alpha cells, and stimulate glucagon production. (Hadley and Levine, 2007).

Glucocorticoids are needed for survival and maintenance of adrenomedullary chromaffin cells, which produce epinephrine (Morita et al., 2007). Glucocorticoids have an action on insulin similar to that of epinephrine. In response to stressful situations, like injury, infections, extreme temperature, or disease, cortisol is secreted to encourage survival (Sapolsky et al., 2000). Glucocorticoids inhibit insulin action (Westerbacka et al., 2003) and boost gluconeogenesis, which encourages glucose disposal to cells in the central nervous system and cells that are not dependent on insulin for glucose uptake (Jazet et al., 2003). Hyperglycemia may be induced, which can cause the mobilization of fats and amino acids for high energy demands (Johnson et al., 2004). This hyperglycemia also guarantees that the brain and other important tissues are supplied with sufficient nutrients in the time of a stressful situation (Johnson et al., 2004). Thus, high amounts of epinephrine cause decreased insulin secretion, which causes an increase in

ACTH, leading to increased cortisol, which can cause insulin resistance as more and more insulin must be produced for the same effect (Morita et al., 2007). Insulin resistance from glucocorticoids may be aggravated by a diminished number of insulin receptors, decreased receptor affinity for insulin, or faulty signaling between cells (Jazet et al., 2003). This can cause a vicious cycle, because high cortisol levels can interfere with sleep patterns, leading to more stress. The high stress levels lead to a suppressed immune system, causing infections, causing higher levels of insulin and more stress (Sapolsky et al., 2000). Furthermore, even with persistent hyperinsulinemia, epinephrine produced from stress can amplify glucose production from the liver (Deibert and Defronzo, 1980). Moreover, in hypoglycemic patients deficient in ACTH, there may be a failure to properly recover from the hypoglycemic effects (Morita et al., 2007).

Confirming these processes, studies have shown that infusion of epinephrine into men induces hyperglycemia (Deibert and Defronzo, 1980). In a study of 23 patients, an insulin-induced hypoglycemic state resulted in decreased epinephrine in all patients (Morita et al., 2007). Studies have also been conducted in the dog (Sherwin et al., 1978; 1979) with similar findings. Exercise studies have been conducted on the horse to evaluate the effect of epinephrine on glucose. In one study, exercising horses were given an oral dose of glucose at 2 g/kg, followed 1 h later by an infusion of epinephrine. Results showed an increase in glycerol and NEFA (Geor et al., 2000). As a result of this process, both endogenous release of epinephrine after stress and exogenous epinephrine infusion can cause diminished glucose tolerance.

Insulin resistance is linked to Cushing's disease, a disease in which high levels of ACTH cause muscle problems. Cortisol is thought to impact insulin resistance by increasing blood glucose through the conversion of protein to glucose via the breakdown of muscle proteins to

amino acids, transporting those amino acids through the blood to the liver, converting these amino acids into glucose, and releasing the glucose into the bloodstream (Schott, 2002). Also, as previously described, high levels of ACTH interfere with insulin action and lead to abnormal cortisol level. This causes higher blood glucose levels, prompting increased insulin production in order to decrease the glucose to normal levels (Hadley and Levine, 2007). This can also be responsible for a decrease in muscle tissue, as cortisol breaks down muscle and decreases muscle synthesis (Schott, 2002). Horses with Cushing's disease typically present with hindered shedding, hirsitism, polyuria and polydipsia, and muscle atropy. Equine metabolic syndrome typically occurs at less than 15 yr of age, while pituitary pars intermedia dysfunction (**PPID**, or equine Cushing's syndrome) occurs in old horses (Frank et al., 2010). Further research needs to be done to determine if there is a correlation between the two diseases (Frank et al., 2010).

Feeding Regimens and Insulin Sensitivity

The effect of nutritional treatments on insulin sensitivity has been studied in many species, typically comparing fed with unfed animals having decreased glucose transport capabilities. In horses, feed deprivation lessens the pool of GLUT4 protein, reduces GLUT4 found in the plasma membrane after insulin stimulation, and causes insulin resistance relatively quickly (Kahn et al., 1988). After feed deprivation for 24 h, ponies showed a delayed peak response to an oral glucose load and delayed return to baseline; after 72 h, there was a delayed return to baseline (Breukink, 1974). Three days of feed deprivation induced insulin resistance to exogenous insulin in donkeys, and was also stressful enough to raise cortisol levels (Forhead and Dobson, 1997).

During feed deprivation, blood glucose concentrations are reduced, and there is a smaller amount of glucose available. Other energy sources must be used instead (such as stored fat).

Otherwise, insulin resistance occurs, as a "thrifty" adaptation to starvation (Forhead and Dobson, 1997). Leptin levels also decrease with feed deprivation (Radin et al., 2009).

Human athletes are commonly put on high carbohydrate diets, because of the high energy content, which may be used to build muscle glycogen and increase endurance (Bosch et al., 1996). High-concentrate diets (typically containing large amounts of corn) also have a huge impact on equine athletes and other animals (Ortigues-Marty et al., 2003). Studies have shown that diets high in nonstructural carbohydrates may decrease insulin sensitivity (Hoffman et al., 2003; Treiber et al., 2005; Pratt et al., 2006). Yet, this seems to only be true in non-exercising horses, as this difference was not seen in horses undergoing training. Thus, physical conditioning alters the effect of diet on insulin sensitivity in horses, most likely due to the changes occurring in muscle in conditioned horses (Geor, 2010).

High fat content diets cause insulin resistance in humans (Reaven, 1988); however, in humans, high fat diets may contain up to 65% fat (Ribero et al., 2004), while high fat diets fed to horses contain only 10 to 15% fat. Adding fats to equine diets has recently become a trend, and fat-supplemented diets may increase fat oxidation during exercise and spare carbohydrates. Fat supplemented horses have shown greater exercise abilities, such as extended run times during incremental speed tests compared to controls (Eaton et al., 1995), lower heart rates and less acidosis during repetitive sprints (Duren et al., 1999), and quicker gallop times over 600 and 1600 m (Harkins et al., 1992). Fat-adapted horses may also have more metabolic flexibility in expending dietary fuels during high and low intensity exercise (Kelley and Mandarino, 2000), which may be due to diminished circulating insulin (Duren et al., 1999) and enhanced insulin sensitivity.

Diets high in fat and fiber fed to horses have been found to result in increased insulin sensitivity when compared to diets rich in sugar and starch, but do not differ from those on a diet of pasture and hay only (Hoffman et al., 2003). This is likely due to the fact that a diet high in fat and fiber more closely mimics the natural state of horses grazing on pasture (Williams et al., 2001). Continued consumption of grain and molasses reduces insulin sensitivity relative to consumption of diets high in fat and fiber (Hoffman et al., 2003).

In a study in which ponies were fed either hay or grain, blood glucose concentrations after feeding were lower on the grain diet as compared to the hay diet (Argenzio and Hintz, 1972). In another study of horses fed grain and hay compared to horses fed hay only, those fed the hay and grain diet had lower blood glucose concentrations (Garcia and Beech, 1986). It is assumed that the lower blood glucose concentrations resulted from enhanced insulin secretion induced by elevated glucose concentrations after the grain consumption.

Cinnamon and Cinnamon Extracts

For centuries, cultures throughout the world have valued certain spices for their medicinal properties (Khan et al., 2003). In humans, some of these spices have ameliorative effects on disease (Khan et al., 2003), with researchers expecting them to have similar effectiveness with a decrease in the side effects commonly caused by orthodox drug management (Kim et al., 2006). Products made from plants, often with antioxidant properties, have been suggested to increase the metabolism of lipids and improve capillary action (Khan et al., 2003).

In a typical animal production system, the secondary metabolites of plants are largely an untapped resource (Greathead, 2003). With animal feed additive legislations constantly changing to prevent microbial resistance to antibiotic drugs, there has been an increased awareness of these plant metabolites as substitute performance enhancers (Greathead, 2003).

Many spices, such as bay leaves, cinnamon, and cloves, promote insulin action in vitro (Khan et al., 2003).

Since 1990, in vitro studies have shown that cinnamon extracts mimic insulin action, potentiating insulin action in isolated adipocytes (Qin et al., 2003). An extract of cinnamon, methylhydroxychalcone polymer (**MHCP**), is thought to be responsible for this insulin mimicry, and thus may be potentially useful in treating insulin resistance through increased glucose utilization (Qin et al., 2003) and through improving the function of insulin receptors (Imparl-Radosevich et al., 1998; Jarvill-Taylor et al., 2001).

In vivo studies of cinnamon extracts have been conducted in humans and rats. Qin et al. (2003) found improvement of in vivo insulin-regulated whole-body glucose utilization in rats treated with cinnamon in a dose dependent manner. These results were further validated through a study by Kim et al. (2006) using a type II diabetic animal model (db/db rats), and indicated that cinnamon extract might suppress blood glucose through improving insulin sensitivity or by reducing the rate of carbohydrate metabolism. The results of Kim et al. (2006) showed that MHCP did not affect body weight or food intake, but greatly decreased blood glucose levels, showing increased glucose disposal. Treated rats also showed a dramatic rise in serum insulin and a decline in triglyceride and total cholesterol levels (Kim et al., 2006). In humans, similar effects have been seen, with cinnamon improving fasting glucose and lipid concentrations and reducing cholesterol and triglyceride concentrations (Khan et al., 2003; Mang et al., 2006).

No refereed journal articles exist at this time on the effect of cinnamon added to the diet of horses, although some popular press articles discuss the possibility that there may be a possible unstudied beneficial effect (Frank et al., 2010). Whether documented by research or not, many horse producers and owners are increasingly relying on herbal remedies and

supplements. This has been noted by many online website and magazines, like wholehorse.com, which suggests the use of supplements like chromium, magnesium, and cinnamon for treatment for equine metabolic syndrome and insulin resistance (Frank et al., 2010).

