SKELETAL MUSCLE GLUCOSE METABOLISM IN A DEXAMETHASONE MODEL OF INSULIN RESISTANCE IN STANDARDBRED HORSES

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ABSTRACT

SKELETAL MUSCLE GLUCOSE METABOLISM IN A DEXAMETHASONE MODEL OF INSULIN RESISTANCE IN STANDARDBRED HORSES

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The purpose of this thesis was to characterize the effects of dexamethasone treatment on 1) insulin sensitivity and glucose dynamics in healthy horses; and 2) selected aspects of insulin signalling and glucose metabolism in equine skeletal muscle. To address 1) six Standardbred horses received either dexamethasone or the equivalent volume of saline every 48 h over a 21-day period in a balanced crossover design. At the end of each treatment period, an insulin modified frequently-sampled intravenous glucose tolerance test with minimal model analysis was administered. To address 2) the same treatment protocol was used and the horses underwent a 2-h euglycemic-hyperinsulinemic clamp (EHC), including muscle biopsies before and after the EHC, to assess insulin sensitivity and measure GLUT-4 protein abundance, muscle glycogen content, glycogen synthase (GS) and hexokinase (HK) enzyme activities and Akt, GSK-3α/β and PP-1α protein abundance and phosphorylation. Dexamethasone treatment significantly decreased insulin sensitivity and increased beta-cell responsiveness, increased HK activity and abrogated the insulin-stimulated increase in GS activity. Decreases in GSK-3α/β $Ser^{21/9}$ phosphorylation may, in part, explain the reduced GS activation.

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LITERATURE REVIEW

General Introduction

Insulin resistance, defined as a decrease in insulin-mediated uptake of glucose by target tissues such as skeletal muscle, has been implicated in the pathogenesis of several conditions of horses and ponies. These conditions include Cushing's syndrome (pituitary gland adenoma), laminitis, hyperlipemia, and the so-called Equine Metabolic Syndrome. For each of these conditions, the antagonistic effect of glucocorticoids (GC) on insulin-mediated glucose metabolism has been proposed as the mechanism of insulin resistance (IR). However, the true role of IR in these conditions is uncertain, in part because nonspecific rather than quantitative measurements have been taken as evidence of IR (Kronfeld *et al.*, 2005). Furthermore, there is little information in horses regarding the specific effects of GC on insulin and glucose metabolism. Based on these observations, the work contained in this thesis was designed to evaluate the effect of excess glucocorticoids on insulin sensitivity and glucose homeostasis as well as various molecules that may be altered in equine skeletal muscle glucose metabolism. It was anticipated that these studies would provide better understanding of changes that may be occurring at the whole-animal and molecular level during states of insulin resistance that are associated with various disease states in horses.

Overview of glucose homeostasis

Regulation of glucose homeostasis within the body is complex, involving interactions between many factors, amongst which are plasma glucose concentration, muscle glycogen content, hepatic glucose output, the secretion and concentration of serum insulin, as well as the overall energy state of the body. Glucose and insulin

regulate glucose uptake by different mechanisms within skeletal muscle, namely glucose-mediated glucose disposal and insulin-mediated glucose disposal. While glucose mediated disposal is less well understood, it is known that an increase in glucose concentration will increase the rate of systemic glucose disposal in normal humans (Lawrence and Roach, 1997). Insulin-mediated glucose disposal involves an increase in glucose transporters at the plasma membrane and a complex signalling pathway that ultimately leads to glycogen synthesis and/or glucose oxidation (Lawrence and Roach, 1997). By either mechanism, once glucose enters the muscle cell it follows one of two pathways, which include oxidization within mitochondria, or storage as glycogen. Storage of glucose as glycogen requires a number of steps. Upon entering the cell via a GLUT-4 transporter, glucose is phosphorylated by the enzyme hexokinase through the addition of a phosphate group, provided by ATP, to form glucose-6-phosphate (G-6-P) (Berg *et al*, 2002). Following an isomerization and phosphorylation step, G-6-P forms glucose-1-phosphate (G-1-P) and finally uridine-diphosphate-glucose (UDP) through the addition of a uridine group (Berg *et al*, 2002). UDP-glucose is then dephosphorylated by glycogen synthase to form glycogen.

As mentioned previously, insulin-mediated glucose metabolism in skeletal muscle involves a complex and incompletely understood array of signalling proteins. In brief, circulating insulin binds to the insulin receptor at the plasma membrane, causing a conformational change in this receptor and its activation (Byrant *et al*, 2002). Upon activation, the receptor phosphorylates insulin receptor substrates 1 & 2, which then recruit other effector molecules such as Akt and phosphatidylinositol 3-

kinase (PI3-kinase) to this insulin receptor complex (Byrant *et al*, 2002). PI3-kinase is responsible for the activation of Akt, a crucial signalling protein involved in both glucose transport and glycogen synthesis (Kandel and Hay, 1999). Akt stimulates glycogen synthesis through the phosphorylation and inactivation of glycogen-synthase kinase-3 (GSK-3), which leads to the dephosphorylation and activation of the glycogen synthase (GS) enzyme (Kandel and Hay, 1999). The dephosphorylation of GS, and overall increase in glycogen synthesis, is also facilitated by protein phosphatase-1 (PP-1), another downstream regulator involved in insulin-stimulated glucose metabolism (Brady and Saltiel, 2001). Alternatively, Akt enhances glucose uptake in insulin-responsive tissues by affecting the glucose transporters GLUT-1, GLUT-3 and GLUT-4. Akt mediates a step in the activation of GLUT-1 and GLUT-3 gene expression, both of which respond to insulin at the transcriptional level (Barthel *et al*, 1999; Kandel and Hay, 1999), while Akt induces the translocation of GLUT-4 to the plasma membrane (Kandel and Hay, 1999). When translocated from intracellular sites to the plasma membrane, the GLUT-4 transporter is responsible for the uptake of glucose into skeletal muscle and adipocytes (Hajduch *et al*, 2001).

Techniques for quantifying insulin sensitivity

Insulin sensitivity is defined as the ability of insulin to produce a normal physiological response and maintain glucose homeostasis (Kirwan *et al*, 2003). The reduction in insulin-mediated glucose uptake by target tissues that is characteristic of IR is particularly evident in skeletal muscle as this tissue accounts for 70-80% of insulin-stimulated glucose uptake in vivo (Garvey *et al*, 1998). Within the insulin signalling cascade there are numerous points where errors, including those that result

in defects in the mechanisms for glucose uptake or glycogen synthesis, can occur (Garvey *et al*, 1998). However, whether IR is associated with a reduction in glucose transport activity or a decrease in intracellular glucose metabolism is not completely understood (Garvey et al, 1998). For example, skeletal muscle IR is generally believed to be caused by a post-receptor defect that leads to impaired GLUT-4 translocation to the plasma membrane and reduced glucose uptake (Krook *et al*, 2004). However, alterations in signalling proteins starting at the insulin receptor and insulin-receptor substrates 1&2 have also been found in the skeletal muscle from insulin resistant and type-2 diabetic individuals (Krook *et al*, 2004)

Two experimental procedures used primarily in humans, and more recently in horses, have become the predominant reference methods for quantifying IR. These procedures include the euglycemic-hyperinsulinemic clamp (EHC) and the minimal model analysis method of glucose and insulin dynamics, which derives plasma glucose and insulin data from a frequently sampled intravenous glucose tolerance test (FSIGT). The EHC, the more widely accepted technique, is designed to maintain a constant plasma glucose concentration during a controlled state of induced hyperinsulinemia that stimulates glucose disposal (Kronfeld *et al*, 2005). In this technique, endogenous glucose production is suppressed by insulin infusion and the glucose disposal rate is calculated as the mean glucose infusion rate during the last 60 minutes of a 120- or 180-minute test (DeFronzo *et al*, 1979; Bergman *et al*, 1979). The EHC has been used extensively to evaluate insulin resistance in various human populations (Ferrannini *et al*, 1997; Stefan *et al*, 2004) and, additionally, to measure changes in insulin sensitivity in response to diet, exercise, obesity and the

development of diabetes (Manco *et al*, 2000; Mingrone *et al*, 1997; Pedersen *et al* 1993; Picchini *et al*, 2005; Paniagua *et al*, 2002). In horses, this method has proven reliable in determining insulin sensitivity (Pratt *et al*, 2005). However, further application of a standardized EHC is still needed to determine reference ranges for insulin sensitivity in horses and ponies (Kronfeld *et al*, 2005).

The FSIGT, a second technique for assessment of IR in humans was proposed over 25 years ago, and is still used today as a clinical tool and as a model to understand the composite effects of insulin secretion and insulin sensitivity on glucose tolerance and risk for type 2 diabetes mellitus (Bergman, 2005). There are four indices produced from minimal-model analysis of glucose-insulin data, namely insulin sensitivity (Si), glucose effectiveness (Sg), acute insulin response to glucose (AIRg) and disposition index (DI) (Bergman, 2005). With minimal alteration to glucose homeostasis, this test allows delineation of the action of insulin on glucose uptake (Si) from Sg, which reflects the capacity of tissues for glucose uptake independent of insulin sensitivity. The AIRg is a measure of insulin secretion during the first ten minutes of the glucose infusion and DI, which is a product of Si and AIRg provides a measure of the combined effect of these two factors (insulin efficiency and insulin secretion) to minimize hyperglycemia. Minimal-model analysis has been used in human research because it provides some information on glucose metabolism, specifically insulin secretion and glucose- and insulin mediated glucose disposal, which cannot be derived from the EHC. In horses, this model has only recently been used to provide another form of analysis to assess insulin sensitivity and pancreatic beta-cell responsiveness (Treiber *et al*, 2005b). For example, this method

has been used to document IR in mature obese horses and demonstrate decreases in insulin sensitivity in weanlings fed a high-glycemic diet (Hoffman *et al*, 2003). Pratt *et al*.(2005), evaluated EHC and minimal model analysis techniques in horses to determine the reproducibility and variability of these tests in determining glucose dynamics and insulin sensitivity. Results of these investigations demonstrated that insulin sensitivity measured by the EHC had lower inter-day variation when compared with the minimal model estimate derived from the FSIGT.

To further understand glucose metabolism in humans, surrogate estimates based on the use of single basal plasma concentrations of glucose and insulin have been developed that utilize mathematical equations to derive indices of insulin sensitivity and beta-cell responsiveness (Fukushima *et al*, 1999; Katz *et al*, 2000). Results derived from surrogate estimates can be compared to reference quintiles for parameters of insulin resistance within a general population (Treiber *et al*, 2005b). The surrogate estimates used most often in human studies include: homeostasis modelling assessment (HOMA), quantitative insulin sensitivity check index (QUICKI), reciprocal inverse square of basal insulin (RISQI) and modified insulin to glucose ratio (MIRG) (Fukushima *et al*, 1999; Katz *et al*, 2000; Uwaifo *et al*, 2002; Gungor *et al*, 2004; Vaccaro *et al*, 2004). Often, surrogate estimates are compared to the results of minimal model analysis to ensure the results accurately assess insulin sensitivity (Vaccaro *et al*, 2004). The use of surrogate estimates to assess insulin resistance in large human populations is highly advantageous, requiring far less time, money and expertise for completion when compared to the EHC and FSIGT.

To date, there have only been a couple of studies in horses in which insulin sensitivity has been assessed using surrogate estimates (Treiber *et al*, 2006a). In the first study by Treiber *et al.*(2005b), the investigators compared several surrogate estimates of insulin sensitivity and beta-cell responsiveness used in human medicine to minimal model analysis of FSIGT data. The results of their study demonstrated a significant correlation between minimal-model analysis of insulin sensitivity (Si) and acute insulin response to glucose (AIRg) with the surrogate estimates RISQI and MIRG. Based on these results, these investigators used RISQI and MIRG surrogates in a further study to assess changes in insulin sensitivity in ponies that may have been at risk for developing pasture associated laminitis (Treiber *et al*, 2006b). Use of surrogate estimates in this study provided a method to assist in the identification of ponies that were at risk for developing laminitis and to establish a set of factors required for the assessment of a pre-laminitic metabolic syndrome.

The role of GLUT-4, Akt, GSK-3 and PP-1 in skeletal muscle

In humans and rodents, there have been numerous studies measuring changes to insulin signalling in insulin resistant skeletal muscle (Krook *et al*, 1998; Kim *et al*, 2000, Delibegovic *et al*, 2003; Christ-Roberts *et al*, 2004). Alterations that occur in upstream mediators of glucose metabolism, such as the insulin receptor, IRS-1&2 and PI 3-kinase in human and rodent skeletal muscle are well defined (Nolan *et al*, 1994; Bjornholm *et al*, 1997; Goodyear *et al*, 1995;Christ-Roberts *et al*, 2004). For example, dexamethasone treatment and excess in rat liver cells and erythrocytes altered the number of insulin receptors and their affinity for insulin (Koricanac *et al*, 2006). In addition, insulin's activation of the IRS-1 PI 3-kinase signalling pathway

has been demonstrated to be reduced in skeletal muscle of rodents and humans with insulin resistance and type-2 diabetes (Anai *et al*, 1998; Goodyear *et al*, 1995; Christ-Roberts *et al*, 2004). However, less is known about what occurs at the level of downstream mediators such as GLUT-4, Akt, GSK-3 and PP-1 in response to changes in insulin sensitivity.

The glucose transporters, and specifically GLUT-4 play a crucial role in facilitating the uptake of glucose into skeletal muscle. GLUT-4 facilitated uptake of glucose across the plasma membrane is stimulated by the release of insulin and by muscle contraction during exercise (Dohm, 2002). Muscle contraction and the release of insulin increase GLUT-4 protein content and translocation by different pathways within skeletal muscle (Kawanaka *et al*, 1996). As mentioned in previous sections, Akt mediates insulin-stimulated translocation of GLUT-4 to the plasma membrane (Cong *et al*, 1997; Brozinick *et al*, 1998), while the mechanisms by which exercise increases GLUT-4 protein expression and translocation are less well understood. It has been shown that exercise increases GLUT-4 transcription and translation, processes which require at least two DNA binding proteins to bind to the promoter, and that the enzyme AMP-activated kinase may also mediate an increase in GLUT-4 expression (Dohm, 2002). During periods of insulin resistance, such as those that may be associated with obesity (Brozinick *et al*, 1994; Christ-Roberts *et al*, 2004), type-2 diabetes (Ryder *et al*, 2000; Koistinen *et al*, 2003; Sano *et al*, 2003) and dexamethasone treatment (Haber and Weinstein, 1992; Dimitriadis *et al*, 1997), a failure in GLUT-4 translocation is one of the mechanisms hypothesized to be responsible for the observed decrease in glucose uptake. Translocation of GLUT-4 to

the plasma membrane and muscle cell surface, especially during hyperinsulinemic conditions, appears to be impaired. However, under these conditions, there is no evidence of decreased GLUT-4 protein content or expression (Dimitriadis *et al*, 1997; Weinstein *et al*, 1998).

Akt, a serine/threonine kinase, has recently come to the forefront as a potential downstream mediator of insulin-stimulated PI 3-kinase activity (Christ-Roberts *et al*, 2004). Of the three Akt isoforms, Akt1 and Akt2 have been shown to be activated in response to insulin in skeletal muscle (Turinsky and Damrau-Abney, 1999). While Akt2 has an integral role in insulin-stimulated GLUT-4 translocation and glucose uptake, both contribute to regulation of GS (Krook *et al*, 2004). This is accomplished through Akt inhibition of GSK-3 activation a critical step in the activation of GS (Kim *et al*, 2000). Further understanding of the role of Akt followed the discovery of AS160. AS160, a protein found in adipocytes, contains a GTPase-activation domain for small G proteins (Rabs) required for membrane trafficking (Krook *et al*, 2004) and is phosphorylated by Akt (Kane *et al*, 2002; Krook *et al*, 2004). When the phosphorylation sites on AS160 are mutated, insulin-stimulated GLUT-4 translocation in 3T3-L1 adipocytes is markedly inhibited (Krook *et al*, 2004). Kane *et al*. (2002) have proposed that AS160 may be a component in the signalling pathway from the insulin receptor to GLUT4 trafficking that is stimulated by Akt phosphorylation and therefore suggesting a crucial role for Akt in the insulin-signalling cascade leading to glucose transport.