Omega-3 Fatty Acids and Fish Oil

Since the 1970's, fish oils have been identified as valuable in alleviating cardiovascular and metabolic diseases as well as some mental illnesses in humans (Horrocks and Yeo, 1999; Kabir et al., 2007). After observing a reduced risk of coronary artery disease in Eskimos, Bang (1973) and Dyerberg (1979) conducted studies on Eskimos living in Greenland with the hypothesis that anti-atherogenic properties might be found in marine oils commonly found in the Eskimo diet. In addition to finding reduced risk of coronary artery disease, researchers found Eskimos to have lower plasma lipid levels despite a high intake of animal fats. In a more recent study, using Alaskan natives consuming seal oil or salmon daily, oil intake was linked with a decreased prevalence of compromised glucose tolerance and diabetes (Adler et al., 1994). Due to studies of this nature, including fish oil in the diet of humans, especially pooled with a low cholesterol, low saturated fat diet (Nordoy et al., 1993), has become of special interest because of the potential benefits in glucose homeostasis and insulin sensitivity (Vessby, 2000; Riserus et al., 2008).

Formerly, the hypolipidemic action of fish oils and the plasma lipid-lowering effect occurring from vegetable oils (containing linoleic acid, omega 6) were considered to have similar properties (Conner et al., 1982). Since then, it has been established that fish oil has low amounts of linoleic acid (found in vegetable oil), and instead contains large amounts of omega 3 fatty acids, predominantly eicosapentanoic acid (**EPA**) and docasahexaenoic acid (**DHA**; Hall et al., 2004; Lombardo et al., 2007). As DHA and EPA increase in serum concentrations

proportionally to the amount of fish oil fed (Ashes et al., 1992), their biological effects can be seen, especially for DHA, which is suspected to be important in setting the pace of animal metabolism. In the nervous system, DHA is highly concentrated in cellular membranes (Turner et al., 2003).

Common effects of fish oil supplementation include a decrease in the manifestation of coronary artery disease and lipid disorders, increased insulin sensitivity, and increased vascular compliance (Mueller and Talbert, 1988). Fish oil supplementation has also been shown to down-regulate enzymes associated with triacylglyceride synthesis (Marsh et al., 1987; Surette et al., 1992), causing a lowering of plasma triacylglycerol (Baltzell et al., 1991; Saynor and Gillot, 1992; Suzukawa et al. 1995) and often a lowering of plasma cholesterol (Singer et al. 1985; Baltzell et al., 1991). Some of these effects generated by fish oil supplementation may improve symptoms of metabolic syndrome and insulin resistance, given that insulin resistance (the link between many symptoms of metabolic syndrome) has been associated with a reduction in the amount of DHA found in the diet of both rats and humans (Turner et al., 2003). Baltzell et al. (1991) suggested that the ingestion of fish oil may alter how insulin is metabolized.

With increasing consumption of soft drinks, there has been a dramatic increase in high fructose corn syrup in the diet (Faeh et al., 2005) that causes a huge carbohydrate load, assuredly a factor in the current prevalence of obesity, metabolic syndrome, and diabetes (Faeh et al., 2005). Fructose is transformed into glucose and then to glycogen, thus leading to a great amount of glycogen stores (Mayes, 1993). This causes hyperglycemia, which leads to a compensatory secretion of insulin, and a down regulating of the insulin receptor (Huang et al., 1997). A certain ratio of fatty acids in cellular membranes could impact insulin action (Lardinois et al., 1987; Lombardo et al., 1996; Riccardi et al., 2004), encouraging triglyceride metabolism, which

improves glucose use and insulin sensitivity and secretion (Huang et al., 1997) at the molecular level. This is accomplished by changing the amounts of fatty acids in the membrane phospholipids of insulin target tissues, which alters biological steps enabled by membranes such as insulin transduction signals (Vessby, 2000). This effect of fatty acids accelerates ideal exchanges between the lipids and proteins within membranes, preserving regular processes of the insulin receptor bound to the membrane (Turner et al., 2003).

In addition to omega-3 fish oils being suggested as long-term additions to the diet of humans to improve glucose metabolism, they have also been shown to have effects in other species. Fish oils have been documented as impeding progress of insulin resistance in rats with dietary-induced insulin resistance (Delarue et al., 2006; Riserus et al., 2008), enhancing insulin sensitivity in pigs (Behme, 1996), reducing plasma triglycerides in pigs (Huff and Telford, 1989), reducing cholesterol levels in hypercholesterolemic animals (Warner et al., 1989), and tending to slow rate of weight gain in animals (Rizkalla et al. 1993; Huang et al., 1997).

Congruent with human studies, in rodents, a decrease in serum triacylglycerides is the most commonly described outcome of fish oil treatment (Baltzell et al., 1991; Fickova et al., 1998). Additional effects have been noted as well though. Addition of omega 3 polyunsaturated fatty acids has been shown to lessen fat matter in both rats and mice (Kabir et al., 2007) and may aim at adipose tissue secretion factors, due to the variation of circulating concentrations of leptin, adiponectin, and adipose tissue gene expression in rodents fed omega 3 (Lombardo et al., 2007). Another effect of fish oil seen in rodents is a decrease in TNF-alpha (a factor expressed in white adipose tissue and associated with insulin sensitivity), protecting against the development of diet-induced insulin resistance. Despite being preventative against developing insulin resistance, fish oil did not improve prior diet-induced insulin resistance in mice fed a high fat diet over 20

wk (Muurling et al., 2003). Podolin et al. (1998) confirmed the preventative effect of fish oil on insulin resistance and also that the supplement would not alter a pre-existing condition, concluding that the prior ailment prevents proper action of fish oil as a supplement.

Research with horses indicates similar responses to fish oil treatment as in other species: an increase in the concentration of EPA and DHA and an altered fatty acid profile (O'Conner et al., 2007; Vervuert et al., 2010), decreased triacylglyceride concentrations (Orme et al., 1997; Geelan et al., 1999), reduced serum cholesterol levels (only as compared to levels with the addition of vegetable oil; Siciliano and Wood, 1993; Orme et al., 1997), and changes in membrane composition (Portier et al., 2006). In addition, O'Conner (2007) found that horses fed an omega 3 supplement tended to have lower heart rates, lower hematocrits, and lower serum insulin concentrations. Fish oil fed to horses may also be beneficial to a syndrome that plagues horses- laminitis. Neelley and Herthel (1997) found that horses given an omega 3 supplement over a month did not develop laminitis even after being fed a high carbohydrate diet. O'Neill (2002) proposed omega 3 fatty acids as an inflammation reducer in horses, followed by the proposal by Vick et al. (2007) that omega 3 fatty acids may lessen laminitis symptoms by impeding inflammatory intermediaries.

Fatty acids may also be beneficial in the diet of horses in other ways, as fats can provide dense energy (Meyer et al., 2002), improve utilization of energy causing a reduction in heat production during exercise (Kronfeld et al., 1994), cause a reduction in carbon dioxide production in turn causing less effort to breathe (Ferrante et al., 1993), maintain greater stores of glycogen in the muscle (Hamilton et al., 1980), and allow less glucose to be used during exercise (Treiber et al., 2006). An issue to deliberate when considering adding fat or oil to the diet of horses is that weight gain may ensue, a predisposing factor to insulin resistance (Tinworth et al.,

2009), but Schmidt et al. (2001) found fish oil may improve how enzyme systems dispose of lipids in horses, and within a certain range, the composition of the fat matters more than the actual amount (Riccardi et al., 2004). Changes within a practical deviation of overall fat ingestion are not likely to have a dramatic influence on insulin resistance (Riccardi et al., 2004).

Rationale for Present Experiments

Caltabilota (2009) used intravenous injection of bovine insulin at two doses (20 and 100 mU/kg BW) to study the possible dose-response relationship of glucose to insulin injection in horses. Although not commonly used as a means of assessing insulin sensitivity in horses for reasons listed in previous sections, the approach of Caltabilota (2009) did in fact detect differences between hyperleptinemic horses (thought to be insulin insensitive but not proven to be) and horses with normal leptin concentrations. Those changes were consistent with the hypothesis that the hyperleptinemic horses were insulin insensitive; i.e., they had a reduced glucose response to a fixed dose of insulin administered intravenously relative to normal horses.

An important factor revealed by the research of Caltabilota (2009) was that insulin dose is a critical factor in the usefulness of the data. The high dose of insulin administered in the winter was too high, and blurred the information that was available from the lower dose. Later in the year, when the horses were apparently less sensitive as a group, the low dose of insulin was less useful in differentiating between hyperleptinemic and normal horses. Thus, the first phase of the research described herein was designed to further study the use of intravenous insulin injection as a means of measuring insulin sensitivity in horses. The goal was to develop a simple, on-farm technique that could be used by clinicians and researchers without complicated equipment, laboratory procedures, or expensive software. The second phase of the research described herein studied factors that might affect insulin sensitivity, or the estimates of

sensitivity, so that standard application protocols could be developed. Lastly, the resulting technique was applied to hyperleptinemic and normal horses to confirm the preliminary results of Caltabilota (2009), and then in the study of two potential treatments for the alleviation of insulin insensitivity in horses, supplementation with cinnamon extract and omega 3-rich fish oil.

CHAPTER 3

DEVELOPMENT OF A METHOD FOR ASSESSMENT OF INSULIN SENSITIVITY FROM GLUCOSE RESPONSES TO INSULIN INJECTION: EFFECT OF HYPERLEPTINEMIA IN MARES AND GELDINGS

Introduction

Total insulin production loss (type 1 diabetes) is rarely seen in horses, but insulin resistance can still cause major problems for horses, such as laminitis, pituitary adenoma, hyperlipidemia, osteochondritis dissecans, reproductive inefficiency, and an impaired ability to exercise properly (Kronfeld et al., 2005; Frank et al., 2006). Due to these problems in the horse, there is a need for practical methods of measuring insulin sensitivity.