In early rat studies, insulin stimulation of Akt was found to be significantly decreased in non-obese spontaneously diabetic Goto-Kakizaki rats when compared to

nondiabetic animals (Krook *et al*, 1997). Further studies using rat muscle have demonstrated that insulin resistance induced by hyperglycaemia is associated with decreased Akt kinase activity (Krook *et al*, 1997; Kim *et al*, 1999a; Kim *et al*, 1999b; Kurowski *et al* 1999, Song *et al*, 1999). Additionally, in a study by Shao *et al.* (2000), using obese, insulin resistant type-2 diabetic mice (db/db phenotype), maximal Akt-Ser ^{473} phosphorylation was decreased by 32% in muscle tissue of diabetic mice when compared to controls, despite equivalent total Akt protein expression in both groups. This decreased Akt phosphorylation in muscle of db/db mice corresponded with a significant decrease in maximal Akt kinase activity (Shao *et al*, 2000). The effects of excess glucocorticoids on Akt protein content and phosphorylation state has been examined in a limited number of studies in rodents. Of the two that have been performed, each has demonstrated reduced insulin-stimulated Akt (PKB in rodents) phosphorylation, but no change in Akt expression (Ruzzin and Jensen, 2005; Ruzzin *et al*, 2005).

Studies investigating insulin resistant and type 2 diabetic humans have shown conflicting results as to whether defective Akt signalling is involved in the development of these disease states. For example, in a study by Krook *et al.* (1998), insulin-stimulated maximal Akt activity was reduced by 66% in muscle from Type 2 diabetics while the total Akt protein concentrations were unchanged. This finding is in contrast to a study by Kim *et al.* (1999b), in which no impairment of insulin-stimulated Akt activation in intact skeletal muscle from type- 2 diabetic humans was reported. To the author's knowledge, there have been no *in vitro* or *in vivo* studies in humans investigating the effects of excess glucocorticoids on Akt protein content and

phosphorylation. Therefore, further studies are required to understand the possible alterations in Akt signalling in association with glucocorticoid excess and states of insulin resistance, such as type-2 diabetes.

GSK-3, another serine/threonine kinase, consisting of highly homologous α- and β- isoforms, phosphorylates and inactivates GS, resulting in reduced glycogen synthesis (Dokken *et al*, 2005). Over activity and inadequate inhibitory control of GSK-3 has been linked to impaired insulin action (Dokken *et al*, 2005). For example, GSK-3 activity is increased in the skeletal muscle and adipose tissue in obese rodents (Bronzinick *et al*, 2000) and in skeletal muscle of obese humans with type 2 diabetes, and this elevated GSK-3 activity is associated with decreased GS activity, glycogen synthesis and insulin sensitivity (Nikoulina *et al*, 2000). Furthermore, *in vitro* assays have demonstrated that GSK-3 causes phosphorylation of IRS-1 on serine residues (Eldar-Finkelman and Krebs, 1997). The resulting decrement in insulin action on glucose transport suggests that GSK-3 elicits a negative effect on the insulin-signalling pathway via impaired IRS-1 tyrosine phosphorylation. This is supported by the finding that pharmacological inhibition of GSK-3 improved glucose disposal and increased GS activity in the liver and muscles of Zucker diabetic fatty rats (Cline *et al*, 2002) and in human skeletal muscle (Nikoulina *et al*, 2002).

Changes in glucose metabolism at the level of insulin signalling have also been associated with a change in protein phosphatase-1 (PP-1) activity in insulin resistant and diabetic animals. Specifically, PP-1 has also been implicated in the regulation of several rate-limiting enzymes in both glucose and lipid metabolism. For example, two pharmacological studies have shown that PP-1 catalyzes the insulin-

dependent dephosphorylation of glycogen synthase, hormone-sensitive lipase and acetyl Co-A carboxylase (Haystead *et al*, 1989; Hess *et al*, 1991). The actions of PP-1 are enabled by glycogen-targeting subunits, which were initially proposed to mediate the compartmentalized activation of PP-1 by insulin (Newgard *et al*, 2000). However, recent data indicate that these targeting proteins actually act as "molecular scaffolds" by allowing the enzyme and its substrate to interact, and in the process exerting profound effects on PP-1 activity and glucose metabolism (Crosson *et al*, 2003). In the skeletal muscle of humans, the glycogen-targeting subunit is called G_M/R_{GI} (Newgard *et al*, 2000). Furthermore, recent research has demonstrated that PP-1 catalyzes the dephosphorylation and inactivation of glycogen phosphorylase, further contributing to the stimulation of glycogen deposition (Newgard *et al*, 2000). A number of studies in rodents have investigated the relationship between PP-1 activity and states of insulin resistance and type-2 diabetes. For example, in a study by Delibegovic *et al.* (2003), the action of insulin on glycogen-targeted forms of PP-1 was assessed to further examine the physiological role of G_M, the most abundant glycogen-targeting subunit in rat muscle. To do this they disrupted the G_M gene in mice through a knockout mutation, resulting in animals that became obese, glucose-intolerant and insulin-resistant as adults. In a similar study by Suzuki *et al.* (2001), mice possessing a homozygous deletion of the G_M gene displayed reduced skeletal muscle glycogen stores and attenuated activation of GS in response to contraction, without changes in glucose homeostasis or insulin sensitivity. Protein targeting to glycogen (PTG), another scaffolding protein associated with PP-1, targets PP-1 to glycogen and enzymes involved in GS (Crosson *et al*, 2003). Mice in which the gene

for PTG was deleted had reduced glycogen stores in adipose tissue, liver, heart and skeletal muscle and a decreased rate of GS activity (Crosson *et al.* 2003). These data imply that PTG, in addition to $G_{M,}$ plays a critical role in bringing together PP-1 with its particular enzymes and substrates to maintain the appropriate metabolic balance between storing and utilizing glycogen.

Hexokinase and Glycogen synthase enzyme activity in skeletal muscle

Insulin resistance in skeletal muscle is characterized by decreased insulin-stimulated glucose disposal, as well as decreased hexokinase (HK) and glycogen synthase (GS) enzyme activities (Christ-Roberts *et al*, 2004). Within skeletal muscle and adipose tissue, hexokinase 1 and 2 isozymes are expressed, although HK2 is the more predominant form (Wilson, 1995). Hexokinase activity is primarily regulated by glucose concentration within the muscle cell, as well as by the phosphorylation and dephosphorylation state of the enzyme, which is influenced by the action of insulin. Additionally, hexokinase 2-gene transcription is induced by insulin, in turn increasing HK mRNA and the rate of protein synthesis and glucose phosphorylation (Osawa *et al*, 1996).

Humans with impaired insulin sensitivity in association with diabetes have decreased HK responsiveness to insulin, resulting in a lesser increase in G-6-P within the muscle following hyperinsulinemia (Rothman *et al*, 1992). Furthermore, HK activity in insulin resistant muscle may be lessened due to a decrease in glucose transport into the muscle as a result of reduced GLUT-4 translocation to the plasma membrane (Rothman *et al*, 1992). Thus, when glucose transport into the muscle cell is reduced,

fewer glucose molecules enter the cell, and less glucose is converted to G-6-P by hexokinase (Rothman *et al*, 1992; Christ-Roberts *et al*, 2004).

Glycogen synthase activity on the other hand is stimulated by the action of insulin and regulated by UDP-glucose concentration. Glycogen synthase activity is further regulated through interaction with a number of protein phosphatases and kinases. For example, GSK-3 is responsible for the phosphorylation and inactivation of GS, resulting in decreased GS activity and glycogenesis (Dokken *et al*, 2005). Inactivation of GSK-3 by Akt dephosphorylates GS, causing its activation and an increase in glycogen synthesis. PP-1 also plays a regulatory role in GS activity and glycogen synthesis. Specifically, the insulin-dependent dephosphorylation and concomitant activation of GS is catalyzed by PP-1. During periods of hyperglycaemia, glucose binds to phosphorylase, relieving the allosteric inhibition of glycogen-targeted PP-1, allowing it to dephosphorylate GS and increase glycogen synthesis (Brady and Saltiel, 2001). During periods of insulin resistance, GS activity is decreased in skeletal muscle. This decrement in GS activity has been observed in human and rodent studies in which GS activity was measured in response to dexamethasone treatment (Dimitriadis *et al*, 1997; Leighton *et al*, 1987; Ruzzin and Jensen, 2005) and in patients and rodents that were pre-diabetic and diabetic (Kelley *et al*, 1992; Christ-Roberts *et al*, 2004; Dokken *et al*, 2005).

General Effects of Glucocorticoids

Glucocorticoids (GC) are a class of steroid hormones characterised by an ability to bind with the cortisol receptor and trigger similar effects. Glucocorticoids have major effects on protein, carbohydrate and fat metabolism, as well as potent

anti-inflammatory and immunosuppressive properties (Grahame-Smith and Aronson, 2002). These effects are particularly evident when GC's are administered at pharmacologic doses, but also is apparent during normal immune responses. Glucocorticoids function by binding to the cytosolic glucocorticoid receptor, which is then activated. Subsequently, the newly formed receptor-ligand complex translocates itself into the cell nucleus, where it binds to many glucocorticoid response elements (GRE) in the promoter region of the target genes (Grahame-Smith and Aronson, 2002). Glucocorticoids are widely used as drugs to treat inflammatory conditions such as arthritis, dermatitis and as adjunction therapy for conditions such as autoimmune disease. A variety of synthetic glucocorticoids, some far more potent than cortisol, have been created for therapeutic use. They differ in the pharmacokinetics (absorption factor, half-life, volume of distribution, clearance) and in pharmacodynamics (for example the capacity of mineralocorticoid activity: retention of sodium (Na+) and water) (Grahame-Smith and Aronson, 2002). As glucocorticoids are absorbed well through the intestines, they are primarily administered *per os* (by mouth), but are also administered topically, intravenously or inhaled. Cortisol (or hydrocortisone) is the glucocorticoid against which all other glucocorticoids are compared to in potency, and as such it receives a potency rating of 1. The following is a list of some typical glucocorticoids and their potency rating: Prednisone (3.5), Prednisolone (4), Methylprednisolone (5), Triamcinolone (5), Betamethasone (25) , Dexamethasone (25) and Beclomethasone (50) (Grahame-Smith and Aronson, 2002). Following therapy with these drugs, a number of adverse side affects can occur, which is related to drug potency, dosage and the duration of

treatment.. Suppression of the pituitary-adrenal axis occurs inevitably, resulting in decreased cortisol secretion; severity depends on duration of treatment (Grahame-Smith and Aronson, 2002). In humans, administration of glucocorticoids can also cause the onset of Cushing's syndrome-like symptoms, including hirsutism, impaired glucose tolerance, hypertension, weight gain and osteoporosis (Grahame-Smith and Aronson, 2002).

The effect of glucocorticoids on insulin sensitivity, glucose metabolism and GLUT-4 expression

The glucocorticoids, particularly cortisol, play a number of important roles in the body including the stimulation of gluconeogenesis, lipolysis and differentiation of pre-adipocytes into functional fat cells, and promotion of triglyceride storage, particularly in visceral adipose tissue (Hauner *et al*, 1987; Andrews and Walker, 1999; Whorwood *et al*, 2002). Endogenous glucocorticoids also regulate hepatic glucose output, stimulating gluconeogenesis by increasing release of gluconeogenic substances from adipose tissue and skeletal muscle and increasing intra-hepatic gluconeogenic enzymes (Dirlewanger *et al*, 2000). GC can also antagonize insulin action. In humans and rodents, it is well recognized that excess GC's results in IR. In humans, administration of the synthetic GC, dexamethasone, results in decreased whole-body insulin-mediated glucose uptake, oxidation and storage (Schneiter and Tappy, 1998). The mechanism of this IR has not been fully elucidated but may involve insulin antagonism at one or more steps distal to the insulin receptor. This antagonism of insulin was demonstrated in studies in which IR was induced by cortisol infusion in healthy humans or observed in subjects with Cushing's Syndrome,

a chronic disease in humans characterized by excess cortisol production, which causes glucose intolerance, obesity and hypertension (Rizza *et al*, 1982; Nosadini *et al*, 1983; Andrews and Walker, 1999). In these studies, the reduced insulin action at insulin-sensitive tissues was explained on the basis of a post-receptor defect, based on a decrease in maximal glucose disposal observed during a EHC without a decrease in the insulin receptor binding capacity (Nosadini *et al*, 1983; Rizza *et al*, 1982). In addition, there may be a decrease in insulin-induced stimulation of muscle blood flow that impairs glucose utilization because of the decreased delivery of both glucose and insulin to insulin-sensitive tissues (Dimitriadis *et al*, 1997). In humans and rats, IR develops early (within 1-2 days) during treatment with GC and may persist after the withdrawal of drug therapy (Dimitriadis *et al*, 1997; Nicod *et al*, 2003; Schneiter and Tappy, 1998). In humans, hepatic IR can also arise as a result of GC treatment due to impairment of insulin-induced suppression of glucose production in the liver (Rizza *et al*, 1982; Dirlewanger *et al*, 2000). The subsequent rise in glucose production may be the result of several mechanisms including enhanced gluconeogenic precursors released in extra-hepatic tissues, stimulation of hepatic uptake of gluconeogenic substrate and gluconeogenic efficiency, and stimulation of G-6-P (Geley *et al*, 1996). Increased glucose production by the liver is also accompanied by increased glucose cycling, which can be attributed to an enhanced flux of substrate in the G-6-P reaction as a result of mechanisms mentioned above (Wajngot *et al*, 1990). Stimulation of glucose re-cycling has been proposed as a mechanism to limit hyperglycemia in response to GC-induced hepatic insulin resistance. (Wajngot *et al*, 1990).

Glucocorticoid treatment, and specifically dexamethasone, is believed to affect glucose uptake in skeletal muscle by reducing GLUT-4 recruitment to the cell surface (Weinstein *et al*, 1995; Dimitriadis *et al*, 1997; Weinstein *et al*, 1998). *In vivo* studies in rats have demonstrated that dexamethasone treatment significantly reduces insulin-stimulated skeletal muscle glucose uptake by inhibiting the recruitment of GLUT-4 to the cell surface, but has no effect on total GLUT-4 protein content (Dimitriadis *et al*, 1997; Weinstein *et al*, 1998). In these studies using intact rat muscle cells, a combination of photolabelling and immunoprecipitation techniques were used to identify only the glucose transporters that were capable of transporting glucose across the plasma membrane, ie. transporters at the cell surface that are physically accessible to glucose (Weinstein *et al*, 1998). These findings are in contrast to studies in which, using subcellular fractionation to identify the location of GLUT-4 transporters, dexamethasone treatment was not found to affect subcellular distribution of GLUT-4 (Venkatesan *et al*, 1996). Thus, the technique used to identify and quantify glucose transporters at the plasma membrane is crucial in determining whether the transporters are accessible to and capable of transporting glucose. This information is essential to assessing the effect of dexamethasone treatment on GLUT-4 recruitment.

There is much species variation regarding the influence of GC on glucose metabolism with respect to preprandial blood glucose concentrations, gluconeogenesis and peripheral glucose utilization (Moore, 1993). For example, in rats, rabbits, guinea pigs and cats, hyperglycemia develops after steroid administration; this effect is much less pronounced in dogs and humans (Abelove and

Paschkis, 1954; Asuma and Eistenstein, 1964). Impaired glucose tolerance has been reported in dogs with endogenous GC excess (Peterson *et al*, 1984a & 1984b) and preprandial hyperinsulinemia has been confirmed after intramuscular administration of methylprednisone for 3-4 days (Altszuler *et al*, 1974). In contrast, in a study in which the GC prednisone was administered orally to dogs for 4 weeks, there was no effect on insulin sensitivity or glucose tolerance (Moore, 1993). Although these findings suggest that dogs are resistant to GC-induced alterations in glucose homeostasis, the results of this study need to be considered in this context of prednisone being a much less potent glucocorticoid than dexamethasone or methylprednisone.