Insulin sensitivity in horses is typically measured via one of 2 standard methods: 1) hyperinsulinemic-euglycemic clamp, in which insulin is infused at one or more fixed doses and sufficient glucose is infused to maintain euglycemia (the clamp; Kaske et al., 2001; Powell et al., 2002; Rijnen and van der Kolk, 2003), or 2) minimal modeling of the insulin-modified, frequently sampled intravenous glucose tolerance test (**FSIGT**; Bergman et al., 1987), in which a bolus of glucose is administered at time 0, and then a bolus of insulin is administered 20 min later (Hoffman et al., 2003; Treiber et al., 2005). Although intravenous insulin injection has been used in various experimental settings with horses and mules (Silver et al., 1987; Alexander et al., 1997; Forhead and Dobson, 1997), it has not been routinely used as a method of estimating insulin sensitivity, either clinically or experimentally. Concerns include: insulin overdose, which might lead to hypoglycemic shock (Given et al., 1988); and there is also a chance of inducing laminitis with repeated injections (Asplin et al., 2007). However, doses of insulin up to 0.4 USP units (**U**) per kg BW were administered to feed-deprived donkeys without any reported serious side-effects (Forhead and Dobson, 1997). Similarly, no detrimental effects in horses

administered a single intravenous dose of insulin of 0.1 U/kg BW were noted (Gentry et al., 1999).

The purpose of the present series of experiments was to develop a safe, direct assessment of insulin sensitivity that could be applied experimentally, and perhaps clinically, to horses. We hypothesized that hyperleptinemic horses would have a reduced insulin sensitivity relative to normal horses, because they display elevated insulin concentrations and exaggerated insulin responses to glucose infusion (Cartmill et al., 2003), which are indicative of insulin insensitivity. To date, there is no report indicating that these horses have a reduced insulin sensitivity.

Materials and Methods

Experiment 3.1. Experiment 3.1 was designed with the goal of developing a repeatable protocol of insulin injections for assessing insulin sensitivity across a wide range of sensitivities. Starting in June of 2008, 15 mares from the resident herd at the Louisiana Agricultural Experiment Station Horse Unit were selected with the following characteristics: low leptin concentrations and low BCS (**LL/LBCS**; n = 5), low leptin concentrations and high BCS (**LL/HBCS**; n = 5), and high leptin concentration and high BCS (**HL/HBCS**; n = 5). The mares were maintained on native grass pastures, which were predominantly Bermuda grass, bahiagrass, and dallis grass.

Originally, the experiment was designed as a replicated Latin square with 3 doses of human recombinant insulin (20, 50, and 125 mU/kg BW; Sigma, cat#I2643, 27.5 U/mg). Human recombinant insulin, rather than pancreatic bovine insulin used by Caltabilota (2009), was chosen for two reasons: 1) potentially greater long-term availability, and 2) potentially greater consistency of the product over time. After treating one replicate of mares, in which a LL/HBCS mare was treated with the 125 mU/kg BW dose and displayed signs of lethargy, it was decided

that a gradual ramping up from the lowest dose (20 mU/kg BW) would be necessary from a safety standpoint. Over the next several weeks, mares were injected in small groups with doses of insulin of 8, 20, 50, or 125 mU/kg BW, depending on upon their responses. That is, all mares received the 20 and 50 mU/kg BW dose, and the percent decreases were calculated. Percent decreases were calculated in two steps. First, by taking the two negative samples and averaging the two values to determine a baseline mean. Then, the 40 and 60 min times were subtracted from this baseline. The time (40 or 60 min) causing the greatest drop in glucose was considered the percent decrease in response to insulin injection. The 125 mU/kg BW dose was administered only to mares not experiencing at least a 50% decrease in glucose to the lower doses. The 8 mU/kg BW dose was added primarily for mares experiencing a 50% decrease or greater to the 20 and 50 mU/kg BW doses, but was subsequently administered to all mares.

Blood sampling was via jugular venipuncture through 20-gauge needles; approximately 3 mL of blood was drawn into 5-mL syringes at -10, 0, 40, 60, 90, 120, 180, and 240 min relative to insulin injection. After approximately 1 mL of blood was expressed from the syringe, a drop of whole blood was used to estimate plasma glucose concentration with a Precision Xtra glucometer. Estimates were generally based on 1 glucometer strip reading; replicate readings were conducted whenever a value seemed unreasonable (about 5% of readings). An earlier assessment (Caltabilota, 2009) of the glucometer (Precision Xtra, Abbott Laboratories) for duplicate readings of 15 blood samples between 77 and 335 mg/dL resulted in a regression equation of: second estimate = 1.03 x (first estimate) + 3.4 mg/dL ($r = 0.98$).

The percent decrease in glucose concentrations was calculated for all injections and plotted against the natural log (ln) of the insulin dose for each mare. In general, these plots resembled a typical dose-response curve, with a linear portion between 20 and 60%. Linear

regression analysis was used to calculate the regression equation for each mare [x = ln(dose) and y = % decrease], and the ln of the dose of insulin resulting in a 50% decrease in glucose concentration [ln(ED50)] was estimated from that equation; ED50 was calculated by taking the antilog of ln(ED50). Estimates of ln(ED50) and ED50 were based on at least 3 doses of insulin. In 4 of the 5 HL/HBCS mares, all % decreases were less than 50%, thus the estimate of ln(ED50) was an extrapolation to 50%.

Glucose concentrations were analyzed separately for each leptin status-BCS group by one-way ANOVA with repeated sampling, with dose as the main effect and blood sampling times as the repeated effect. From that analysis, comparisons of post-injection glucose concentrations were compared (LSD-test) to the mean at time 0 to determine when they were no longer different; this was considered to be the time of recovery for the sake of discussion. Body condition scores and the ln(ED50) and ED50 estimates were analyzed by one-way ANOVA, and differences among groups assessed with the LSD-test (Steel et al., 1997).

Experiment 3.2. Experiment 3.2 was performed with two main objectives: 1) to determine if the insulin injection scheme could be streamlined and standardized, and 2) to determine the repeatability of the ln(ED50) estimates obtained in Experiment 3.1. Twelve mares previously tested (6 LL/HBCS and 6 HL/HBCS) were retested during October of 2009, with 1 d of no treatment between each day of insulin injection. Although the quality and quantity of pasture grasses in October would not be expected to be identical to those in the summer, it was assumed that the relative insulin insensitivity displayed by hyperleptinemic mares in Experiment 3.1 would persist, because we have observed that the hyperleptinemic condition itself persists over years.

The standard approach was to treat each mare with the 50 mU/kg BW dose of recombinant human insulin on the first day. The mares were deprived of feed overnight (with ad libitum access to water) and treated the following morning between 0700 and 0900 h. Blood samples were collected via jugular venipuncture (as described for Experiment 3.1) at -10, 0, 40, and 60 min relative to intravenous injection of human recombinant insulin. Plasma glucose concentrations were estimated with the glucometer as described in Experiment 3.1.

Depending upon the % decrease in glucose concentration for a given mare, the second injection 2 d later was either 32 mU/kg BW for those mares exhibiting a 50% decrease or greater to the 50 mU/kg BW or 79 mU/kg BW for those exhibiting less than 50% decrease. The dose on the third day was either 20 or 125 mU/kg BW. The goal was to bracket the approximate 50% point; if the first two injections were on each side of 50%, the selected third dose chosen was on the lower, rather than higher, end of the dose-response curve. The third dose was administered 2 d after the second, so that the entire 3-injection protocol was completed in 5 d.

The ln(ED50) and ED50 values were calculated for each mare as described in Experiment 3.1. These data were analyzed by one-way ANOVA to test the effect of leptin status. In addition, the % decrease in glucose values in response to the 50 mU/kg BW dose of insulin and the calculated ln(ED50) values from Experiment 3.1 were compared to those obtained in this experiment by linear regression analysis as an assessment of the repeatability of the estimates.

Results

Experiment 3.1. Glucose concentrations in response to various doses of recombinant human insulin in mares with LL/LBCS, LL/HBCS, and HL/HBCS are presented in Figure 3.1. In LL/LBCS mares (Figure 3.1A), given 8, 20, and 50 mU/kg BW, mean dose-dependent

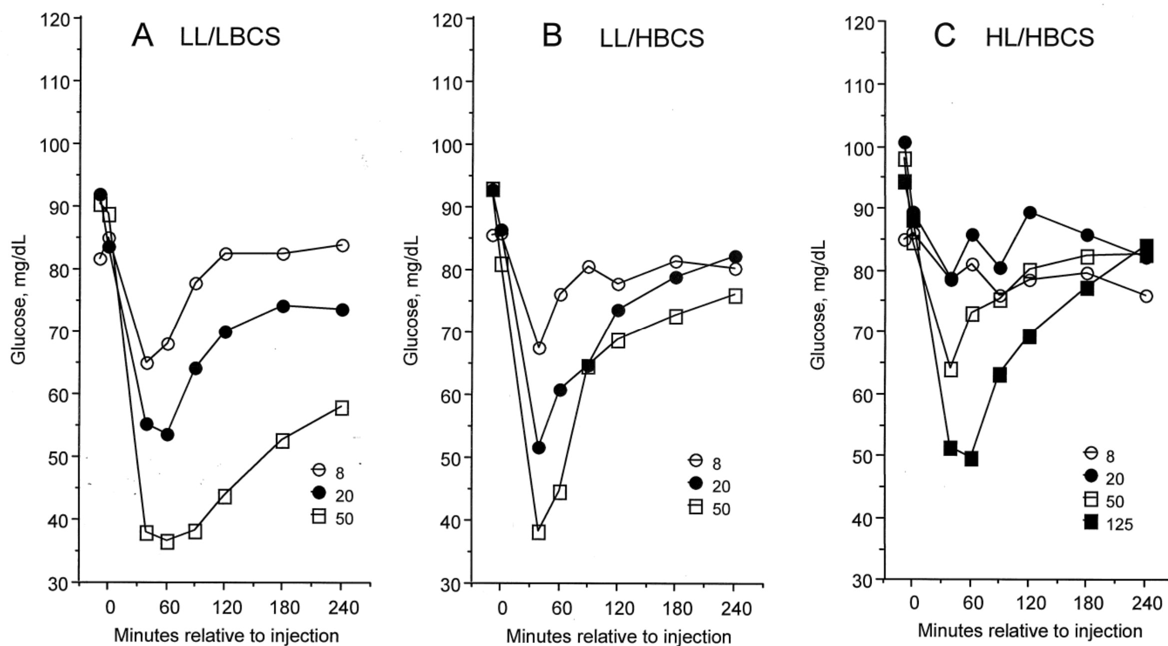


Figure 3.1. Mean glucose concentrations in mares with low leptin concentrations (LL) and low BCS (LBCS; Panel A), LL and high BCS (HBCS; Panel B), and high leptin concentrations (HL) and HBCS (Panel C) administered recombinant human insulin at 8, 20, 50, or 125 mU/kg BW during June and July in Experiment 3.1. The doses administered were based on an individual's response to the 20 mU/kg BW dose; HL/HBCS mares received the 125 mU/kg BW dose because none of them had a decrease in glucose concentrations of 50% or greater after the 50 mU/kg BW dose. Pooled SEM were 7.9, 7.1, and 8.4 mg/dL for glucose concentrations in LL/LBCS, LL/HBCS, and HL/HBCS mares, respectively.

decreases ($P < 0.001$) in glucose concentrations of 23.4, 43.1, and 64.3% respectively, were produced (SEM = 6.2%). Likewise, injection of the same doses in mares with LL/HBCS (Figure 3.1B) produced mean dose-dependent decreases ($P < 0.001$) in glucose concentrations of 26.8, 41.2, and 54.8%, respectively (SEM = 7.3%). Injection of doses of 8, 20, 50, and 125 mU/kg BW to mares with HL/HBCS (Figure 3.1C) produced mean dose-dependent decreases ($P < 0.01$) in glucose concentrations of 9.0, 16.6, 32.6, and 47.5%, respectively (SEM = 5.4%).

In addition to the initial percent decrease in glucose concentrations (40 or 60 min after injection), there were differences among groups in the recovery of glucose concentrations back to pre-injection concentrations. Mares with LL/LBCS displayed delayed recovery ($P < 0.05$) at the 50 mU/kg BW dose; in general, the recoveries were similar for all other doses in all three groups.

Mean BCS (Figure 3.2A) of mares with LL/HBCS and HL/HBCS were similar, but were both greater ($P < 0.05$) than mean BCS of mares with LL/LBCS. Mean ln(ED50) and ED50 were similar for mares with LL/LBCS and LL/HBCS (Figures 3.2B and 3.2C); both were less ($P < 0.01$) than the respective means for mares with HL/HBCS.

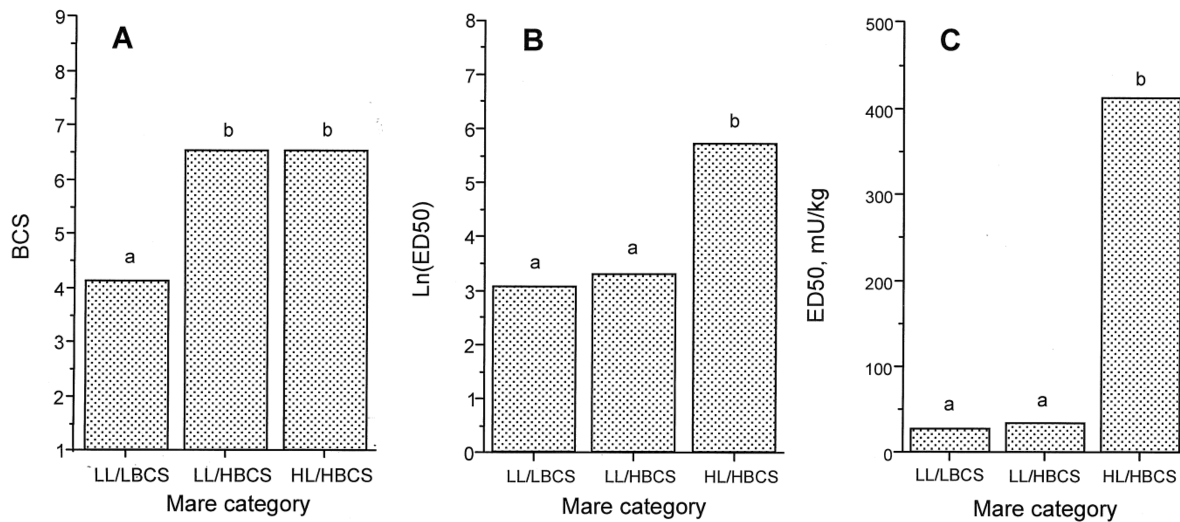


Figure 3.2. Mean BCS (Panel A), natural log (ln) of the dose of insulin that caused a 50% decrease in glucose concentrations (ED50; Panel B), and ED50 (Panel C) for mares with low leptin concentrations (LL) and low BCS (LBCS), LL and high BCS (HBCS), and high leptin concentrations (HL) and HBCS in Experiment 3.1. Means with no like superscript differ ($P < 0.05$). Pooled SEM were 0.42 for BCS, 0.51 for the ln(ED50), and 96 mU/kg BW for ED50.

Experiment 3.2. Estimates of ln(ED50) and ED50 based on the standardized approach in October in the 12 mares that had been assessed during the previous summer are presented in Figure 3.3A and 3.3B. As in the summer, LL/HBCS mares had lesser ($P < 0.001$) values in both cases relative to HL/HBCS mares. All mares were first administered the 50 mU/kg BW dose of recombinant human insulin. The decrease in glucose concentrations for those injections were highly correlated ($P < 0.01$; $R^2 = 0.847$; % decrease in October = 0.72 x % decrease in summer + 6.98%) to the responses obtained earlier (Figure 3.4A). Estimate of the ln(ED50), calculated after the subsequent injection of higher (79 and 125 mU/kg BW) or lower (20 an 32 mU/kg BW) doses, as appropriate, were also highly correlated ($P < 0.01$; $R^2 = 0.822$; ln(ED50) in October = 0.77 x ln(E50) in summer + 1.4) with those obtained earlier (Figure 3.4B).

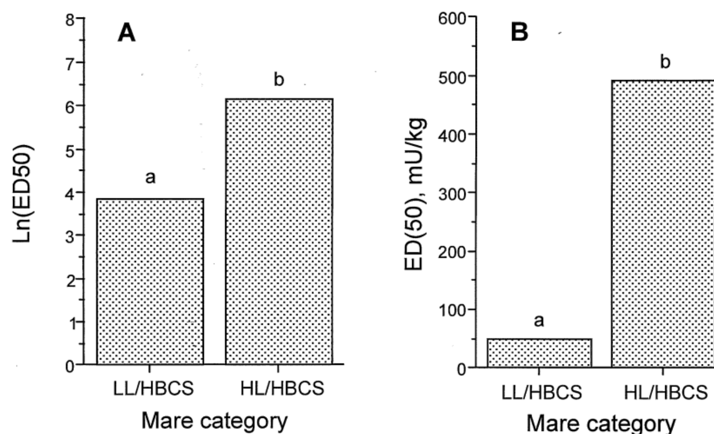


Figure 3.3. Mean natural log (ln) of the dose of insulin that caused a 50% decrease in glucose concentrations (ED50; Panel A) and ED50 (Panel B) for 6 mares with low leptin concentrations (LL) and high BCS (HBCS) vs. 6 mares with high leptin concentrations (HL) and high BCS (HBCS) originally assessed for insulin sensitivity in Experiment 3.1 and re-assessed in October in Experiment 3.2. Means with no like superscript differ ($P < 0.05$). Pooled SEM were 0.22 for the ln(ED50) and 74 mU/kg BW for ED50.

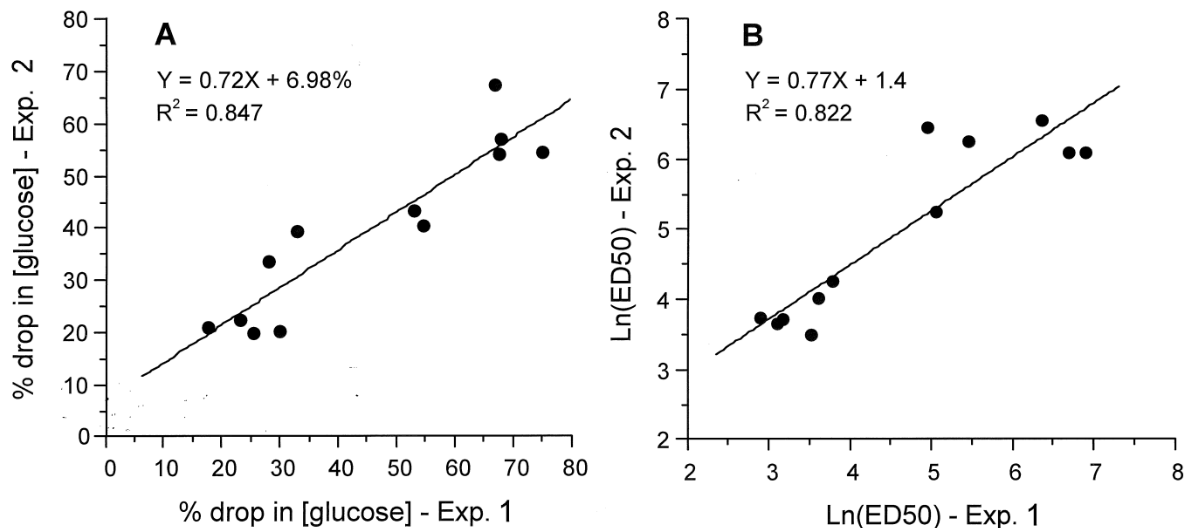


Figure 3.4. Regression analysis for the % decrease in glucose concentrations (Panel A) and the natural log (ln) of the dose of insulin that caused a 50% decrease in glucose concentrations (ED50) for data collected in Experiment 3.1 vs. 3.2 from 6 mares with low leptin concentrations and high BCS and 6 mares with high leptin concentrations and high BCS. In each case, the data were highly correlated ($R^2 > 0.8$; $P < 0.001$).