Most studies in humans and some medium-to-large animal species have only measured the effects of glucocorticoid excess and the resultant insulin resistance at the whole-animal level. However, to accurately quantify the development of insulin resistance in skeletal muscle in response to glucocorticoid treatment, it is advantageous to assess the changes occurring at the molecular level in the insulin-signalling pathway and, to date, this work has primarily been attempted in rodent skeletal muscle. At the tissue level, dexamethasone treatment in rodents has been shown to decrease sensitivity of glucose uptake to insulin (Weinstein *et al*, 1995;Dimitriadis *et al*, 1997; Ruzzin and Jensen, 2005; Ruzzin *et al*, 2005). Some studies have focused on changes at the insulin receptor and its substrates while others have narrowed their attention to downstream effectors of glucose metabolism in response to exogenously administered dexamethasone. In the former category, a study by Saad *et al.* (1993), found that dexamethasone treatment resulted in no change in

insulin receptor level or phosphorylation, and no change in IRS-1 phosphorylation. However, IRS-1 and PI3-kinase levels, and insulin stimulated IRS-1 associated PI3-kinase activity was decreased. Studies investigating a possible mechanism for the decrease in glucose transport following dexamethasone treatment have primarily focused on GLUT-4 protein level and recruitment to the plasma membrane. In these investigations, there was no reduction in the total amount of GLUT-4 protein within the muscle cell, but a decrease in cell surface GLUT-4, suggesting impaired glucose trafficking (Dimitriadis *et al*, 1997; Weinstein *et al*, 1998). Dexamethasone treatment also decreases glycogen synthase fractional activity and glycogen synthesis, which occurs through the inability of insulin to dephosphorylate glycogen synthase (Ruzzin *et al*, 2005). Furthermore, it appears that insulin-stimulated Akt and GSK-3 phosphorylation are reduced in dexamethasone-treated rat skeletal muscle, with no change in Akt or GSK-3 expression (Ruzzin and Jensen, 2005; Ruzzin *et al*, 2005). To date there have been no studies in humans evaluating the effect of dexamethasone treatment on GSK-3 protein content or phosphorylation or PP-1 activity.

The effects of glucocorticoid excess in the horse

In horses, there have been very few controlled studies investigating the effects of GC excess, and its relationship to aspects of altered glucose metabolism, including insulin resistance. However, there are two syndromes in horses that describe the possible relationship between GC excess and IR, and its association with the development of obesity and laminitis.

Equine Cushing's syndrome is a chronic progressive disease in older horses caused by a tumour of intermediate lobe (pars intermedia) of the pituitary gland (Loeb

et al, 1966; Love, 1993; McCue, 2002). The adenoma results in increased production of a variety of peptides including α-melanocyte stimulating hormone, corticotropin like intermediate lobe peptide and adrenocorticotropin (ACTH), all of which are cleaved from a common precursor protein known as pro-opiomelanocortin (POMC) (Love, 1993). The abnormal secretory patterns and/or excessive plasma levels of these peptides may contribute to the observed clinical signs (Love, 1993). However, it seems that the ACTH-induced adrenocortical hyperplasia, with resultant increased synthesis and secretion of cortisol from the adrenal cortex, is the pivotal event in the pathogenesis of Cushing's disease, including development of insulin resistance (Love, 1993; McCue, 2002). Insulin resistance in horses with Cushing's syndrome is suggested by the presence of hyperglycemia, glucose intolerance, and hyperinsulinemia, and it has been proposed that these disturbances in glucose homeostasis develop due to the antagonistic effects that cortisol has on the action of insulin (Garcia and Beech, 1986; Masuzaki *et al*, 2001; Rask *et al*, 2001). Chronic laminitis is also fairly common presenting sign of equine Cushing's disease, which is postulated to arise through the effects of excessive endogenous cortisol on the laminar vasculature (Eyre and Elmes, 1979). While a satisfactory explanation for the development of laminitis in horses with equine Cushing's syndrome is lacking, it is hypothesized that both glucose dysregulation and hyperadrenalcorticism may contribute to this disease process (Johnson, 2002b).

The second condition has been termed Equine Metabolic Syndrome because of similarities to the human form of metabolic syndrome which refers to a collection of metabolic abnormalities including IR, obesity, dyslipidemia, hyperglycemia and

hypertension (Grundy *et al*, 2004). Clinical features of the equine condition include obesity, chronic laminitis, hyperinsulinemia and glucose intolerance (Johnson, 2004b).It has been hypothesized that excess GC have an important role in the development of IR and obesity in human metabolic syndrome (Fraser *et al*, 1999; Reynolds *et al*, 2001; Reinehr and Andler, 2004). A study in the general population demonstrated that even modestly increased cortisol levels contributed to obesity, and that insulin resistance is positively associated with cortical activity (Fraser *et al*, 1999). Furthermore, in the adipose tissue of metabolic syndrome patients, there is evidence of enhanced activity of 11β-hydroxysteroid dehydrogenase (11β-HSD), the enzyme that converts inactive cortisone to the biologically active cortisol (Rask *et al*, 2001; Johnson, 2002b). The enhanced 11β-HSD activity may result in excess cortisol production, which is associated with the development of obesity and IR due to the antagonistic effects of cortisol on insulin action (Wang, 2005). Similarly, GC excess due to increased 11β-HSD activity in adipose tissue has been proposed to underlie development of IR in obese horses, but evidence to support this hypothesis is currently lacking.

In addition to the two syndromes mentioned above, glucocorticoid excess also has been implicated in the pathogenesis of laminitis in horses (Pass *et al*, 1998; French *et al*, 2000). Supporting this possibility are clinical observations that ponies and horses with hyperadrenocortism (Cushing's disease) are predisposed to development of laminitis. Excess GC's have been postulated to cause degenerative connective tissue changes in the hoof lamellar interface, which may lead to the development of laminitis (Johnson, 2002b). It is possible that IR associated with high

cortisol plays a role in the development of laminitis (Freestone *et al*, 1991; Jeffcott *et al*, 1986; Jeffcott and Field, 1985). Lastly, it is hypothesized that changes in local 11β-HSD-1 activity occurring during laminitis could affect the extent to which glucocorticoids are involved in furthering its development (Johnson *et al*, 2004a). Johnson *et al.* (2004a) showed that acute laminitis is accompanied by increased 11-ketoreductase and 11β-HSD-1 activity in the both the skin and lamellar tissues and an increase in local GC activity with conversion of cortisone to cortisol. The increase in cortisol may then further the development of laminitis in the acutely induced laminitic horses. A key feature in the development of laminitis in horses is the separation of the secondary epidermal and dermal lamellae of the hoof (Pass *et al*, 1998). It has been proposed that ischaemia, resulting from vasoconstriction in the hoof, is the primary affliction, leading to the lamellar separation (Hood *et al*, 1993). Only one *in vitro* study has measured the direct effects of hydrocortisone and betamethasone on equine vasculature, reporting additional vasoconstriction of equine digital blood vessels following the addition of these glucocorticoids to the medium (Eyre *et al*, 1979). However, in a more recent study, *in vivo* dexamethasone administration lowered local skin temperature as measured by thermographic imaging (Cornelisse *et al*, 2006). The investigators suggested the reduction in skin temperature could reflect reduced blood flow to the hoof and be a factor in the development of laminitis. In contrast, other studies have demonstrated vasodilation and an increase in hoof blood flow during the development of laminitis (Trout *et al*, 1990; Pollitt and Davies, 1998). Alterations in whole body glucose metabolism due to carbohydrate overloading, septic metritis, and hyperlipaemia have also been implicated in the onset of laminitis (Pass *et al*, 1998).

There are only limited data on the metabolic effects of exogenous GC treatment in horses. The effect of dexamethasone treatment on insulin sensitivity was assessed in Quarter horses with polysaccharide storage myopathy (PSSM) a hereditary glycogen storage disorder, characterized by the accumulation of glycogen, glucose-6-phosphate, and abnormal polysaccharide inclusions in skeletal muscle (Firshman *et al*, 2005; Valberg *et al*, 1993 and 1999). Results of this study showed that dexamethasone treatment significantly decreased whole-body insulin-stimulated glucose uptake, measured during an EHC. However, dexamethasone treatment did not alleviate exertional rhabdomyolysis in these horses. In another study, the administration (IV or IM) of triamcinolone acetonide was used to test its effects on glucose metabolism in healthy horses (French *et al*, 2000). The triamcinolone treatment resulted in a sustained period of hyperglycaemia, hyperinsulinemia and hypertriglyceridemia with the observed hyperglycaemia believed to be a result of decreased glucose utilization by tissues and increased gluconeogenesis. While the authors of this study suggest that these metabolic alterations were consistent with glucocorticoid-induced IR, there was no quantification of IR and the data did not provide a mechanistic explanation for the observed changes in glucose homeostasis. Lastly, in a study by de Graaf-Roelfsema *et al.* (2005), the effect of a single hydrocortisone injection on the sensitivity of peripheral tissues to insulin in healthy horses was quantified by an EHC. The investigators found that the single dose of hydrocortisone caused an increase in glucose use and insulin sensitivity in peripheral tissues and suggested that the increase in glucose metabolism was purely a reflection of an increase in hydrocortisone-induced glucose use in peripheral tissues. Although

this study quantified the effects of short-term synthetic hydrocortisone treatment on insulin sensitivity, the results cannot be compared directly to previous studies in which more potent glucocorticoids were administered over a longer period of time. Furthermore, none of these studies have provided a mechanistic explanation for the observed changes in glucose metabolism in response to glucocorticoid treatment. Therefore, further studies are necessary to assess the effects of long-term synthetic glucocorticoid treatment (e.g. dexamethasone) on aspects of insulin signalling and glucose metabolism in insulin sensitive tissues such as skeletal muscle.

RATIONALE, HYPOTHESES AND OBJECTIVES

In a number of species, administration of synthetic glucocorticoid has been demonstrated to affect insulin signalling and glucose uptake in insulin sensitive tissues. While elevated concentrations of endogenous glucocorticoids have been implicated in the development of IR and laminitis in horses, there is little published data quantifying the effects of glucocorticoids on insulin sensitivity. Furthermore, the effects of glucocorticoid treatment on insulin-mediated glucose metabolism in skeletal muscle have not been studied in the horse. In the present study, dexamethasone was chosen to address the issues mentioned above, as it has been the glucocorticoid used in equine studies that have examined its effects on insulin sensitivity (Firshman *et al*, 2005) and skin blood flow (Cornelisse *et al*, 2006).

Therefore, we hypothesize that:

Dexamethasone treatment will result in a profound state of insulin resistance accompanied by changes in skeletal muscle glucose metabolism including no change in total GLUT-4 protein content or muscle glycogen concentration, but insulin-stimulated phosphorylation of Akt, GSK-3, and PP-1 and increases in GS and HK activity will be reduced

The specific objectives of this study are to:

1. **Characterize the effects of chronic, low-dose dexamethasone treatment on insulin sensitivity and glucose homeostasis by measurement of:**
    a) basal plasma glucose, insulin, free fatty acid and triglyceride concentrations at intervals during a 21-day treatment period;

b) insulin-stimulated glucose uptake during a euglycemic-hyperinsulinemic clamp (EHC); and

c) insulin sensitivity, glucose effectiveness, and insulin secretory response (minimal model analysis) during an insulin-modified frequently sampled intravenous glucose tolerance test.

2. **To examine the effects of dexamethasone treatment on selected aspects of skeletal muscle glucose metabolism via measurement of the following in muscle samples obtained before and after a 2-h period of sustained hyperinsulinemia:**

   a) GLUT-4, Akt, GSK-3 and PP-1 protein content by western immunoblotting;

   b) the activities of glycogen synthase and hexokinase;

   c) phosphorylation state of the insulin signalling proteins Akt , GSK-3 and PP-1; and

   d) muscle glycogen content.

CHAPTER 1

THE EFFECTS OF DEXAMETHASONE ON INSULIN SENSITIVITY AND GLUCOSE DYNAMICS IN STANDARDBRED HORSES

SUMMARY

Reasons for performing the study: Increased risk of laminitis in horses has been associated with exogenously administered glucocorticoids. One possible mechanism linking glucocorticoid therapy and laminitis is the development of insulin resistance. In several species, both excess endogenous and exogenous glucocorticoids induce insulin resistance, but few studies have quantified the effects of glucocorticoids on insulin sensitivity and glucose homeostasis in horses.

Objective: To determine the effects of dexamethasone on glucose dynamics and insulin sensitivity in healthy horses.

Methods: In a balanced crossover study, 6 Standardbred horses received either dexamethasone (DEX)(0.08 mg/kg bwt i.v. every 48 h) or the equivalent volume of saline over a 21-day period (CON), with a 3-week washout between treatments. Blood samples were taken on days –2, 1, 7, 14 and 21 in each period for measurement of plasma glucose and serum non-esterified fatty acids (NEFA), triglycerides (TG), cortisol and insulin concentrations. A 3-h frequently sampled intravenous glucose tolerance test (FSIGT) was administered 2 days after the end of each treatment period. Minimal model analysis of the glucose and insulin data from the FSIGT was used to estimate insulin sensitivity (Si, x 10^{-4} L•min^{-1}•mU^{-1}), glucose effectiveness (Sg, x 10^{-2} min^{-1}), acute insulin response to glucose (AIRg, mU/L•min^{-1}) and the disposition index (DI, dimensionless = Si x AIRg). Proxies for insulin sensitivity (RISQI: reciprocal of the inverse square of basal insulin) and beta cell responsiveness (MIRG: modified insulin to glucose ratio) were calculated from basal plasma glucose and serum insulin concentrations.

Results: Mean basal serum insulin concentrations significantly increased following 7, 14, and 21 days of DEX treatment, while there was no change in mean plasma glucose concentrations in DEX, except on day 14. Minimal model analysis of FSIGT data demonstrated a significant decrease in Si and a significant increase in AIRg following DEX, with no change in Sg or DI. Mean RISQI was significantly lower in DEX than CON on days 7, 14, and 21, while MIRG was higher in DEX than CON on these sample days.

Conclusions: The study demonstrated marked insulin resistance in horses after 21 days of dexamethasone treatment. The decrease in insulin sensitivity was partially compensated by enhanced beta cell responsiveness and insulin secretion, such that there was no significant change in disposition index. Furthermore, onset of moderate basal hyperinsulinemia without concomitant hyperglycaemia was also consistent with the development of compensated insulin resistance.

Potential Relevance: As insulin resistance has been associated with predisposition to laminitis, a glucocorticoid-induced decrease in insulin sensitivity may increase risk for development of laminitis in some horses and ponies.

INTRODUCTION

Glucocorticoids have widespread use in veterinary medicine as anti-inflammatory and immunosuppressive agents. However, chronic glucocorticoid therapy can result in adverse side effects. For example, an increased risk of laminitis has been associated with exogenously administered glucocorticoids in horses, perhaps by inducing insulin resistance (Johnson *et al*, 2002b, 2004a&b). Several mechanisms have been proposed to explain glucocorticoid-induced laminitis in horses, including

alterations in vascular function with reduced blood flow, weakening of the lamellar attachment interface through dermo-epidermal atrophy, or via induction of insulin resistance (Cornelisse *et al*, 2006; Johnson *et al*, 2004a&b). Induction of insulin resistance may be an important mechanism given the strong association between insulin resistance and predisposition to laminitis in ponies (Treiber *et al.* 2006a&b) and possibly horses (Johnson *et al.* 2002b, 2004a&b).

There is conflicting information regarding the effect of exogenous glucocorticoids on insulin sensitivity in horses. In one study of Quarter horses with polysaccharide storage myopathy, chronic dexamethasone administration resulted in decreased insulin sensitivity, measured by the euglycemic-hyperinsulinemic clamp (EHC) method (Firshman *et al*, 2005). Similarly, French *et al.* (2000) reported that administration of a single dose of triamcinolone acetonide to healthy horses resulted in sustained hyperglycemia, hyperinsulinemia and hypertriglyceridemia. In contrast, de Graaf-Roelfsema *et al.* (2005) recently reported that a single dose of hydrocortisone increased insulin sensitivity in healthy horses. Differences in study design, including type of glucocorticoid, dosing regimen and method for assessment of insulin sensitivity, could account for these conflicting results. Nonetheless, it appears that further studies are needed to examine the effects of glucocorticoids on insulin sensitivity in horses.