Discussion

Caltabilota (2009) reported that intravenous insulin injection might be useful for estimating insulin sensitivity in horses. In that report, he also showed that hyperleptinemic mares, as described by Cartmill et al. (2003) and Huff et al. (2008), had a reduced glucose response to insulin injection compared to mares with normal leptin concentrations. Because hyperleptinemic mares make up approximately 20% of foaling broodmares (Huff et al., 2008) and 30% of non-foaling mares with high BCS (Henneke, 1983) their identification is important for several reasons. First, they are likely more predisposed to problems such as laminitis (Bailey, 2008; Treiber et al., 2006), due to their constant hyperinsulinemia (Cartmill et al., 2003). Second, their presence, if unknown, can skew data in experiments involving insulin, leptin, or thyroid

hormone measurements (Gentry et al., 2002; Cartmill et al., 2003). Third, their identification is necessary for the development of potential ameliorating therapies or management changes.

In the course of these experiments, many insulin injections were given to mares. The only indication of any side effects to treatment was that described for the LL/HBCS mare that was treated with the 125 mU/kg BW dose of human insulin early in Experiment 3.1. Based on that experience, it was decided to modify the originally planned procedure such that all horses first received a moderate dose of insulin, and subsequent injections were based on an individual's response to that dose. Again, as a safety factor, no horse was administered a dose above 125 mU/kg BW, even when that dose did not reduce glucose concentrations at least 50%. This forced us to extrapolate beyond the actual data to calculate ln(ED50) for the least sensitive mares. Because of this extrapolation, the estimate of an actual ED50 above 125 mU/kg BW could be expected to be less precise than for those estimates below 125 mU/kg BW; however, a horse with an ED50 above 125 mU/kg BW would be considered insensitive regardless. Moreover, the repeatability of the ln(ED50) estimates in Experiment 3.1and 3.2 would indicate that this is likely not a serious limitation to the estimation procedure.

The glucose response to insulin injection seems to be comprised of two phases, the second of which is only noticeable at higher insulin doses relative to the sensitivity of the horse. The first and immediate component is the decrease from time of injection to the occurrence of the nadir in glucose concentrations, usually within the first 60 min. This is assumed to be primarily due to the uptake of glucose by peripheral tissues, mostly skeletal muscle, but also liver and adipose tissue. After small doses of insulin, glucose concentrations rapidly recover and return to pre-injection concentrations by approximately 90 to 120 min postinjection. The second phase, observed after the highest doses of insulin, is a slow recovery, such that glucose

concentrations stay depressed longer and return to baseline 60 to 90 min later than after the lower insulin doses. This latter effect likely reflects continued suppression of liver output of glucose from glycogenolysis, gluconeogenesis, or both. Because the two standard methods of assessing insulin sensitivity (the clamp and FSIGT) supposedly measure primarily or solely the first component (muscle, liver, and adipose tissue uptake), it was finally decided that the response in the first 40 to 60 min after injection was the best indicator of that event. The occurrence of the glucose nadir at 40 min was about equal to that at 60 min (48 vs 52% of all responses, respectively).

The proper dose of insulin is important for gaining meaningful information about apparent insulin sensitivity. That is, insulin doses too low or too high on the dose-response curve, if administered as a single dose, are less able to differentiate between horses of low and high insulin sensitivities (Caltabilota, 2009). Points between the 20 and 60% decrease in glucose concentrations provided the most reliable regression lines. Because of this, smaller increments in the insulin doses (the 32 and 79 mU/g BW doses) were added for assessments in Experiment 3.2. These doses allowed for closer bracketing of the 50% decrease point for horses exhibiting % decreases in glucose concentrations close to 50% after administration of the 50 mU/kg BW dose (starting dose).

In addition to these two experiments, another 9 mares and geldings were tested as part of a comprehensive assessment of the LSU herd. Throughout all the tests, the greatest % decrease in glucose concentrations observed was 78% in a gelding administered insulin at 125 mU/kg BW. Percent decreases >70% were obtained on a few other occasions, and it is possible that the upper limit, without noticeable side effects, may be around 80%. It was decided to use ED50 as the standard due to its common use in classical dose-response (sigmoidal curve) analyses.

However, it is based on the assumption that % decreases in glucose concentrations can range from 0 to 100%, which is unlikely from a physiological standpoint. A truer ED50 point might be based on a decrease in 40% of pre-injection values (i.e., 50% of 80%); however, a retrospective recalculation of the data from these two experiments based on a 40% decrease as a reference point did not alter the results (differences among groups or correlations).

The calculation of ln(ED50) and ED50 is these experiments was based on regression analysis of the ln of the insulin dose and the % decrease in glucose concentrations, and in general, three doses of insulin provided linear regression equations with high correlation coefficients. In the process of developing a standardized procedure for estimating ED50, the results from the first two doses of insulin were compared to see if they would be predictive of the final estimates based on 3 doses; the conclusion was that 2 doses provided good estimates in most cases in which the ED50 was low, but were less adequate for horses of low insulin sensitivity. Data from a single dose of insulin (50 mU/kg BW) does seem to provide a close approximation of an animal's sensitivity to insulin; however, to be applicable across a wide range of sensitivities, it was felt that the procedure with three insulin doses provided the most reliable and repeatable information.

Pratt et al. (2005) assessed the repeatability of the clamp technique and FSIGT methods of estimating insulin sensitivity in horses by administering each test twice to 6 horses in a 4-wk period. The inter-day of CV insulin sensitivity estimates averaged 14.1% (range, 7 to 20%) and 23.7% (range, 9 to 35%) for the clamp and FSIGT tests, respectively. For comparison, a similar calculation for the data in Experiment 3.1 and 3.2 (actually a month or more apart) resulted in an average within-horse CV of 8.9% (range, 2.3 to 18.8%). Pratt et al. (2005) concluded that the inter-day CV for the clamp technique was lower than for the FSIGT; thus, the repeatability of the

intravenous insulin injection method is at least equivalent, if not better, than that of the clamp technique.

One limitation of the present approach to estimating ED50 is the time involved. The final 3-injection regimen established in these experiments takes 5 d to complete, given the 1 d rest (nontreatment) period between injections. Whether the injections could be done in 3 successive days, or even closer together, needs to be determined. The potential carry-over from one injection to the next also needs to be studied. Also, horses used in the current experiments had pre-injection glucose concentrations within the normal range for feed-deprived horses; it is not known whether this approach would be applicable to horses with severe hyperglycemia (e.g., glucose concentrations of 200 mg/dL and greater). Thirdly, the assessments of possible detrimental effect were limited to external signs, and would not detect microscopic changes in hoof lamellar tisues, such as those reported by Asplin et al. (2007). For comparison, the ponies treated by Asplin et al. (2007) had mean insulin concentrations of 1036 mU/L over a 72-h infusion period; peak concentrations expected in the horses in this experiment at the 125 mU/kg BW dose would be approximately 1900 to 2500 mU/L in the first 10 min after injection (assuming a 5 to 7% of BW plasma volume), which would decay back to normal within a few hours (Gentry et al., 1999; Cartmill, 2004). Using area under the curve (concentration x hours) as an index of exposure to insulin, the highest dose used herein produces less than 7500 area units, whereas the ponies in Asplin et al. (2007) experienced an average of 74,592 area units, or 10 times more than the highest dose used herein.

Although much of the data reported herein concerns the development of the approach of direct assessment of insulin sensitivity by intravenous administration of insulin, the experiments agree with previous data showing hyperleptinemic horses have a reduced insulin sensitivity

(higher ED50) relative to horses with normal or low leptin concentrations (Caltabilota, 2009). Attempts to measure hyperleptinemic and normal horses via the clamp and FSIGT techniques had been variable and indicated no difference between horses in different leptin concentrations (Cartmill, 2004), sexes, or body weights, even though insulin concentrations in response to glucose infusion in the FSIGT were exaggerated, indicative of insulin resistance. The reason for this lack of detection of difference is unclear, but may be in part due to technician experience, variation among horses used in those trials, or to relative sensitivities of the detection methods.

Cartmill et al. (2003, 2005) reported that hyperleptinemic horses had elevated insulin concentrations, and Storer et al. (2007) confirmed that this elevation in insulin concentrations persisted in hyperleptinemic horses even when they were maintained solely on grass hay. Given that leptin can be stimulated directly by insulin infusion (while maintaining glucose concentrations within normal limits; Cartmill et al., 2005), it is likely that the hyperleptinemic condition is a result of reduced insulin sensitivity, which equates to long-term elevations of insulin concentrations and hence a long-term stimulation of adipose tissue by leptin. Although most of the hyperleptinemic horses studied over the years have had high BCS, Huff et al. (2008) reported that 11 of 24 hyperleptinemic mares (post-foaling and lactating) had BCS between 4 and 5.5. Thus, the hyperleptinemic condition is not always associated with obesity (BCS of 7 and above), and, as Huff et al. (2009) reported, is not associated with alteration of the base sequence of the exon 2 of the equine leptin gene.

In conclusion, dose-response analysis of glucose responses to intravenous insulin injections seems to be a useful approach for assessing insulin sensitivity in horses with relatively normal pre-injection glucose concentrations. Based on this approach, it was concluded that hyperleptinemic horses, which are also hyperinsulinemic and have exaggerated insulin responses

to glucose injection, are indeed less sensitive to insulin than normal horses with low leptin concentrations of the same body condition.