Therefore, the objective of the study reported herein was to characterize the effects of dexamethasone on insulin sensitivity and glucose homeostasis in horses by measurement of: a) basal plasma glucose and serum insulin, free fatty acid and triglyceride concentrations at intervals during a 21-day treatment period and b) insulin

sensitivity, glucose effectiveness, and insulin secretory response by minimal model analysis of an insulin-modified frequently sampled intravenous glucose tolerance test (FSIGT).

MATERIALS AND METHODS

Horses

The study was carried out with the permission of the University of Guelph's Animal Care Committee, from mid-May to mid-July of 2005. Six mature Standardbred horses (2 mares, 4 geldings, 4-5 years old, mean [± S.D] body weight 443.2 ± 4.7 kg) that had been paddock-rested for 1-month were used in this study. Horses were housed in box stalls with approximately 4 hours/day of turnout into a small paddock. Throughout the study, horses were fed approximately 12 kg of grass hay daily. Horses had access to fresh water and a salt block at all times. As an increase in dietary hydrolysable carbohydrate is one factor that may contribute to altered insulin sensitivity (Treiber *et al*, 2005a; Pratt *et al*, 2006), no grain-concentrate was fed. Muzzles were applied to the horses during turnout to prevent grazing.

Experimental design

The design of this study was based on a previous study by Firshman *et al.* (2005) that examined the effects of dexamethasone treatment on insulin sensitivity in horses with polysaccharide storage myopathy (PSSM). A balanced crossover design was used, with horses receiving either dexamethasone (DEX) (0.08 mg/kg body weight) or the equivalent volume of saline (CON) by i.v. injection every 48 hours for 3 weeks (a total of 11 treatments). The dexamethasone dosage is consistent with dosing regimens used for treatment of inflammatory conditions in horses, such as

recurrent airway obstruction (heaves). Following a 3-week washout period, the experimental protocol was repeated with horses receiving the opposite treatment. Horses were randomly assigned to treatments, such that horses 1, 4 and 6 received saline in the first treatment period, while horses 2, 3, and 5 received dexamethasone. Treatments were administered at 0800 h. Blood samples were taken 2 days before and at 1, 7, 14 and 21 days after the start of treatment for measurement of plasma glucose and serum non-esterified fatty acids (NEFA), triglycerides (TG), and insulin concentrations. Serum cortisol was measured in blood samples obtained on days -2, 7 and 21 of treatment. A 3-h frequent sample intravenous glucose tolerance test (FSIGT) was administered 2 days after the end of each treatment period.

Frequent sample intravenous glucose tolerance test (FSIGT)

Feed was withheld for 12 h prior to the procedure. At 0700 h on the day of the FSIGT horses were weighed (± 0.5 kg, KSL electronic scale, KSL Inc., Kitchener, Ontario, Canada) and a catheter (14g x 5¼", BS Angiocath, Sandy Utah, USA) was inserted in one of the jugular veins after aseptic preparation and desensitization of the overlying skin. After a 30 min period of rest, during which baseline blood samples were obtained (-30, -15 and -1 min relative to glucose injection), a glucose (50% w/v dextrose) load of 0.3 g/kg was injected over 30 s via the jugular catheter. Further blood samples were collected at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 19, 22, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150 and 180 minutes after glucose administration for measurement of plasma glucose and serum insulin concentrations. At 20 minutes following the glucose bolus, insulin (Humulin-R, Eli-Lily, Indianapolis, Indiana, USA) was administered via the jugular catheter at a rate of 30

mU/kg bodyweight. Horses stood in stocks for the first 40 min of the FSIGT, after which they were loosely restrained in a 3.5 m × 4 m stall.

Blood analyses

Samples for assay of plasma glucose concentration were placed into evacuated tubes containing EDTA, while samples for measurement of serum insulin, cortisol, NEFA and TG concentrations were placed into evacuated tubes containing no additive. All sample tubes were centrifuged for 15 min (1,600 × g), after which plasma and serum were harvested and stored at -20°C until analysis. Plasma glucose was measured in triplicate spectrophotometrically by use of a microplate reader and a commercially available kit (Infinity Reagent, Thermo Electron, Waltham, Massachusetts, USA). Serum TG and NEFA concentrations were measured in a commercial laboratory (Animal Health Laboratory, University of Guelph) by use of enzymatic colorimetric methods and an automated analyzer (Hitachi 911, Roche). Serum insulin and cortisol concentrations were measured in duplicate by use of commercially available radioimmunoassay kits (Coat-a-Count, Diagnostic Products Corporation, Los Angeles, California, USA) that have been validated for the horse (Freestone et al. 1992). The intra- and inter-assay CV's for the insulin and cortisol assays were, respectively, 2.9% and 4.8%, and 3.3% and 5.7%.

Computer analysis

The plasma glucose and serum insulin concentration data from the FSIGT were analyzed by the minimal model method using a computerized algorithm (MinMod Millenium, Version 5.15). This analysis provides an estimate of the relationship between the time course of changes in plasma glucose and insulin

concentration after IV glucose administration (Bergman *et al*, 1979). The insulin sensitivity index (Si, x 10^{-4} $L \cdot min^{-1} \cdot mU^{-1}$) represents changes in net fractional glucose clearance rate per unit change in insulin after IV glucose administration. Glucose effectiveness, or Sg (x 10^{-2} min^{-1}), represents net fractional glucose clearance rate independent of insulin. The acute insulin response to glucose (AIRg, $mU/L \cdot min^{-1}$) was estimated by calculation of the incremental area under the insulin curve generated from data in the first 10 minutes after glucose administration before insulin is given. Finally, the disposition index (DI, dimensionless = Si x AIRg) represents the appropriateness of the pancreatic β-cell response relative to the degree of insulin resistance in the tissues. Resting values for plasma glucose and serum insulin, measured on days -2, 1, 7, 14 and 21 of treatment, were used to calculate proxies for insulin sensitivity (the reciprocal of the square root of basal insulin concentration, RISQI) and pancreatic beta-cell responsiveness (modified insulin-to-glucose ratio, MIRG), as previously described (Treiber *et al*, 2005b):

$$RISQI = 1/\sqrt{[insulin]} = [insulin]^{-0.5} \text{ (units = } mU/L^{-0.5})$$

$$MIRG = [800 - 0.30 \cdot (insulin - 50)^2]/(glucose - 30) \text{ (units =}$$

$$mU_{insulin}^2/[10 \cdot L \cdot mg_{glucose}])$$

Statistical analysis

Values are expressed as means ± s.e. The effects of dexamethasone on minimal model parameters were evaluated by paired Student's *t* test. Results for basal plasma glucose, serum insulin, cortisol, TG and NEFA, and proxies (RISQI and MIRG), and glucose and insulin during the FSIGT were compared using analysis of variance with repeated measures. When a significant ($P<0.05$) F ratio was obtained,

means were compared by the Bonferroni *t* test. Spearman rank correlation coefficients were calculated to evaluate relationships between minimal model parameters and values for the proxies obtained on day 21. Statistical analyses were conducted with the use of computer software programs (Systat, Version 11.0 and Sigmastat, Version 3.5; SPSS Inc.). For all analyses, significance was accepted at $P<0.05$.

RESULTS

Mean serum cortisol concentration did not differ between CON and DEX on day -2, but was significantly lower ($P<0.05$) in DEX than in CON on days 7 and 21 (Table 1.1). Plasma glucose concentration was significantly higher in DEX than CON on day 14, while serum insulin concentration was significantly higher in DEX than in CON on days 7, 14 and 21 (Fig. 1.1A and B). Mean serum TG did not differ between DEX and CON on any day. Mean serum NEFA was higher ($P<0.05$) in DEX than in CON on day -2 only (Table 1.1).

The glucose and insulin data from the FSIGTs are shown in Figure 1.2. Mean plasma glucose (mmol/L) was not significantly different between treatments at any time point during the 3-hr FSIGT (Fig. 1.2A). The average of the mean serum insulin concentration in pre-FSIGT samples (i.e. the average of values obtained at -30, -15 and -1 minutes) was higher ($P<0.05$) in DEX than in CON (14.30 ± 3.53 vs. 9.84 ± 4.59 μU/ml). Mean serum insulin concentrations were higher ($P<0.05$) in DEX from the -30 minute sample to the 20 minute sample and from the 40 minute sample to the 150 minute sample inclusively (Fig. 1.2B).

The minimal model was successfully applied to the FSIGT glucose and insulin data for all horses in CON and DEX ($r^2 = 0.971 \pm 0.004$). The insulin sensitivity

index was lower ($P<0.01$) in DEX than CON, while the acute insulin response to glucose was higher ($P<0.01$) in DEX than CON (Table 1.2). There was no significant difference in glucose effectiveness or disposition index min between treatments (Table 1.2).

The proxy for insulin sensitivity (RISQI) was significantly lower in DEX than CON on days 7, 14 and 21 of treatment (Fig. 1.3A). On day 21, RISQI was approximately 50% lower ($P<0.01$) in DEX (0.214 ± 0.028 $mU/L^{-0.5}$) than in CON (0.413 ± 0.049 $mU/L)^{-0.5}$. Conversely, MIRG, the proxy for beta cell responsiveness, was higher ($P<0.05$) in DEX than CON on days 7, 14, and 21 of treatment (Fig. 1.3B). Plots of RISQI concentration versus minimal model Si, and MIRG concentration versus AIRg are shown in Figure 1.4 (A and B, respectively). Spearman rank correlation analysis demonstrated significant relationships between RISQI and Si ($r = 0.846$; $P<0.001$) and between MIRG and AIRg ($r = 0.683$; $P = 0.014$).

DISCUSSION

The present study examined the effects of dexamethasone (0.08 mg/kg bwt) treatment over a 21-day period on insulin sensitivity and glucose dynamics in Standardbred horses. The main findings of the study were: 1) an approximately 4-fold decrease in insulin sensitivity and 2-fold increase in acute insulin response to glucose measured by minimal model analysis of an FSIGT, 2) an approximately 2-fold increase in basal serum insulin concentration without an increase in plasma glucose concentration following dexamethasone treatment, and 3) a 2-fold decrease in RISQI and 2-fold increase in MIRG by day 21 of treatment measured by proxy analysis.

These observations are consistent with the development of compensated insulin resistance in dexamethasone treated horses.

Minimal-model analysis was used to quantify insulin sensitivity, based on plasma glucose and insulin data from an insulin-modified FSIGT. This model is a physiological compartmental representation of the glucose and insulin regulatory system and includes four indices (insulin sensitivity [Si], glucose effectiveness [Sg], acute insulin response to glucose [AIRg], and disposition index [DI]) derived from minimal model analysis of glucose-insulin data. In human subjects, this clinical tool is used to understand the composite effects of insulin secretion and insulin sensitivity on glucose tolerance and risk for type 2 diabetes mellitus (Bergman, 2005). This is the only method that differentiates between the action of insulin on glucose uptake (Si) and glucose effectiveness (Sg), which reflects the capacity of tissues for glucose uptake independent of insulin stimulation (Treiber *et al*, 2006a). The AIRg is a measure of insulin secretion during the first ten minutes after glucose injection, while DI, which is a product of Si and AIRg, provides a measure of the combined effect of these two factors (insulin efficiency and insulin secretion) to minimize hyperglycemia. This technique has been used to assess glucose dynamics and insulin sensitivity in response to changes in exercise and diet in horses (Hoffman *et al*, 2003; Treiber *et al*, 2005a; Treiber *et al*, 2005b;Treiber *et al*, 2006a) and to assess changes in insulin sensitivity in horses and ponies predisposed to development of laminitis (Treiber *et al*, 2005b, 2006b).

In the current study, minimal-model analysis demonstrated a significant decrease in insulin sensitivity after dexamethasone treatment. Despite a decrease in

insulin sensitivity, there was no significant change in glucose effectiveness or disposition index, the latter most likely reflecting an increase in insulin secretion (i.e. compensation). Our values for minimal model parameters were within the 95% reference interval based on data from a population of 48 healthy Thoroughbred horses (Treiber *et al*, 2005b). However, for all horses in DEX, values for Si were in the lowest quintile whereas all values for AIRg fell within the highest quintile (Treiber *et al*, 2005b).

To assess changes in insulin sensitivity and insulin response during dexamethasone treatment we applied proxy analysis of basal data for plasma glucose and insulin, a method recently developed for use in horses and ponies (Treiber *et al*, 2005b). The first proxy, RISQI (the reciprocal of the square root of basal insulin) provides a measure of insulin sensitivity, while the second, MIRG (modified insulin-to-glucose ratio) determines beta cell responsiveness; each requires a single baseline blood sample (Treiber *et al*, 2005b; Treiber *et al*, 2006b). These proxies have been used to evaluate insulin resistance and compensation in ponies predisposed to pasture-associated laminitis (Treiber *et al*, 2006b). Proxy analysis of basal blood samples collected throughout the treatment period of our study further supported the finding of compensated insulin resistance derived from the minimal-model analysis, with evidence of insulin resistance and increased pancreatic insulin response after 7 days of dexamethasone treatment. The moderate correlations between the one-sample basal proxies and the minimal model parameters are consistent with results of studies in healthy horses and ponies predisposed to laminitis (Treiber *et al*, 2005b).

The decrease in insulin sensitivity following dexamethasone treatment in our study is similar to the findings by Firshman *et al.* (2005) in which the same treatment protocol was used to evaluate insulin sensitivity in Quarter horses with polysaccharide storage myopathy (PSSM). In the study by Firshman *et al.* (2005), dexamethasone treatment significantly reduced whole-body insulin-stimulated glucose uptake measured by use of a EHC. Interestingly, de Graaf-Roelfsema *et al.* (2005) reported an increase in the sensitivity of peripheral tissues to exogenous insulin in Standardbred horses following a single dose of hydrocortisone. An apparent increase in glucose use as a result of cortisol excess has also been reported in dogs with hyperadrenocorticism and in healthy humans (Peterson *et al*, 1984a&b; Rizza *et al*, 1982). Using 6-^{3}H-glucose as a tracer to measure glucose uptake in dogs with hyperadrenocorticism, Peterson *et al.* (1984b) determined that the mean rates of hepatic glucose production and peripheral tissue utilization of glucose were increased suggesting a cortisol-induced a state of hepatic insulin resistance.

The increase in resting serum insulin concentration observed following 7 days of dexamethasone treatment is also consistent with the results of French *et al.* (2000) who examined the effects of acute triamcinolone acetonide administration on glucose and lipid metabolism in horses. When administered intravenously or intramuscularly at 0.05mg/kg bwt triamcinolone acetonide induced hyperglycemia, hyperinsulinemia and hypertriglyceridemia which persisted for 3-4 days (French *et al*, 2000). Increases in triglyceride or non-esterified fatty acid concentration were not evident in the present study following dexamethasone treatment, and hyperglycemia was not evident except on day 14 of the treatment period. The more extensive changes in

glucose and lipid metabolism observed following triamcinolone treatment when compared to dexamethasone may be attributable to the type and dose of glucocorticoid administered. The results of this study provide evidence for a significant impact of glucocorticoid treatment on aspects of insulin sensitivity and glucose dynamics in horses. The extent to which such glucocorticoid-associated insulin refractoriness could contribute to increased risk for laminitis in horses is currently unknown (Johnson, 2002b) and little quantitative evidence at the whole-animal level exists to support possible mechanisms for development of laminitis in horses in response to exogenously administered glucocorticoids. Following a 21-day dexamethasone treatment regimen, there was no evidence for the development of laminitis in the horses used in the current study and this is consistent with observations from other studies in which dexamethasone or triamcinolone acetonide were administered (Freestone *et al*, 1991; French *et al*, 2000; Firshman *et al*, 2005). Interestingly, in a recent study by Cornelisse *et al* (2006), dexamethasone treatment of horses resulted in an apparent decrease in vascular perfusion of the skin, evidenced by decreased skin temperature. Furthermore, dexamethasone treatment potentiated decrements in skin temperature in response to dermal administration of the α_1-adrenoceptor agonist phenylephrine. The authors of this study hypothesized that similar alterations in the function of digital blood vessels after glucocorticoid therapy could contribute to the pathogenesis of laminitis, particularly in animals with disease states that are characterized by peripheral hypoperfusion and/or increased circulating catecholamines (e.g, sepsis, colic) (Cornelisse *et al*, 2006).

This study quantified the significant decrease in insulin sensitivity in Standardbred horses following 21-days of dexamethasone treatment. The decrement in insulin sensitivity was partially compensated by an increase in beta cell response and insulin secretion such that no change in disposition index was evident. The significant decrease in RISQI and increase in MIRG detected following chronic dexamethasone administration further corroborated the compensated insulin resistance determined using minimal-model analysis of FSIGT data.

TABLE 1.1: Basal serum cortisol, triglyceride (TG) and non-esterified fatty acid (NEFA) concentrations at intervals throughout the 21-day treatment period in the control and dexamethasone treatments.

Sample Day	-2	1	7	14	21
Cortisol (nmol/L)					
CON	5.78 ± 0.9	N/D	4.87 ± 1.57	N/D	3.61 ± 1.29
DEX	6.72 ± 0.83	N/D	0.96 ± 1.24*	N/D	0.32 ± 1.34*
TG (mmol/L)					
CON	0.25 ± 0.02	0.31 ± 0.02	0.27 ± 0.01	0.32 ± 0.02	0.38 ± 0.06
DEX	0.24 ± 0.04	0.26 ± 0.04	0.34 ± 0.03	0.38 ± 0.06	0.38 ± 0.06
NEFA (mmol/L)					
CON	0.15 ± 0.03	0.06 ± 0.03	0.13 ± 0.02	0.13 ± 0.05	0.20 ± 0.07
DEX	0.28 ± 0.07	0.08 ± 0.04	0.10 ± 0.03	0.20 ± 0.06	0.10 ± 0.04

Data are mean ± s.e. for 6 horses. DEX, dexamethasone; CON, control; N/D, not done.

*P<0.05 vs. CON.

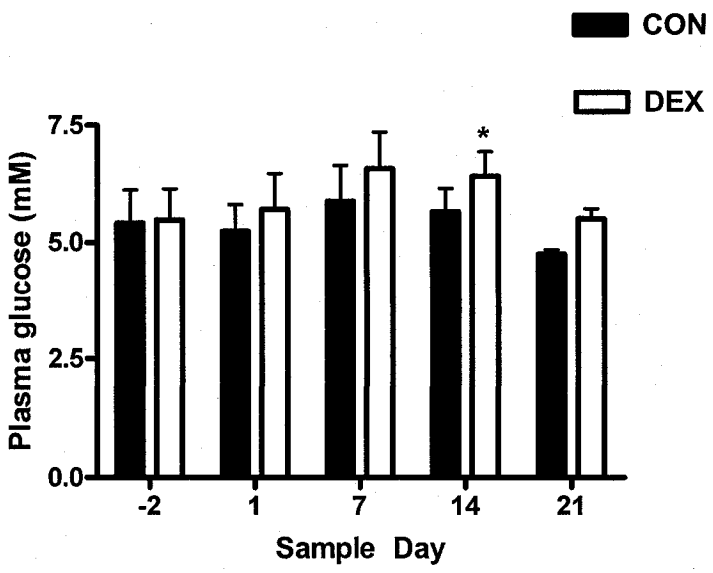
TABLE 1.2: Minimal model analysis of the insulin-modified frequently-sampled intravenous glucose tolerance test (FSIGT) in control and dexamethasone treatments.

VARIABLE	CON	DEX
Si **($\times 10^{-4}$ L•min^{-1}•mU^{-1})**	2.59 ± 0.45	0.53 ± 0.13*
Sg **($\times 10^{-2}$ min^{-1})**	1.59 ± 0.20	1.39 ± 0.11
AIRg **(mU/L•min^{-1})**	153.8 ± 38.9	398.1 ± 26.5*
DI **($\times 10^{4}$)**	240 ± 45	155 ± 42

Data are mean ± s.e. for 6 horses. DEX, dexamethasone; CON, control; Si, insulin sensitivity; Sg, glucose effectiveness; AIRg, acute insulin response to glucose; DI, disposition index.

*P<0.05 vs. CON.

A



B

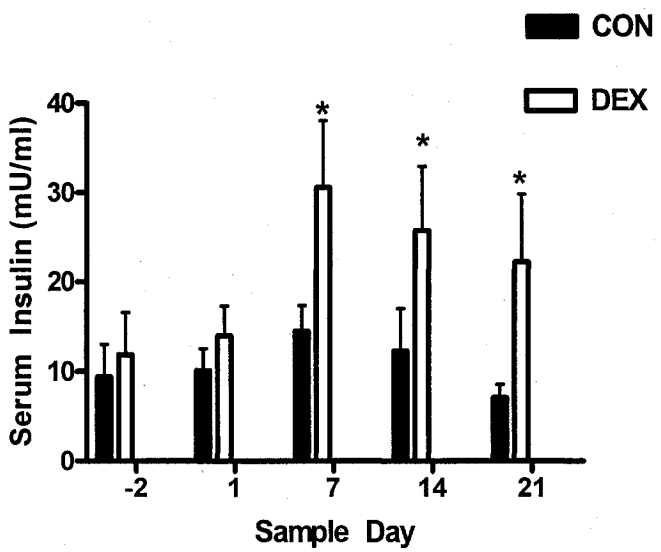
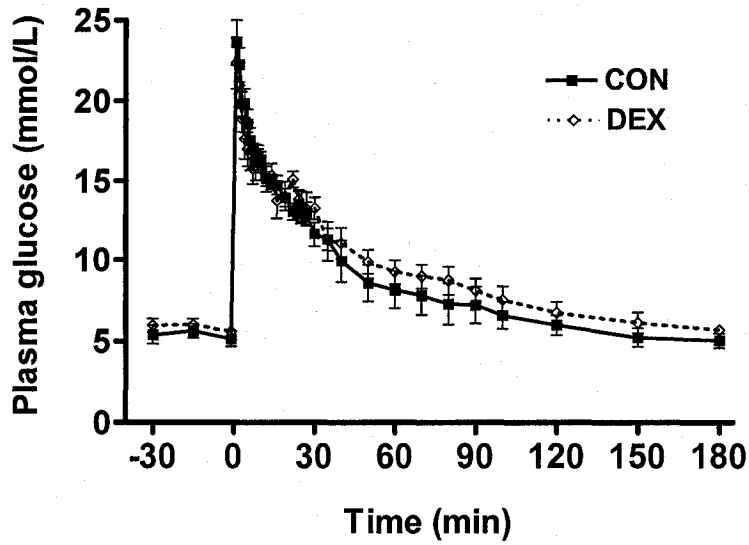


FIGURE 1.1: Plasma glucose concentrations (**A**) and serum insulin concentrations (**B**) at intervals during the treatment period in the control (CON) and dexamethasone (DEX) treatments. Plasma glucose concentration was significantly higher ($P<0.05$) in DEX than in CON on day 14 of treatment, while serum insulin concentration was higher ($P<0.01$) in DEX than in CON on days 7, 14 and 21.

A



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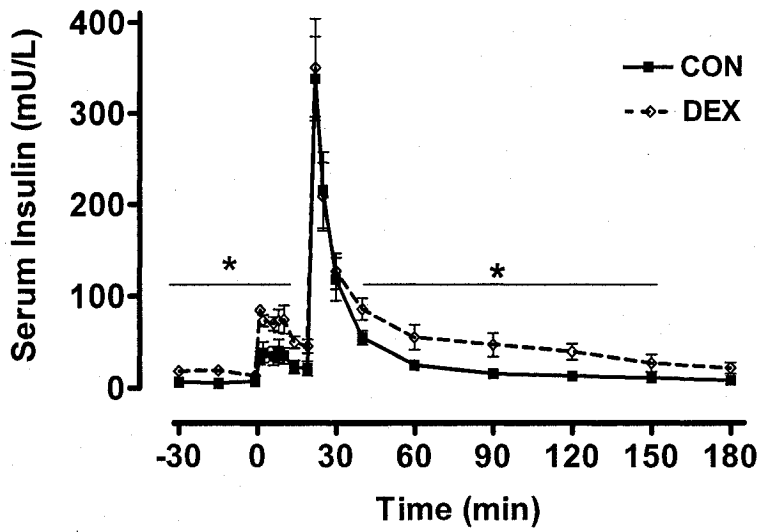
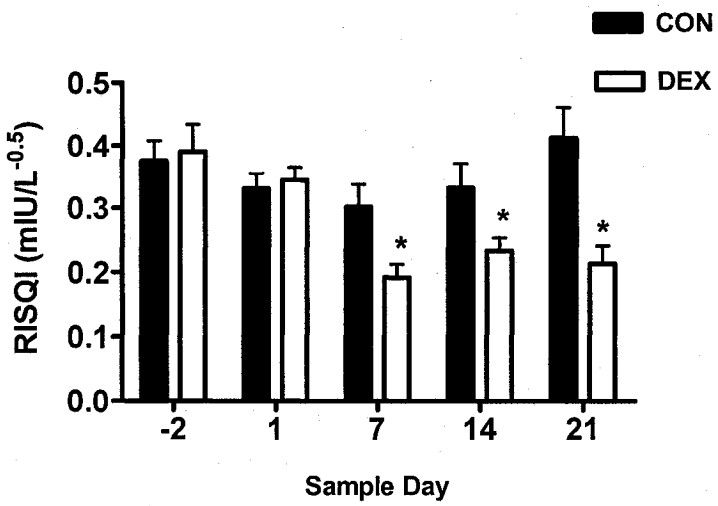


FIGURE 1.2: Plasma glucose (**A**) and serum insulin (**B**) concentrations during the insulin-modified frequently-sampled intravenous glucose tolerance test (FSIGT) in control (CON) and dexamethasone (DEX) treatments. Mean plasma glucose did not differ between treatments at any time point. However, serum insulin was significantly higher ($P<0.05$) in DEX than in CON from –30 to 20 minutes and from 40 to 150 minutes (asterisks and horizontal bars).

A



B

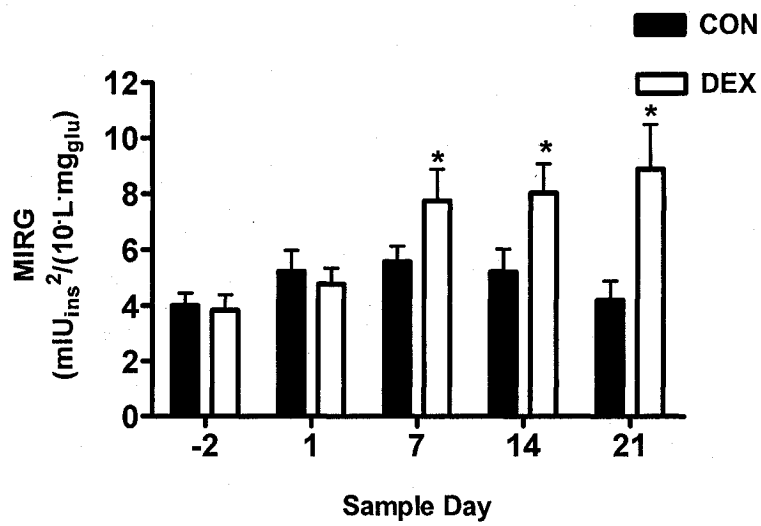
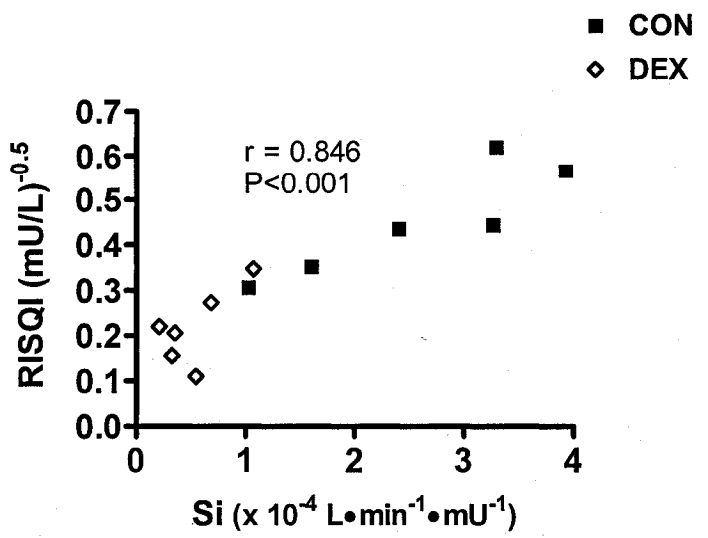


FIGURE 1.3: Proxies for insulin sensitivity (RISQI = reciprocal of the square root of insulin; **A**) and pancreatic beta cell responsiveness (MIRG = modified insulin to glucose ratio; **B**) in the control (CON) and dexamethasone (DEX) treatments, based on basal plasma data. Mean RISQI was significantly lower ($P<0.05$) in DEX than in CON on days 7, 14 and 21. Conversely, mean MIRG was higher ($P<0.05$) in DEX than CON on these sample days.

A



B

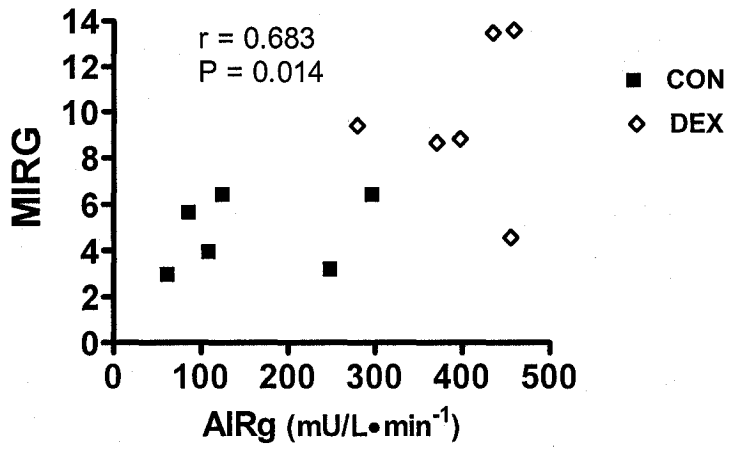


FIGURE 1.4: Plots of reciprocal of the inverse square of basal insulin (RISQI) concentration versus. minimal model insulin sensitivity (Si; **A**) and modified insulin-to-glucose ratio (MIRG) versus beta cell response (acute insulin response to glucose, AIRg; **B**). Both relationships were statistically significant (Spearman correlations).

CHAPTER 2

COMPONENTS OF INSULIN SIGNALLING AND GLUCOSE METABOLISM IN EQUINE SKELETAL MUSCLE AFTER DEXAMETHASONE-INDUCED INSULIN RESISTANCE

SUMMARY

Reasons for performing the study: Studies in humans and several animal species have demonstrated that glucocorticoid treatment results in insulin resistance with decreased glucose uptake in insulin sensitive tissues such as skeletal muscle. Few studies in horses have quantified the extent of glucocorticoid-induced insulin resistance and there have been no reports of the effects of glucocorticoid treatment on mechanisms of glucose metabolism in skeletal muscle.

Objectives: To examine the effects of dexamethasone treatment on selected aspects of skeletal muscle insulin signaling and glucose metabolism in samples obtained before and after a 2-h euglycemic-hyperinsulinemic clamp (EHC).

Methods: In a balanced crossover study, 6 Standardbred horses received either dexamethasone (DEX; 0.08mg/kg bwt i.v. every 48h) or the equivalent volume of saline (CON) i.v. for 21 days (11 treatments). One day after the end of each treatment period, a 2-h EHC was administered for measurement of insulin sensitivity. Percutaneous middle gluteal muscle biopsies obtained before and after the EHC were analysed for GLUT-4, Akt, GSK-3α, GSK-3β, and PP-1α protein content and phosphorylation state (Akt Ser^{473}, GSK-3α/β $Ser^{21/9}$ and PP1-α Thr^{320}), glycogen synthase (GS) and hexokinase (HK) enzyme activities, and muscle glycogen content.