CHAPTER 4

FACTORS AFFECTING THE GLUCOSE RESPONSE TO INSULIN INJECTION IN MARES: EPINEPHRINE, SHORT AND LONG TERM PRIOR FEED INTAKE, AND SUPPLEMENTATION WITH CINNAMON EXTRACT OR OMEGA-3 FATTY ACID-RICH FISH OIL

Introduction

Caltabilota (2009), using intravenous injection of insulin, reported that that mares with hyperleptinemia had a lesser glucose response to fixed insulin doses, as was predicted by the fact that hyperleptinemic horses also have elevated insulin concentrations (Storer et al., 2007) and an exaggerated insulin response to administered glucose (Cartmill et al., 2003). In the experiments described in the previous chapter (Experiments 3.1 and 3.2), it was confirmed that intravenous injection of appropriate insulin doses can be used to estimate insulin sensitivity in horses, and that hyperleptinemic horses have reduced insulin sensitivity relative to horses with normal leptin concentrations.

Poor insulin sensitivity in horses has been associated with laminitis and the metabolic syndrome (Treiber et al., 2006; Bailey 2008). Exercise has been reported to improve insulin sensitivity in horses (Powell, 2002; Stewart-Hunt, 2006), and both cinnamon extract (Anderson, 2008) and omega-3 fatty acid (via fish oil) consumption (Popp-Snijders, 1987; Oh et al., 2010) improves insulin sensitivity in various species.

The series of experiments reported herein were conducted with two objectives. The first was to determine the effects of elevated epinephrine (as would occur in stressed horses) and prior feed intake in the short (feed deprived, overnight hay, and pasture) and long term (10 d of pasture vs. hay in a dry lot) on the insulin-induced decrease in glucose concentrations in horses.

The second objective was to determine whether supplementation with cinnamon extract or fish oil would improve the insulin sensitivity of hyperleptinemic (insulin insensitive) mares.

Materials and Methods

The Animal Care and Use Committee of the LSU Agricultural Center approved all experimental procedures. Mares chosen from the resident herd in Baton Rouge were light horse mares between 13 and 23 yr old, weighing between 463 and 648 kg with BCS (Henneke, 1983) between 5 and 8. These mares were kept on native grass pastures throughout the year. During the winter, they were also supplemented with native grass hay (round bales) as needed to maintain body condition. All mares had been previously categorized with regard to mean leptin concentration (relative to other mares; i.e., either hyperleptinemic or normal) and insulin sensitivity (sensitive or insensitive as determined by their responses to insulin injection).

Experiment 4.1. Effects of pretreatment with epinephrine. Experiment 4.1 was designed to determine the effect of pre-injection with epinephrine on the glucose response to a single dose of recombinant human insulin in sensitive versus insensitive mares (determined in Experiments 3.1 and 3.2). Four insulin sensitive and 4 insensitive mares were used. The experiment was performed as a single switch-back, with 2 mares within each sensitivity group exposed to epinephrine on the first day (December 5, 2009; the rest received saline), and the other mares receiving epinephrine on the second day (December 7, 2009). Epinephrine was administered i.v. at a dose of 5 μg/kg body weight (Sticker et al., 1995) in saline at a volume of 0.01 mL/kg; control injections were the same volume of saline only. For each treatment day, mares were brought in from pasture and were deprived of feed overnight (approximately 13 h) in a dry lot paddock but had ad libitum access to water. Injections started at approximately 0800 the next morning. Jugular blood samples were collected for glucose determination via a hand-

held glucometer (Precision Xtra, Abbott Laboratories, Abbott Park, IL; Eiler et al. 2005; Caltabilota, 2009) at -10 and 0 min before epinephrine or saline injection. Subsequent samples were collected at 20 min (followed by the insulin injections) and 30, 40, 60, and 80 min. Insulin was administered intravenously after the 20-min sample at 50 mU/kg of body weight for sensitive mares and 125 mU/kg of body weight for insensitive mares. These doses had been found previously (Chapter 3) to produce decreases in blood glucose of approximately 50% for the mares in the respective categories. Once sampling had been completed, mares were returned to pasture.

Blood glucose concentrations were analyzed by ANOVA (SAS Instit., Inc, Cary NC) as a replicated 2 x 2 Latin square design with a 2 x 2 factorial arrangement of treatments (epinephrine treatment and insulin sensitivity category).

Experiment 4.2. Effect of overnight feeding regimen. Experiment 4.2 was designed to determine the effects of overnight feed intake on the glucose response after an injection of human recombinant insulin in sensitive versus insensitive mares. The experiment was performed as a replicated 3 x 3 Latin square. The three treatments groups were: feed deprived overnight, ad libitum access to grass hay overnight, and pastured overnight. All groups of horses had access to water ad libitum. Twelve mares were used: 6 insulin insensitive mares and 6 insulin sensitive mares (determined in Experiments 3.1 and 3.2). Within each phase, 2 mares of each category were managed overnight as described in the treatments, and then tested the following morning. The test days were July 16, 18, and 20, 2010. No later than noon on each test day, all mares were returned to pasture. For overnight feed deprivation, mares were brought in from the pasture at approximately 1900 h the day before and kept in a dry lot with ad libitum access to water. For the hay-fed treatment, mares were brought in from pasture the same way, but placed in a dry lot

with ad libitum access to grass hay and water overnight. For the pastured group, mares were left in the pasture until the morning of testing, and were brought up at approximately 0700 h.

On each day of testing, mares were tethered loosely inside an open-sided barn at 0700 and two blood samples were drawn by jugular venipuncture 10 min apart. Insulin was administered intravenously at a dose of 50 mU/kg BW for sensitive mares and 125 mU/kg BW for insensitive mares. Subsequent blood samples were collected at 40 and 60 min after insulin injection. Glucose concentration was determined in all blood samples with the glucometer described previously. The maximum % decrease in blood glucose concentrations was calculated for each mare on each occasion by first averaging the two pre-insulin blood glucose concentrations. The blood glucose values at 40 and 60 min were subtracted from this mean, and the net decrease was then expressed as a percentage of the pre-injection mean. The largest % decrease of the two (at 40 and 60 min) was used as the data point for that mare on that occasion.

When all phases were complete, the % decreases were analyzed by ANOVA as a replicated (4 squares) Latin square design (SAS Instit., Inc., Cary, NC). Treatment effects were arranged as a 2 x 3 factorial (2 sensitivity categories and 3 overnight feeding regimens). Differences between means were assessed by the least-significant difference (LSD) test (Steel et al., 1997).

Experiment 4.3. Effect of 10-d acclimatization to pasture vs. hay. Experiment 4.3 was conducted in October and November, 2010, as a replicated 2 x 2 Latin square design to test the effect of long-term feeding regimen (10 d) on the % decrease in blood glucose concentrations after a standard dose of insulin in sensitive versus insensitive mares. Procedures were similar to those in Experiment 4.2, except that mares were acclimated for 10 d to either native grass hay fed in a dry lot with ad libitum access to water, or maintenance on pasture with ad libitum access

to water. All mares were feed-deprived overnight (minimum of 12 h) before each insulin injection. Doses of insulin used at the end of each period were the same as in Experiments 4.1 and 4.2. Additionally, a second injection of insulin was used 2 d later so that ED50 values could be estimated. The ED50 value, as defined in Experiment 3.1, was the calculated dose of human recombinant insulin that causes a 50% decrease in blood glucose concentrations in 40 to 60 min after intravenous injection, and was estimated by linear regression of at least 2 different doses of insulin falling on the linear portion of the insulin-glucose dose response curve (natural log of insulin dose in mU/kg BW on the x-axis and % decrease in blood glucose concentrations on the y-axis). Data were analyzed by ANOVA as a replicated 2 x 2 Latin square design with a 2 x 2 factorial arrangement of treatments (insulin sensitivity classifications and feeding regimens); differences between means were assessed by the LSD test.

Experiment 4.4. Effect of cinnamon extract on insulin sensitivity. Experiment 4.4 was conducted in April and May, 2010. Ten mares with reduced insulin sensitivity were used. Each mare was randomly allotted to one of two treatments: cinnamon extract (Cinnulin PF, Integrity, Spring Hill, TN) or vehicle (controls). The cinnamon extract was prepared as an aqueous solution. Cinnulin (10 mL) was given orally via a 30-mL syringe twice daily (at approximately 0800 and 2000 h). Control mares were given 10 mL water twice daily in the same manner as the treatment mares.

Mares were first assessed for insulin sensitivity by intravenous insulin injection; a total of 3 doses of insulin (between 32 and 125 mU/kg BW) were injected on 3 consecutive days for assessment of ED50. Treatments were then administered for 10 d and the insulin injections for the sensitivity assessments were repeated on the 8th, 9th, and 10th day. The ED50 values pre- and post-treatment were compared by one-way ANOVA. In addition, blood samples were

collected by jugular venipuncture from all mares on d 5 through 10 of treatment for measurement of insulin and leptin concentrations. Insulin was measured with commercially available reagents (Diagnostic Systems Laboratory, Webster, TX) and leptin was measured with a previously validated radioimmunoassay (Cartmill et al., 2003). Hormonal data were analyzed with repeated measures ANOVA in SAS.

Experiment 4.5. Effect of omega-3 fatty acid-rich fish oil on insulin sensitivity.

Experiment 4.5 (June, 2010) was conducted in a similar manner as Experiment 4.4 after re-randomization of the mares (one mare was replaced). Treated mares (n = 5) received 10 mL of an omega-3 fatty acid-rich fish oil (Wellpride; http://www.wellpride.com) twice daily (morning and evening) top-dressed on 0.5 kg of sweet feed, and controls (n = 5) received the sweet feed only. Insulin sensitivity was assessed as described in Experiment 4.4, but on alternate days rather than successive days, and mares were then supplemented for 13 d. Post-treatment assessments of insulin sensitivity were conducted in the last 5 d of supplementation.

Results

Experiment 4.1. There was an effect ($P = 0.002$) of epinephrine pre-treatment on the blood glucose responses to insulin injection in Experiment 4.1, as well as a treatment x time interaction ($P < 0.001$; Figure 4.1). There was no effect of insulin sensitivity status (due to the different doses of insulin used) or any interaction with treatment or time. Blood glucose concentrations decreased by approximately 30% at 60 min after insulin injection relative to pre-injection concentrations in mares of both sensitivity groups. Prior administration of epinephrine completely abolished the decrease in blood glucose concentrations, and concentrations were higher ($P < 0.05$) at 20, 30, 40, and 60 min after injection relative to pre-injection concentrations.