Results: Dexamethasone treatment resulted in marked insulin resistance evidenced by resting hyperinsulinemia and a more than 3-fold decrease in glucose infusion rate during the EHC. In skeletal muscle, dexamethasone treatment resulted in increased HK activity, abrogation of the insulin-stimulated increase in GS fractional velocity, decreased phosphorylation of GSK-3α Ser^{21} and GSK-3β Ser^{9}, and increased total PP-1α protein

content, but had no effect on GLUT-4 content and glycogen, or Akt protein content and phosphorylation state.

Conclusions: This study demonstrated marked insulin resistance in horses after 21 days of dexamethasone treatment. Glycogen synthase activity did not increase with insulin stimulation following dexamethasone treatment, which could reflect impairment of GSK-3 phosphorylation.

Potential Relevance: An increased risk of laminitis has been associated with exogenously administered glucocorticoids in horses, perhaps by inducing insulin resistance. Studies are warranted to examine the effects of dexamethasone on insulin signaling and glucose metabolism in tissues of the foot (digital blood vessels, laminar epithelium).

INTRODUCTION

In humans and rodents, it is well recognized that excess glucocorticoids (GC) antagonize the action of insulin, resulting in decreased glucose uptake in insulin sensitive tissues, particularly skeletal muscle (Schneiter and Tappy, 1998). The mechanism by which glucocorticoids induce insulin resistance has not been fully elucidated but may involve insulin antagonism at one or more steps distal to the insulin receptor (Nosadini *et al*, 1983; Rizza *et al*, 1982). In different rodent studies, dexamethasone treatment has been shown to decrease GLUT-4 translocation to the plasma membrane, reduce activation of phosphatidylinositol 3-kinase (PI 3-K) after insulin stimulation, decrease phosphorylation of protein kinase B (PKB) and glycogen synthase kinase-3 (GSK-3), and decrease expression of IRS-1 (Dimitriadis *et al*, 1997; Saad *et al*, 1993; Schneiter and Tappy, 1998; Ruzzin and Jensen, 2005). These alterations in insulin signalling and GLUT-4 translocation could contribute to decreased insulin-mediated glucose uptake following GC treatment.

In a clinical setting, glucocorticoids are potent anti-inflammatory drugs used in equine medicine for treatment of a variety of conditions, including inflammatory airway disease, osteoarthritis and immune-mediated diseases (Johnson *et al*, 2004a&b; Cornelisse *et al*, 2006). However, the administration of glucocorticoids to horses and ponies has been implicated in the development of laminitis (Johnson *et al*, 2002b, 2004a&b; Cornelisse *et al*, 2006). It also has been suggested that glucocorticoid treatment increases risk of laminitis via the induction of insulin resistance (Johnson *et al*, 2004a&b) and recent studies have provided evidence for development of insulin resistance following glucocorticoid treatment (Firshman *et al*, 2005; Tiley *et al*, 2006 [Chapter 1]).

To date, however, there have been no studies in horses that have examined the effects of glucocorticoid treatment on mechanisms of insulin signalling and glucose metabolism in insulin sensitive tissues such as skeletal muscle.

Therefore, the objective of the study reported herein was to characterize the effects of dexamethasone on selected aspects of insulin signalling and glucose metabolism in skeletal muscle. Specifically, we measured the protein abundance of GLUT-4, Akt, GSK-3α/β and PP-1α, the phosphorylation state of Akt Ser- ^{473}, GSK-3α/β Ser- $^{21/9}$ and PP-1α Thr- ^{320}, the activities of glycogen synthase and hexokinase, and glycogen content in muscle samples taken before and after a 2-h period of hyperinsulinemia (euglycemic-hyperinsulinemic clamp). It was hypothesized that dexamethasone treatment would result in no change in total GLUT-4 protein or glycogen contents, but would result in decreased insulin-stimulated activation of glycogen synthase and hexokinase and phosphorylation of Akt Ser^{473}, GSK-3α/β Ser$^{21/9}$ and PP-1α Thr^{320}.

MATERIALS AND METHODS

Horses

The studies were conducted according to procedures reviewed and approved by the University of Guelph's Animal Care Committee, from mid-May to mid-July of 2005. Six mature Standardbred horses (2 mares, 4 geldings, 4-5 years old, mean [± S.D] body weight 443.2 ± 4.7 kg) that had been paddock-rested for 1 month were used in this study. Horses were housed in box stalls with approximately 4 hours/day of turnout into a small paddock. Horses were fed mixed grass hay at approximately 2.5% bwt, with access to fresh water and a salt block at all times. As an increase in dietary hydrolysable carbohydrate is one factor that may contribute to altered insulin sensitivity (Treiber *et al*,

2005a) and expression of GLUT-4 and insulin signalling proteins, no grain-concentrate was fed and horses wore muzzles during turnout to prevent grazing.

Experimental design

The experimental design is the same as previously described in Chapter 1, with the following modification, one day after the final treatment a 2 h euglycemic-hyperinsulinemic clamp (EHC) was administered, which included muscle biopsies, both pre-and post insulin stimulation.

Euglycemic- hyperinsulinemic clamp (EHC)

The EHC was undertaken as previously described (Stewart-Hunt *et al*, 2006). Feed was withheld overnight (approximately 10 hours) prior to each EHC. On the morning of the procedure (0800 h), the horses were weighed (± 0.5 kg, KSL electronic scale, KSL Inc., Kitchener, Ontario, Canada) and catheters (14g x 5¼", BS Angiocath, Sandy Utah, USA) were inserted into each jugular vein following aseptic preparation and desensitization of the overlying skin. One of the catheters was used for infusion of glucose (50% w/v dextrose) and insulin while all blood samples were obtained via the catheter in the opposite jugular vein. Blood was collected at the beginning of the experiment for the determination of baseline blood glucose and serum insulin concentrations, and to harvest serum (2 ml) to be used for preparation of the insulin infusate. A muscle biopsy was also taken, after which the horses were returned to their stalls for a 1-h recovery period. Thereafter, horses were positioned in stocks, a priming dose (18 mU/kg bwt) of insulin (Humulin-R, Eli-Lily, Indianapolis, Indiana, USA) was administered, and simultaneous infusions of 50% (w/v) dextrose and insulin (3 μU/kg bwt/min) were started. A syringe pump (Precision Syringe Pump, KD Scientific, Kansas

City, Missouri, USA) was used for infusion of dextrose, while insulin was administered via a peristaltic pump (Vet IV 2.2, Heska Corp., Denver, Colorado, USA). Blood glucose concentration was measured every 5 min using an automated analyzer (YSI 2300, Yellow Springs Instruments, Yellow Springs, Ohio, USA) and the rate of dextrose infusion was adjusted to maintain blood glucose concentration at approximately 5 mmol/l. Additional blood samples were collected every 15 minutes for subsequent measurement of serum insulin concentrations. Samples were placed into evacuated tubes containing no additive and centrifuged for 15 min (1,600 × g), after which serum was harvested and stored at -20°C until analysis. A second biopsy sample was taken from the contralateral middle gluteal muscle at the end of the EHC, after which glucose and insulin infusion was stopped.

The blood glucose and serum insulin data were used to calculate two measures of insulin sensitivity. Calculations were made using data from the last 60 min of the EHC, as the first hour was used as an equilibration period to achieve steady state euglycemia. The mean rate of glucose infusion (GIR, mg/kg/min) was calculated for each 5 min interval and averaged over the last 60 min of the EHC (M_{60}). A second measure of insulin sensitivity was derived from the ratio of M to the prevailing insulin concentration (I_{60}) during the last 60 min of the EHC (M/I_{60}). This ratio reflects the rate of glucose disposal per unit of insulin and is an index of tissue sensitivity to exogenous insulin (De Fronzo *et al*, 1979; Rijnen *et al*, 2003). Insulin clearance during the last 60 min of the EHC (MCR_I) was calculated on the basis of the insulin infusion rate and the change in serum insulin concentration (DeFronzo *et al,* 1979).

Muscle biopsy

Muscle biopsies were obtained at a standardized site and depth (8 cm) in the middle gluteal muscle using the percutaneous needle biopsy technique (Lindholm and Piehl, 1974). The muscle sample (approximately 500 mg wet weight) was quickly blotted free of excess blood and immediately immersed in liquid nitrogen. All muscle samples were stored at -80°C until analysis.

Serum immunoreactive insulin

Serum insulin concentrations were measured in duplicate by use of a commercially available radioimmunoassay kit (Coat-a-Count, Diagnostic Products Corporation, Los Angeles, California, USA), validated for the horse (Freestone *et al.* 1992). The intra- and interassay CVs for the insulin assay were, respectively, 2.9% and 4.8%.

Glycogen synthase activity

Glycogen synthase (GS) activity was determined as previously described (Pehleman *et al*, 2004). Frozen muscle samples (20-30 mg) were homogenized in ice-cold homogenizing media (50mM Tris-HCL, 5mM EDTA, 20mM sodium fluoride and 5mM DTT, pH = 7.4) until all fibrous material was broken down into solution. The homogenates were centrifuged at 4°C for 5 minutes at 7000rpm, after which 100 µl of each homogenate's supernatant was removed and added to 450 µl of incubation media (50mM Tris-HCL, 2mM EDTA, 10mM sodium fluoride, 10mM glycogen, 0.5mM DTT, 0.02% bovine serum albumin, ph = 7.4) containing either no G-6-P or 10 mmol/L G-6-P. These homogenate solutions were used for the determination of glycogen synthase (GS) I form (active) or GS_0, and GS I+D form (total) or GS_{10}, respectively. Uridine diphosphate (UDP) glucose (8 mmol/L) was added to catalyze the incorporation of glucose from

UDP-glucose to glycogen, in turn releasing UDP. Samples were incubated at 37°C for 45 min. To stop the reaction, the samples were placed in a 90°C hot water bath for 2.5 minutes, and then centrifuged at 7000 rpm for 5 minutes at 4°C. A set of UDP standards (25-150μU) was also prepared. Fluorescence readings were taken in the presence of UDP reagent (20mM Tris, 30mM KCL, 0.02% BSA, 2mM MgCl2, 0.4mM PEP, 3.8mM NaDH and 0.4 U/ml LDH, pH= 7.6) prior to the addition of 3.0 U/ml pyruvate kinase (PK). Samples were then allowed to stand at room temperature for 15 min until a second fluorescence reading was taken. Glycogen synthase I form and the I + D form activity was determined by the difference in NAD^+ fluorescence measured in the samples before and after the addition of PK and against NAD^+ fluorescence in the UDP standards. Glycogen synthase activity was then expressed as fractional velocity (GS_{FV}), or the activity of GS at 0 mmol G-6-P divided by the activity at 10 mmol G-6-P. The intra- and interassay CVs were, respectively, 5.9% and 6.4%.

Hexokinase activity

20mM imidazole-HCL and 0.02% BSA Hexokinase activity was determined using the protocol of Phillips *et al.* (1996). Frozen muscle samples (20-30mg) were homogenized in ice-cold phosphate buffer (pH= 7.4) comprised of 50% glycerol, 20mM KH_2PO_4, 5mM ß-mercaptoethanol, 0.5mM EDTA and 0.02% BSA. Homogenates were centrifuged at 4°C for 5 minutes at 7000rpm, and each was diluted (2.5 fold) in a buffer (20mM imidazole-HCL, 0.02% BSA, pH= 7.0). 20 μl of the diluted homogenate was then added to 100 μl of a reagent (pH = 8.1) containing 100mM Tris-HCL, 5mM glucose, 5mM ATP, 2mM $MgCl_2$, 0.5mM NAD^+, 0.5% Triton, 0.05% BSA and 2 U/ml G-6-P dehydrogenase. Final samples were incubated at 25°C for 1 h after which 10 μl of 1M

HCL was added and samples were incubated for 3 min at 95°C to stop the reaction. Subsequently, 1ml of a second reagent (50mM imidazole, 100μM NADP+, 30mM sodium acetate, 5mM MgCl2, 5mM EDTA and 0.1 U/ml phosphogluconate dehydrogenase, pH = 7.0) was added to the samples, the reaction allowed to proceed for 15 min at 25°C, following which fluorescence readings were taken. The intra- and interassay CVs were, respectively, 1.7% and 2.1%.

Western immunoblot analysis of GLUT-4, Akt, GSK-3 and PP-1

Western immunoblot was used to assess GLUT-4, Akt, GSK-3α/β and PP-1α protein abundance and the phosphorylation state of Akt Ser^{473}, GSK-3α/β $Ser^{21/9}$ and PP-1α Thr^{320} in muscle homogenates, as previously described (McCutcheon *et al.*, 2002, 2006). Frozen wet muscle biopsy sections (30 mg) were homogenized on ice in protein lysis buffer [10mMTris-HCL, 5mM EDTA, 50mM NaCl, 30mM $Na_4P_2O_7$, 1% Triton-X, 1mM PMSF, 5μg aprotinin/ml, 1μg pepstatin-A/ml, 2μg leupeptin/ml, 50mM NaF, and 300μM Na_3VO_4]. Samples were then centrifuged at 14,000 rpm for 20 min at 6°C after which the protein lysate layer was carefully extracted. Protein concentration of the homogenates was determined using a commercially available kit (Pierce BCA protein assay, Rockford, Illinois, USA). An aliquot of homogenate containing 40μg of protein was mixed with sample loading buffer (187.5mm Tris-HCL, 6% SDS, 30% glycerol, 0.03% bromophenol blue, and 50mM DTT) and subjected to Tris-glycine SDS-polyacrylamide gel electrophoresis for 90 min at 125 volts (10% resolving gel), and then transferred to a nitrocellulose membrane (Hybond™ ECL™, Amersham Biosciences, UK) for 90 min at 25 volts. All samples were run in duplicate and a broad range pre-stained protein marker (New England Biolabs, Ipswich, Massachusetts, USA) was also

run in the first well of all gels to confirm binding of all antibodies at the correct molecular weight.

Following gel transfer, membranes were blocked for 2 h in 5% skim milk TBST solution. After blocking, membranes were incubated with a polyclonal, anti-GLUT-4 (Biogenesis, UK), anti-Akt (which detects total Akt-1, Akt-2, and Akt-3), anti-phospho Akt-Ser^{473} and anti-phospho GSK-3α/β Ser$^{21/9}$ (Cell Signaling Technology, Beverly, Massachusetts, USA), or monoclonal anti-GSK-3α/β (Upstate Biotechnology, Lake Placid, NY, USA), followed by an anti-rabbit horseradish peroxidase (HRP)-linked immunoglobulin-G (IgG) antibody (Cell Signaling Technology, Beverly, Massachusetts, USA). All primary antibodies were diluted 1:1000 while the secondary antibody was applied in a 1:2000 dilution. For determination of total and phosphorylated Akt and GSK-3α/β, it was necessary to probe for the phosphorylated form first. The antibodies were then stripped from the membrane by applying a stripping buffer (comprised of 7.9µl/ml β-mercaptoethanol, 1M Tris and 10% SDS) for 15 min in an incubated rocker set at 50°C, followed by a series of washes in TBST, 2 h of blocking and application of the antibodies against total Akt and GSK-3. Protein bands were visualized after application of a chemiluminescence reagent (Western Lightning^{TM}, Perkin Elmer Life Sciences, Boston, Massachusetts, USA) and signals quantified by densitometry using the Alpha Innotech FluorChem^{TM} IS-9900 imaging system. Intensity of Glut-4, Akt and GSK-3α/β protein bands were corrected to tubulin protein bands imaged on the same membrane. A mouse monoclonal anti-α tubulin antibody (Sigma-Aldrich, St. Louis, Missouri, USA) was applied overnight (4˚C) at a dilution of 1:40,000. Anti-mouse HRP-linked IgG

antibody (Cell Signaling Technology, Beverly, Massachusetts, USA) was applied for 1 h at a dilution of 1:2000 before imaging using the aforementioned system.

For detection of total (polyclonal anti-PP-1α, Cell Signaling Technology, Beverly, Massachusetts, USA) and phospho-PP-1α $Ther^{320}$ (polyclonal anti- PP-1α $Ther^{320}$, Cell Signaling Technology, Beverly, Massachusetts, USA) the protocol was modified by using 5% solution of bovine serum albumin (BSA) in TBST (Sigma-Aldrich, St.Louis, Missouri, USA) rather than 5% skim milk TBST as the blocking solution and for the dilution of both primary and secondary antibodies and by using a Supersignal chemiluminescence solution (Pierce Supersignal Chemiluminescence, Rockford, Illinois, USA) for detection of the protein bands.

Muscle glycogen

Frozen muscle samples (50-60 mg) were freeze-dried and subsequently pulverized using a steel mortar and pestle device. Pulverized samples were dissected free of any visible blood, connective tissue and fat before analysis. Glycogen content (as glucosyl units) was determined via acid extraction following the procedures of Passoneau and Lauderdale. (1974). In brief, a 5-7 mg sample of freeze-dried muscle was added to 500μl of 1M HCL and vortexed and the weight of the tube + sample and HCL was recorded. Samples were then boiled at 100°C on a heating block for 2 hours, with vortexing every 30 minutes. After 2 h, tubes were cooled to room temperature and re-weighed with any changes >50μl corrected by adding the corresponding volume of ddH_2O. Samples were neutralized with 500μl of 1N NaOH and centrifuged at 3000rpm for 10 min. The glucose concentration of the supernatant was measured spectrophotometrically by use of a

commercial commercial kit (hexokinase method; Infinity™ Glucose, Thermo Electron Corporation, Melbourne Australia).

Metabolic Clearance Rate

The mean metabolic clearance rate (MCR) of insulin during the EHC was determined from the following equation, MCR = insulin infusion rate/Δinsulin concentration,

where Δ insulin concentration = steady state mean of insulin – baseline insulin concentration. (Units = ml/kg/min)

Statistical analysis

Descriptive statistics of continuous variables are expressed as mean ± s.e. Normality was tested using the Kolmorgornov-Smirnov statistic. Non-normally distributed variables were $\log_{10}$ transformed to achieve normality (baseline insulin concentration and Western immunoblot densitometry data). Paired variables (e.g. basal insulin concentration, M_{60}, M/I_{60}, MCR of insulin) were analyzed by paired *t* test. Muscle variables (e.g. glycogen content, GLUT-4 protein abundance, GS and HK activities) were analyzed by two-way analysis of variance with repeated measures to evaluate the effects of treatment (DEX vs. CON), time (pre- vs. post-EHC) and their interaction. Pair-wise comparisons were performed by the Bonferroni *t* test. Significance was set at $P<0.05$. Software programs (Systat 11.0, Systat Software, Los Angeles, California, USA) were used for statistical computations.

RESULTS

Euglycemic-hyperinsulinemic clamp and insulin sensitivity

There was no significant difference between treatments for resting blood glucose concentration or mean blood glucose concentration during the last 60 minutes of the EHC (Table 2.1). Mean values for resting serum insulin concentration and I_{60} were significantly higher in DEX than in CON, whereas mean MCR_I did not differ between treatments (Table 2.1). In both treatments, mean GIR was not different when comparing the 60-90 min and 90-120 min periods (Fig. 2.1). Mean values for GIR (Fig. 2.1) and M_{60} (Table 2.1) were more than 3-fold lower ($P<0.001$) in DEX than in CON. Similarly, mean M/I_{60} was approximately 3-to-4-fold lower ($P<0.001$) in DEX than CON (Table 2.1).

Muscle enzyme activities, GLUT-4 and glycogen content

Mean basal glycogen synthase activity (GS_0) and glycogen synthase fractional velocity (GS_{FV}) in pre-EHC samples did not differ between CON and DEX (Table 2.2). In CON, mean GS_0 and GS_{FV} were higher ($P<0.01$) in post- when compared to pre-EHC samples but were unchanged in DEX. There was no difference in total glycogen synthase activity (GS_{10}) between treatments or sampling times. Mean HK activity was higher ($P<0.05$) in DEX than CON in both the pre-and post EHC samples (Table 2.2) with no difference following insulin stimulation in either treatment.

There was no difference in total GLUT-4 protein abundance between treatments or sample times (pre vs. post EHC) (Fig. 2.2). Muscle glycogen content also did not differ between treatments or samples times (Fig. 2.3).

Insulin signalling proteins

Expression of total Akt, GSK-3α and GSK-3β, as determined by densitometric scanning of immunoblots, was similar in the CON and DEX treatments (Figs. 2.4A and 2.5). Total PP-1α was approximately 50% higher ($P<0.05$) in DEX than CON in both the pre- and post-EHC samples (Fig. 2.7A).

Akt Ser^{473} phosphorylation was more than 5-fold higher ($P<0.05$) in post- when compared to pre-EHC samples in both DEX and CON. However, there was no effect of treatment on Akt Ser^{473} phosphorylation (Figure 2.4B). In pre-EHC samples, phosphorylation of GSK-3α Ser^{21} and phosphorylation of GSK-3β Ser^{9} were approximately 2-fold lower ($P<0.05$) in DEX than in CON (Fig. 2.6). In both CON and DEX, phosphorylation of GSK-3α Ser^{21} or GSK-3β Ser^{9} did not change following the 2-h period of hyperinsulinemia (i.e. post- vs. pre-EHC samples) and phosphorylation of GSK-3α Ser^{21} and GSK-3β Ser^{9} remained lower ($P<0.05$) in DEX than CON (Figure 2.6). The phosphorylation of PP-1α Thr^{320} did not differ between treatments or sample times (Figure 2.7B).

DISCUSSION

This study examined the effects of a 3-week period of dexamethasone treatment on insulin-mediated glucose disposal and selected components of insulin signalling and glucose metabolism in skeletal muscle of horses. The main findings of the study were: 1) induction of insulin resistance, evidenced by an increase in resting serum insulin concentration and an approximately 3-fold decrease in the glucose infusion rate required to maintain euglycemia during the EHC, 2) abrogation of the insulin-stimulated increase in muscle glycogen synthase activity; 3) a 2-fold decrease in the phosphorylation of

GSK-3α Ser^{21} and GSK-3β Ser^{9}; 4) a 30-40% increase in hexokinase activity, and 5) a 2-fold increase in PP-1α protein expression. Dexamethasone treatment did not affect muscle glycogen content, the expression of total GLUT-4, Akt and GSK-3α/β, or insulin-stimulated phosphorylation of Akt Ser^{473}.

The dexamethasone-induced insulin resistance observed in the present study is consistent with the findings of one previous study in horses that employed the same dexamethasone treatment regimen (Firshman *et al*, 2005). The means by which glucocorticoids induce insulin resistance in horses remain unclear. Insulin suppression of hepatic gluconeogenesis can be diminished by dexamethasone. Alternatively, glucocorticoids can decrease the rate of glucose uptake and glycogen synthesis in peripheral tissues. A variety of mechanisms have been shown to induce insulin resistance following dexamethasone administration in other species (Dimitriadis *et al*, 1997; McMahon *et al*, 1988; Weinstein *et al*, 1995; Ruzzin *et al*, 2005) including alterations in insulin binding at the plasma membrane or in altering components of the insulin-signalling cascade. Additionally, abnormalities of glucose transporter 4 (GLUT-4) content or distribution within the cell or a combination of these are potential mechanisms of dexamethasone induced insulin resistance. For example, dexamethasone treatment of cultured rat adipocytes significantly decreased insulin binding to its receptor, IRS-1 and IRS-2 expression and PI3-K activity (Buren *et al,* 2002).

Skeletal muscle is the primary tissue of glucose utilization and therefore the major site of differences in insulin sensitivity (DeFronzo *et al*, 1981). Therefore, in the context of the current study, several potential mechanisms for induction of insulin resistance in equine skeletal muscle must be considered as candidates for inducing alterations in

insulin sensitivity evident following administration of dexamethasone. Alterations in the activity of glucose transporters in muscle, specifically GLUT-4, can diminish glucose uptake. Expression of GLUT-4 may be decreased, resulting in a reduction of availability of glucose transporters at the cell membrane. The expression of GLUT-4 and rate of insulin-mediated glucose disposal are closely linked as demonstrated by enhanced insulin sensitivity and glucose transport in skeletal muscle of transgenic mice overexpressing GLUT-4 (Ren *et al*, 1993; Hansen *et al*, 1995; Leturgue *et al*, 1996; Henriksen, 2002). However, other investigators have determined that dexamethasone does not decrease GLUT-4 content or gene expression in rat skeletal muscle (Haber and Weinstein, 1992), and that a reduction in GLUT-4 expression is not necessarily associated with decreased insulin responsiveness in rats (Kahn *et al*, 1991). Alternatively, glucose uptake may be reduced despite adequate GLUT-4 expression when the rate of translocation of GLUT-4 to the sarcolemma is decreased. In rat soleus muscle, glucocorticoid-induced insulin resistance is reported to reflect impairment of insulin-stimulated GLUT-4 translocation (Dimitriadis *et al*, 1997). In the present study, there was no change in GLUT-4 protein following dexamethasone treatment. While decreased GLUT-4 translocation without a decrement in total GLUT-4 protein abundance is possible, no quantification of the membrane-associated fraction of total GLUT-4 was undertaken to determine if changes in GLUT-4 translocation occurred following dexamethasone treatment. Furthermore, evidence for the contribution of decreased GLUT-4 expression and/or activity of signalling proteins to decreased GLUT-4 has not been a consistent finding as GLUT-4 has been shown to increase in rat fast-twitch skeletal muscle following dexamethasone treatment while remaining unchanged in slow twitch fibers (Ruzzin *et al*, 2005).

Glucocorticoid-induced diminishment of insulin action may reflect inhibition of the intermediary signalling pathway between the insulin receptor and glucose transport activation. When insulin binds to its receptor on the cell membrane, the receptor must be tyrosine-phosphorylated in order to activate IRS-1. Subsequently, activated IRS-1 binds the p85α regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase). Then protein kinase B (PKB), activated by phosphorylation at Thr^{308} and Ser^{473}, phosphorylates glycogen synthase kinase-3 (GSK-3) which inactivates the kinase. GSK-3 inactivation contributes to the complex process of increased activation of glycogen synthase (GS). Glycogen synthase activation is a complex process involving phosphorylation of nine amino acids by a variety of kinases and dephosphorylation by protein phosphatase-1 (PP-1) (Lawrence and Roach, 1997; Munro *et al*, 2005; Walker *et al*, 2000; Newgard *et al*, 2000; Brady and Saltiel, 2001). Some studies in rat skeletal muscle have demonstrated that dexamethasone decreases insulin stimulated phosphorylation of tyrosine kinase and impairs the activity of the insulin receptor thereby reducing the rate of glucose uptake without altering the number and relative affinity of the skeletal muscle insulin receptor (Block *et al*, 1989; Coderre *et al*, 1992). Others have reported that, rather than altered tyrosine phosphorylation of the receptor, dexamethasone-induced insulin resistance was associated with inhibition of the subsequent step of insulin-stimulated IRS-1 associated PI 3-kinase activity (Leighton *et al*, 1987; Saad *et al*, 1993). Dexamethasone administration has also been demonstrated to decrease insulin-stimulated PKB and GSK-3α/β phosphorylation without a reduction in PKB or GSK-3α/β expression, potentially contributing to skeletal muscle insulin resistance (Ruzzin *et al*, 2005). However, when GSK-3α/β phosphorylation is inhibited, glycogen synthase fractional activity was

reported to increase in dexamethasone-treated rats (although not to the extent measured in untreated rats) without improving the rate of insulin-stimulated glucose uptake. In the present study, the 2-fold decrease in the phosphorylation of GSK-3α Ser^{21} and GSK-3β Ser^{9} following dexamethasone treatment without a change in total GSK-3α/β abundance is consistent with these recent findings. The absence of an insulin-stimulated increase in GS fractional activity in the dexamethasone-treated horses may reflect a reduction in GSK-3α/β phosphorylation in the middle gluteal muscle examined in this study.

Many findings to date regarding dexamethasone induced insulin resistance argue for impairment of insulin signalling proteins rather than a reduction in the expression of key components such as PKB, GSK-3, and GLUT-4. While reduced expression of PKB or increased expression of GSK-3 has been implicated in insulin resistance (Cushman and Wardzala, 1980; Kono *et al*, 1982; Klip *et al*, 1987), dexamethasone treatment does not necessarily alter expression of these proteins. Protein kinase B (PKB, Akt-Ser^{473}) is one of several kinases in skeletal muscle involved in phosphorylation of GSK-3α and GSK-3β, a process that promotes glucose utilisation via activation of GS (Cross *et al*, 1995; Sutherland *et al* 1993). Following chronic dexamethasone treatment in rats, PKB (Akt Ser^{473}) phosphorylation was reduced by 40% in rat soleus and epitrochlearis muscle (Ruzzin *et al*, 2005). However, a 12 day dexamethasone treatment that resulted in reduced insulin-stimulated PKB Ser^{473} phosphorylation did not alter PKB-β protein abundance (Ruzzin *et al*, 2005; Ruzzin and Jensen, 2005). In the present study, dexamethasone treatment did not alter phosphorylation of PKB (Akt-Ser^{473}) before or after insulin stimulation. One possible explanation for the absence of any change in PKB phosphorylation proposed by Sakoda *et al.* (2000) and others is that dexamethasone-

induced insulin resistance reflects alterations in the signalling pathway distal to or "downstream from" PKB.

Glycogen synthase total activity was similar before and after the EHC following dexamethasone treatment in the present study. Consistent with findings in rats (Ruzzin *et al*, 2005), insulin stimulation did not increase GS fractional velocity (via decreased GS phosphorylation) in the dexamethasone-treated group. Glucocorticoids have been shown to abolish insulin-stimulated dephosphorylation of GS at several sites (Ser^{645}, Ser^{649}, Ser^{653}, Ser^{657}), which could contribute to altering the complex regulation of GS activity. As mentioned earlier, dexamethasone treatment may have abolished an insulin-stimulated increase in GS fractional activity via a reduction in GSK-3α/β phosphorylation. Another strong regulator of GS activity is skeletal muscle glycogen content. Indeed, there is an inverse relationship between muscle glycogen content and GS fractional velocity (Jensen *et al*, 1997). However, as glycogen content did not differ between the dexamethasone treatment group and controls, it is difficult to speculate as to any role for glycogen concentration in the GS activity measured. The failure of insulin to increase GS fractional activity following dexamethasone treatment is more likely to reflect other mechanisms that abrogated the ability of insulin to dephosphorylate and activate GS.

Insulin enhances glycogen storage through dephosphorylation of GS and glycogen phosphorylase. This insulin-dependent dephosphorylation is catalyzed by PP-1 although the mechanisms by which insulin assists activation of glycogen-targeted PP-1 are uncertain (Newgard *et al*, 2000). Importantly, PP-1 is present in almost every cellular compartment and involved in the regulation of a broad range of cellular processes not affected by insulin (e.g. cell division, ion channel function) (Brady and Saltiel, 2001).

Even within the four-member family of glycogen-targeted forms of PP-1, the most abundant subunit in human skeletal muscle ($PP1G_M$) does not appear to be insulin regulated (Munro *et al*, 2002). In the current study, PP-1α protein abundance was increased following dexamethasone treatment in pre-and post-EHC samples with no increase in PP-1α phosphorylation at threonine residue^{320}. Interestingly, when glucose transport was reduced in muscle specific GLUT-4 knockout mice, one of the major alterations was a substantial increase in the abundance of glycogen targeting PP-1 subunits in the skeletal muscle of the muscle-G4KO mice (Kim *et al*, 2005). The lack of a significant change in PP-1 phosphorylation state following dexamethasone treatment might be attributable to the residue we chose to measure its phosphorylation at PP-1. Following further investigation, it was found that phosphorylation of threonine^{320} residue on PP-1 actually plays a role in regulating the cell cycle (Liu *et al*, 1999) and not glycogen synthase regulation.