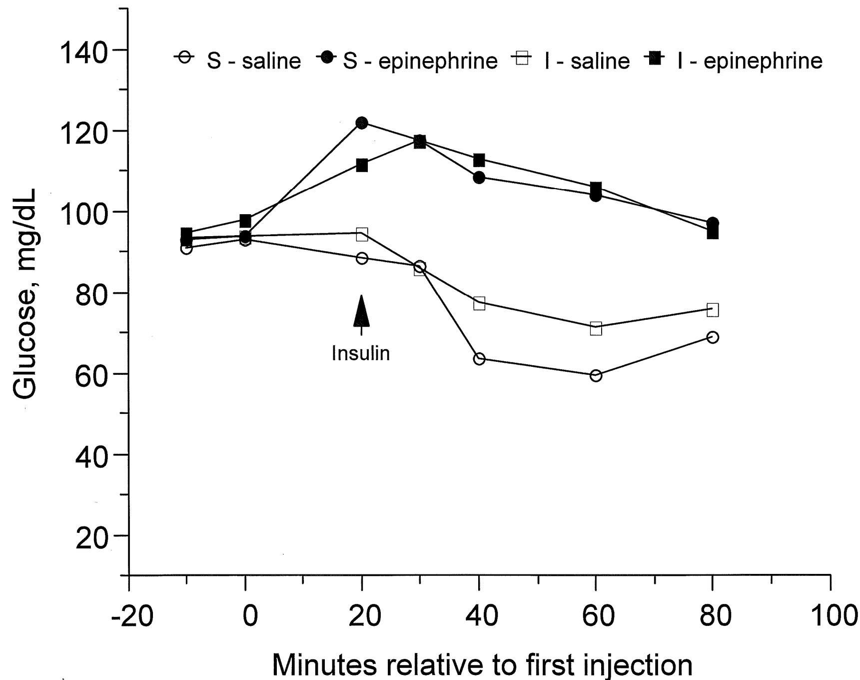


Figure 4.1. Blood glucose concentrations in insulin sensitive (S) and insensitive (I) mares before and after an injection of epinephrine (5 µg/kg BW) or saline, followed by an injection of recombinant human insulin 20 min later. Insulin doses were designed to decrease blood glucose concentrations equally in the two groups, thus sensitive mares were administered insulin at 50 mU/kg BW and insensitive mares were administered insulin at 125 mU/kg BW. Insulin injection decreased ($P < 0.001$) blood glucose concentrations equally in all mares, and prior administration of epinephrine increased ($P < 0.05$) blood glucose concentrations in all mares. Pooled SEM from the ANOVA was 4.4 mg/dL.

Experiment 4.2. Overnight feeding regimen affected ($P = 0.0004$) blood glucose concentrations in both sensitive and insensitive mares (Figure 4.2). The mean percentage decreases in blood glucose concentrations differed ($P < 0.06$) among all treatments (49.8, 58.3, and 70.2% for fasted, hay, and pasture, respectively). The only difference ($P = 0.0854$) between the responses of sensitive and insensitive mares was in the pastured group, when insensitive mares had the greater response.

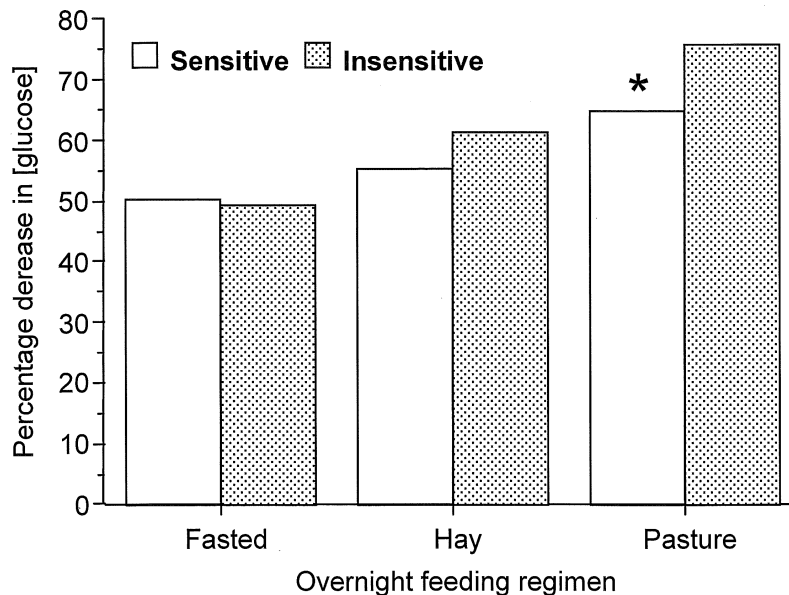
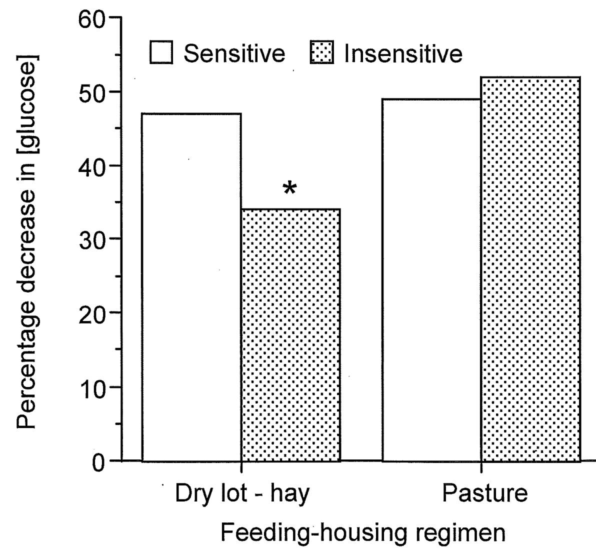


Figure 4.2. Percentage decreases in glucose concentrations for mares predetermined to be insulin sensitive versus insensitive in Experiment 4.2. Mares were housed in stalls and supplied with water but no feed or hay (Fasted) or just hay (Hay) overnight, or housed on pasture and brought in for testing that morning (Pasture). Sensitive mares were administered insulin at 50 mU/kg BW and insensitive mares were administered insulin at 125 mU/kg BW. The mean percentage decreases in blood glucose concentrations differed among all treatments (49.8, 58.3, and 70.2% for fasted, hay, and pasture, $P < 0.06$). The only difference between the sensitive and insensitive mares was for testing after being on pasture (asterisk; $P = 0.0854$). Pooled SEM from the ANOVA was 6.0%.

Experiment 4.3. There was an effect ($P < 0.05$) of 10 d of acclimatization of mares to either pasture or hay in a dry lot and insulin sensitivity groups (Figure 4.3). The % decrease in blood glucose concentrations (Figure 3A) to the first insulin injection (50 and 125 mU/kg BW for the sensitive and insensitive mares, respectively) was similar for both groups when acclimated to pasture, but the decrease was much less ($P = 0.021$) for insensitive mares than sensitive mares when they were kept in a dry lot and fed hay. The ED50 values (Figure 4.3B),



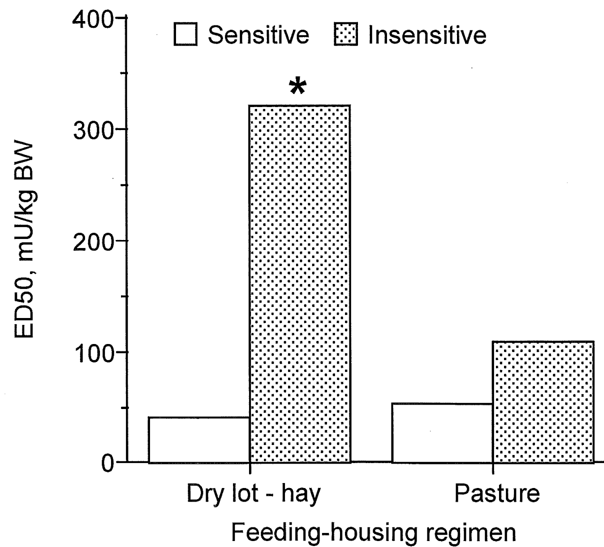


Figure 4.3. Percentage decreases in blood glucose concentrations to the first insulin injection (top panel) and associated ED50 values (bottom panel) for mares predetermined to be insulin sensitive versus insensitive in Experiment 4.3. Mares were kept for at 10 d in a dry lot with ab libitum access to native grass hay and water (dry lot - hay) or kept on pasture with ad libitum access to water (pasture). In each case, they were kept in a dry lot with no access to feed starting approximately 14 h before insulin injection. Sensitive mares were administered human recombinant insulin intravenously at 50 mU/kg BW and insensitive mares were administered insulin at 125 mU/kg BW. Two subsequent injections were used for the calculations of ED50. The asterisk indicates a difference ($P = 0.021$ for percentage decrease; $P \leq 0.073$ for ED50) from all other means. The SEM was 3.4% for percentage decrease and 106 mU/kg BW for ED50.

which are inversely related to insulin sensitivity, indicated the same trend; mean ED50 of insensitive mares on dry lot was greater (P = 0.073) than when they were on pasture as well as from insensitive mares (P = 0.029) in either situation.

Experiment 4.4. Mean ED50 values before treatment was initiated for control mares and those to receive cinnamon extract were 103.4 and 104.6 mU/kg BW (SEM = 23 mU/kg BW). At the end of treatment, mean ED50 values were 98.1 and 75.8 mU/kg BW, respectively (P = 0.53). Cinnamon extract feeding did not affect plasma concentrations of leptin or insulin (data not shown).

Experiment 4.5. Mean ED50 values before treatment was initiated for control mares and those to receive fish oil were 192.3 and 128.0 mU/kg BW (SEM = 44 mU/kg BW). At the end of treatment, mean ED50 values were 128.0 and 83.5 mU/kg BW, respectively (P = 0.32). From this, it was determined that there was no significant effect of fish oil treatment.

Discussion

The experiments conducted herein were designed to better characterize any differential responses of insulin sensitive versus insensitive mares under various conditions that might be encountered when assessing insulin sensitivity in horses. Whatever the assessment method, factors such as stress before testing, or questions as to how best to prepare the mare before testing, need to be clarified so that assessments can be standardized across a spectrum of circumstances. Short term stress, and its associated increase in adrenal catecholamine output, is known to reduce insulin sensitivity in humans (Brandi, 1993; Sherwin, 1984). In contrast, exercise, which also stimulates adrenal catecholamine output, has been shown to increase insulin sensitivity in humans in both the short (Borghouts, 2000) and long term (Soman, 1979; Nuutila,

1994). This contradiction is likely due to the direct effect of exercise on muscle, which increases GLUT-4 (Cortright and Dohm, 1997; Borghouts, 2000) protein in the absence of insulin, in spite of the increase in epinephrine occurring at the same time. Administration of epinephrine alone, without exercise, reduces both whole body insulin sensitivity in humans (Deibert and Defronzo, 1980) and isolated muscle sensitivity in rats (Budohoski, 1987). The stimulation of blood glucose concentrations by epinephrine in Experiment 4.1 was similar in magnitude (about 25% above baseline) to that reported by Sticker et al. (1995) in mares not injected with insulin. Thus, the stimulatory effect of epinephrine at this dose on liver output of glucose was unaffected by insulin injection 20 min later. This is in agreement with reports in humans (Deibert and Defronzo, 1980; Vicini, 2002) and rodents (Budohoski, 1987) showing that epinephrine not only suppresses liver glycogenolysis and gluconeogenesis, but also acts directly on muscle to inhibit the normal response to insulin. The practical implication of these results is that any stimulus that might trigger adrenal output of epinephrine, such as excitement or stress, will alter the assessment of insulin sensitivity and must be avoided before testing.

Traditionally, preparation of subjects for assessment of insulin sensitivity involved a period of feed deprivation before testing (Bergman, 1987; Powell, 2002; Vick et al., 2007). This insured that any insulin response to prior meals would not affect the testing result. In contrast, Hoffman et al. (2003) provided horses hay ad libitum overnight prior to administering a modified FSIGT, because feed deprivation had been reported to reduce tissue sensitivity to insulin action in donkeys (Forhead and Dobson, 1997). However, in that study, Forhead and Dobson (1997) compared only overnight versus 3 d of feed deprivation, which may not provide any insight into the effect of overnight versus no feed deprivation. That is, the elevation of plasma FFA during feed deprivation, which Sessions et al. (2004) reported to be a mediator in the decrease in insulin

sensitivity, are 200% higher after 3 d of feed deprivation but unaffected after approximately 22 h of feed deprivation (Sticker et al., 1995a,b). Thus, it was imperative to directly assess the effect of overnight feed deprivation versus those of ad libitum hay or continued pasture grazing. The results in Experiment 4.2 showed that an overnight period of feed deprivation resulted in a lesser response to injected insulin in both sensitive and insensitive mares. Moreover, overnight availability of hay resulted in a lesser insulin response compared to overnight grazing on pasture. Based on the results of Experiment 3.1 and 3.2, in which all insulin injections were given after an overnight period of feed deprivation, the doses of insulin were adjusted for sensitive (50 mU/kg BW) and insensitive mares (125 mu/kg BW) to provide decreases in blood glucose concentrations of about 50%, because that response best estimates actual ED50 value for a given horse. Further adjustment of the doses downward for testing after pasture grazing may have indicated that the difference between sensitive and insensitive mares was even greater than observed, because responses to either very high or very low insulin doses tend to either diminish or obliterate the differences that are detectable with appropriate insulin doses.

The lowered glucose response to overnight feed deprivation relative to ad libitum hay or pasture grazing is assumed to be due to several intertwined factors: 1) the extended time which endogenous insulin secretion would be minimal, 2) little or no absorption by the gut of digestion products of ingested nonstructural carbohydrates and greater absorption of VFA from the cecum, and 3) greater liver glycogenolysis and gluconeogenesis due to the first two factors. The possibility that a slight increase in FFA concentration, perhaps not detectable under the conditions of the study of Sticker et al. (1995a,b) or an increase in adrenal catecholamine output affecting insulin sensitivity directly at the muscle, deserves further study.

Again, these assessments were made after an overnight period of feed deprivation. It is assumed that consumption of the hay, relative to the pasture, resulted in a lower absorption and utilization of sugars from the digestible carbohydrates, due to losses normally incurred during drying of the grasses to hay (Pelletier et al., 2010). This assumption is supported by the fact that hay versus pasture grazing has been shown to reduce mean insulin and leptin concentrations in both normal and hyperleptinemic horses by 50% (Storer et al., 2007). In addition, consumption of pasture includes simultaneous consumption of water (in the plants), which also tends to increase insulin secretion relative to consumption of dry feed alone (Nadal et al., 1997). Again, greater dependency on VFA from the cecum for energy in horses acclimated to hay would result in a situation similar to that described for Experiment 4.2 for overnight feed deprivation (i.e., low insulin concentrations and greater liver glucogenic activity), and hence reduced insulin sensitivity.

The fact that overnight pasture availability and pasture acclimatization for 10 d both increased insulin sensitivity in mares relative to feed deprivation or hay consumption seems in contradiction to the fact that longer-term acclimatization to carbohydrate-rich diets decreases insulin sensitivity relative to fat and fiber-rich diets (Hoffman et al., 2003; Treiber et al., 2005). Treiber et al. (2005) discussed this point in their report, indicating that insulin resistance is generally associated with decreased energy availability or increased energy demand. They suggested that rapid increases and decreases in blood glucose and insulin concentrations after a high-glycemic meal may in fact trigger similar energy-conserving regulation. An involvement of growth hormone secretion, which is suppressed in humans after a meal and then surges several hours later, may have been implied (but was not stated) by the reference to a report by Yalow et

al. (1969) in that discussion (Treiber et al., 2005). Elevation of growth hormone levels does elicit a diabetic-like situation with insulin resistance in humans (Rosenfeld, 1982).

Neither cinnamon extract nor fish oil supplementation altered the insulin sensitivity of mares with known low sensitivity under the conditions of these experiments. Most of the positive effects of cinnamon ingestion on insulin sensitivity have been reported for humans (Solomon, 2009; Qin, 2010) and rodents (Couturier et al., 2010). In contrast, several studies with horses have shown positive effects of omega-3 polyunsaturated fatty acids on the production of mediators of inflammation (McCann et al., 2000; Hall et al., 2004), immune function in yearlings (Vineyard et al., 2010), heart rate during exercise (O'Conner et al., 2004), serum triglycerides (O'Conner et al., 2007) and stride length (Woodward et al., 2005). Omega-3 fatty acid supplementation has been shown to improve insulin sensitivity in several species (Behme, 1996; Gingras et al., 2007; Anderson et al., 2008; Huang et al., 2010).

In conclusion, insulin sensitivity, as assessed by intravenous insulin injection in mares, is acutely affected by prior epinephrine administration, thus measures need to be taken to avoid excitement or stress of animals before testing is begun. Lastly, two potential supplements shown to improve insulin sensitivity in other species, cinnamon extract and omega-3 rich fish oil, had no effect on insulin sensitivity in mares of known low sensitivity.

SUMMARY AND CONCLUSIONS

The goal of the research conducted herein was to develop a more practical method of measuring insulin sensitivity in horses. Identification of insensitive horses can 1) help producers modify management practices for affected animals to reduce the detrimental impact on their future productivity and 2) aid researchers in developing experimental groups. The method developed is based on the intravenous administration of recombinant human insulin at doses of 8, 20, 50, and 125 mU/kg BW, starting with a dose of 50 mU/kg BW, with the subsequent doses being lower or higher as appropriate to get sufficient data for estimation of ED50. This method was then further refined and validated through practical applications in experiments studying 1) the effect of the hormone epinephrine, 2) the effect of pre-trial feeding regimen, and 3) the possible benefit of two nutritional supplements (cinnamon extract and fish oil).

In the first study, dose-responses in mares of potentially different insulin sensitivities were measured. The ED50 was similar for all mares with normal plasma leptin concentrations, regardless of BCS, and was lower than for mares displaying hyperleptinemia. The second study was conducted in order to determine the repeatability of the results obtained in the first study. Estimates obtained in both experiments were highly correlated, showing equal to or better repeatability as compared to other methods of assessing insulin sensitivity in horses.

After the first two experiments, the practical application experiments helped to refine the procedure. The administration of epinephrine before the injection of insulin elevated blood glucose concentrations and prevented the insulin induced drop in blood glucose concentrations in all mares (both normal and hyperleptinemic). Depriving mares of feed overnight caused a decrease in insulin sensitivity relative to overnight ad libitum access to hay. Both resulted in reduced insulin sensitivity relative to overnight pasture availability, and both sensitive and

insensitive mares responded similarly except when kept on pasture. When confined to a dry lot for 10 d with only access to hay, relative to pasture grazing, insensitive mares demonstrated reduced insulin sensitivity, but sensitive mares did not. Neither supplementation with cinnamon extract nor omega-3 fatty acid-rich fish oil had an effect on insulin sensitivity of mares with known low insulin sensitivity under the conditions of these experiments.

In conclusion, dose-response analysis of glucose responses to intravenous insulin injections seems to be a useful approach for assessing insulin sensitivity in horses with relatively normal pre-injection glucose concentrations. This method also seems to be relatively safe, as no negative physical effects were noted once the procedure was standardized. While it does take a few days to complete, it is relatively easy to perform, does not require skilled technical ability, and does not require expensive software or complex mathematical calculations. This research should be beneficial to researchers and producers.

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VITA

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