The first committed step in glucose uptake in skeletal muscle is the phosphorylation of glucose to glucose-6-phosphate (G-6-P) by hexokinase (HK). In human skeletal muscle, two isoforms (HKI and HKII) have been identified of which only HKII is regulated by insulin in skeletal muscle (Mandarino *et al*, 1995). In EHC's performed in human subjects, HKII activity was significantly increased after 240 min (Mandarino *et al*, 1995) to 360 min (Vogt *et al*, 2000) of insulin stimulation, changes that were sustained for a further 360 min and associated with increased HKII mRNA expression. In the present study, no change in HK activity was noted following insulin stimulation. Importantly however, these measurements did not extend beyond 120 min. It is possible that a longer period of measurement in our EHC might have yielded more

information regarding skeletal muscle HK activity in the dexamethasone treated group. The absence of any insulin-stimulated increase in HK has also been reported in human studies (Kelly *et al*, 1996) although in this instance there was no attempt to separate the activities of HKI and HKII or to subfractionate biopsy extracts into soluble and particulate fractions, making it more difficult to assess small changes in activity. An interesting finding in the present study was the increase in HK following dexamethasone treatment. As insulin is likely a physiological regulator of HK expression in skeletal muscle (Vogt *et al*, 2000), potentially the chronic state of hyperinsulinemia invoked by dexamethasone could account for the changes in this enzyme observed at the end of the treatment period.

The present study demonstrated that 21 days of dexamethasone treatment induced insulin resistance in Standardbred horses, as indicated by the marked decrease in glucose infusion rate during the euglycemic-hyperinsulinemic clamp. Although several alterations in important proteins and enzymes involved in skeletal muscle glucose metabolism were noted following the period of induced insulin resistance, the effects of dexamethasone on other aspects of insulin signalling should be investigated. Furthermore, in addition to direct effects of dexamethasone on components of insulin signalling and glucose metabolism, the importance of other physiological mechanisms must also be considered. Importantly, dexamethasone has also been demonstrated to potentiate vasoconstriction in equine digital vessels. Cornelisse *et al.* (2006) demonstrated that dexamethasone administered intravenously (0.10 mg/kg bodyweight) decreased baseline skin temperature, reflecting a reduction in skin perfusion. It is therefore also possible that alterations in glucose and insulin delivery to skeletal muscle as a result of reduced blood

flow to the periphery are an important mechanism in dexamethasone-induced insulin resistant in horses.

TABLE 2.1: Measures of glucose metabolism and insulin sensitivity during the euglycemic-hyperinsulinemic clamps (EHC).

	CON	DEX
Resting blood glucose (mmol/l)	3.58 ± 0.34	4.14 ± 0.27
Resting serum insulin (μU/ml)	4.5 ± 1.5	54.2 ± 10.7*
Blood glucose 60 min (mmol/l)	5.11 ± 0.24	5.84 ± 0.48
MCR_I (μU/min/ml)	0.0092 ± 0.0005	0.0095 ± 0.0013
I_{60} (μU/ml)	322.3 ± 29.1	393.1 ± 38.9*
M_{60} (mg/kg/min)	7.73 ± 1.04	2.15 ± 0.44#
M/I_{60} ($x10^{-2}$ mg/kg/min per mU/ml)	2.35 ± 0.002	0.58 ± 0.001*

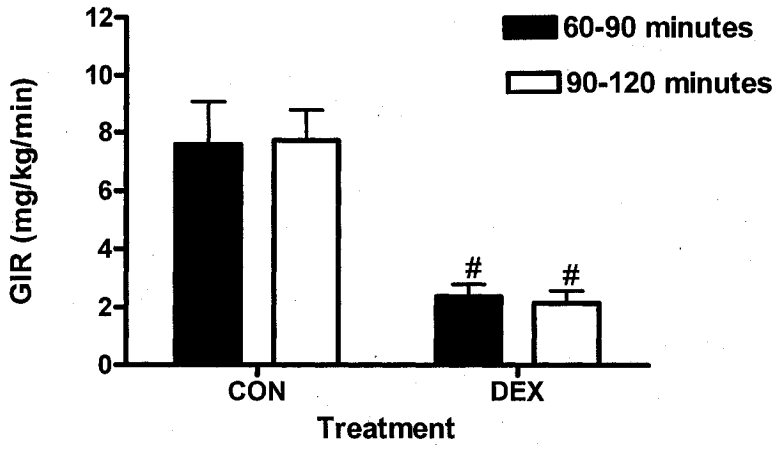
Data are mean [± s.e.] for 6 horses. DEX, dexamethasone; CON, control. Blood glucose 60 min, average blood glucose concentration during the final 60 min of the EHC; MCR_I, metabolic clearance rate of insulin; I_{60}, serum insulin concentration averaged over the final 60 min of the EHC; M_{60}, glucose infusion rate averaged over the last 60 min of the EHC; M/I_{60}, insulin sensitivity index averaged over the last 60 min of the EHC.
* $P<0.05$ vs CON. # $P<0.001$ vs CON.

TABLE 2.2: The activities of hexokinase and glycogen synthase in middle gluteal muscle before (Pre) and after (Post) the euglycemic-hyperinsulinemic clamp (EHC).

ENZYME ACTIVITY		CON	DEX
GS_0 (nmol/mg muscle/h)	**Pre EHC**	46.65 ± 12.41#	37.25 ± 6.25*
	Post EHC	100.94 ± 12.72	46.20 ± 15.03*
GS_{10} (nmol/mg muscle/h)	**Pre EHC**	150.82 ± 6.06	150.79 ± 8.14
	Post EHC	152.95 ± 6.07	150.44 ± 5.60
GS_{FV} (GS0/GS10)	**Pre EHC**	0.31 ± 0.08#	0.25 ± 0.04*
	Post EHC	0.67 ± 0.08	0.31 ± 0.09*
HK (nmol/μg protein/h)	**Pre EHC**	7.44 ± 0.96	12.34 ± 1.08*
	Post EHC	9.01 ± 0.97	12.55 ± 1.58*

Data are mean [± s.e] for 6 horses. CON, control; DEX, dexamethasone; GS_0, active form of glycogen synthase; GS_{10}, total glycogen synthase activity; GS_{FV}, glycogen synthase fractional velocity; HK, hexokinase activity.
# indicates significant difference ($P<0.05$) between Pre and Post EHC measurements within treatment; * indicates significant difference ($P<0.05$) from CON.

A



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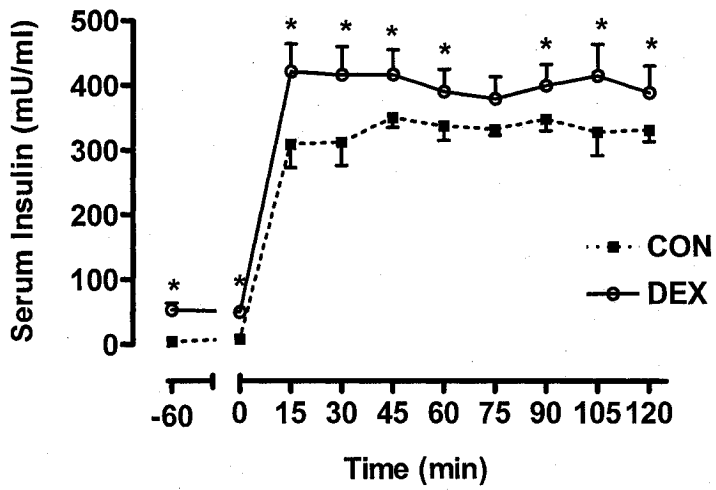


FIGURE 2.1: Mean glucose infusion rate (GIR; **A**) and serum insulin concentration (**B**) during the euglycemic-hyperinsulinemic clamp (EHC) in CON and DEX treatments. Mean GIR was significantly ($P<0.001$) lower in DEX than in CON during the 60-90 and 90-120 minute periods. Serum insulin concentrations were higher ($P<0.05$) in DEX than in CON at rest and at most time points during the EHC.

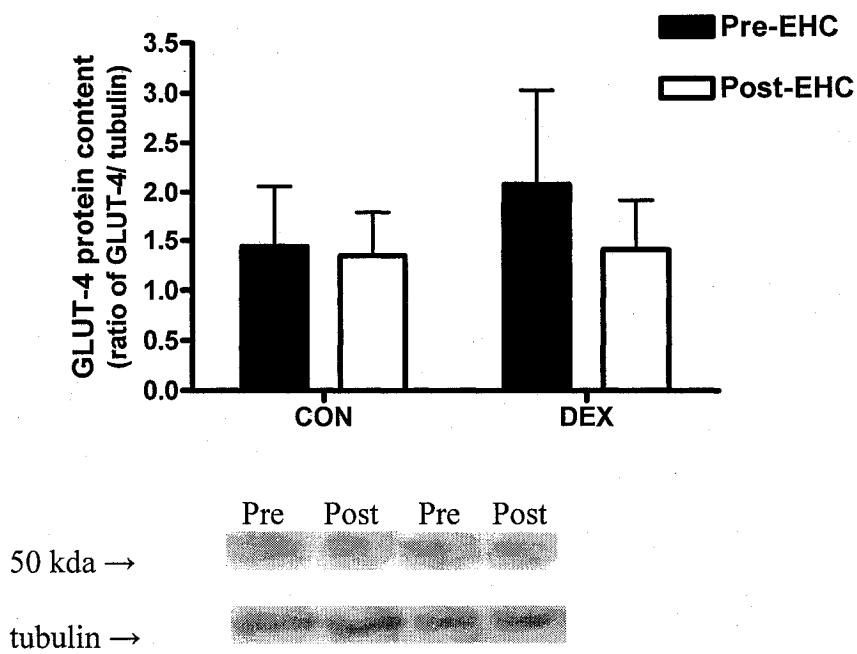


FIGURE 2.2: Mean [± s.e] GLUT-4 protein expression in lysates of middle gluteal muscle measured before (Pre) and after (Post) an EHC. There was no significant difference between treatments or sample times. Representative immunoblots for GLUT-4 and tubulin (loading control) are shown in the lower panel.

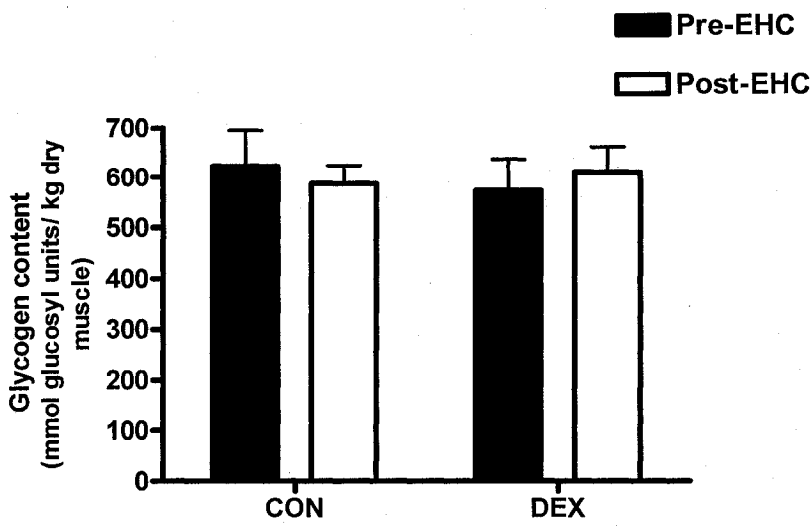
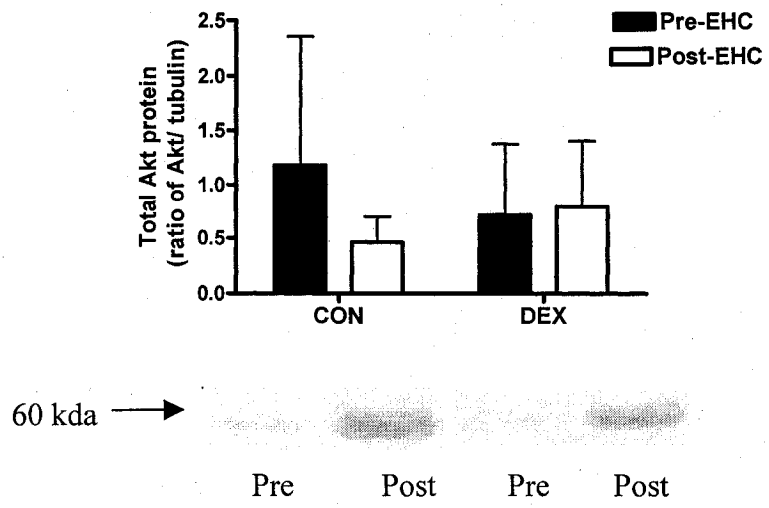


FIGURE 2.3: Mean [± s.e] glycogen content of middle gluteal muscle samples taken before (Pre) and after (Post) an EHC. There was no significant difference between treatments or sample times

A



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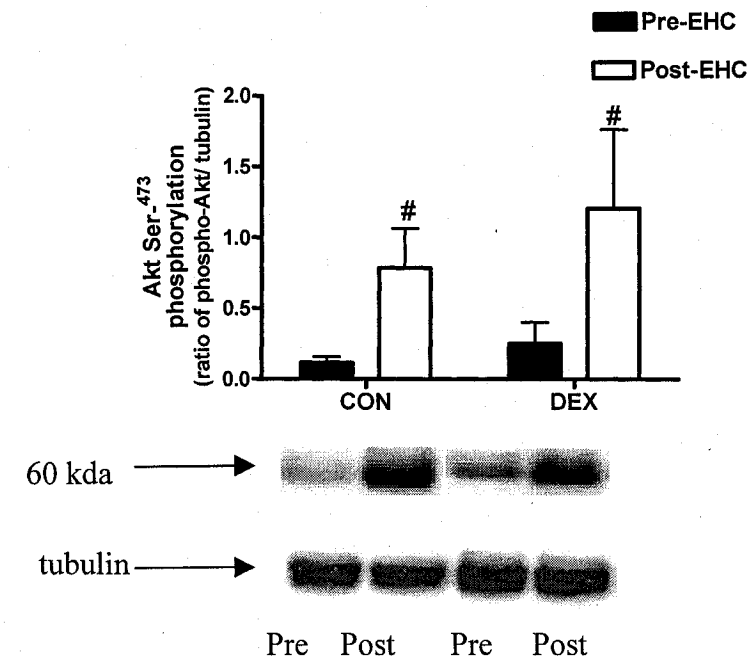
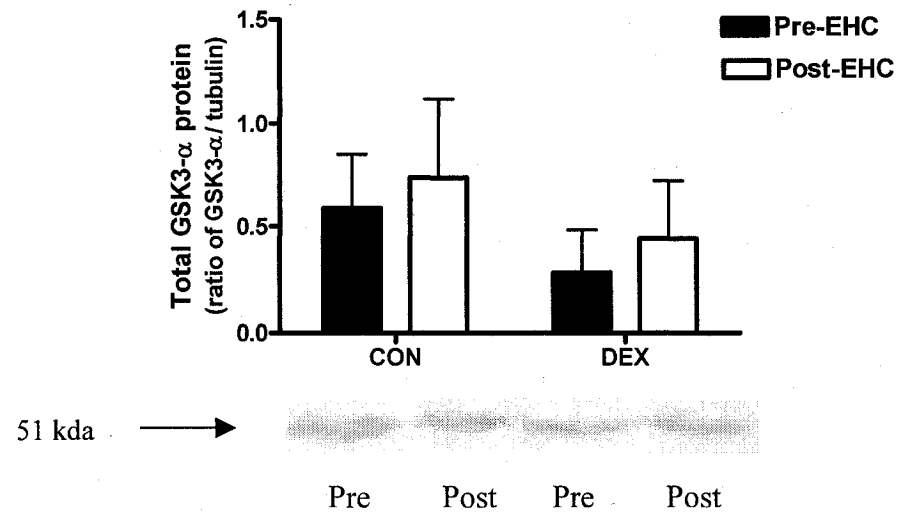


FIGURE 2.4: Muscle tissue protein lysates resolved by SDS-PAGE and immunoblotted with anti-Akt total protein antibodies (**A**) and anti-phospho Akt-Ser^{473} antibodies (**B**) and expressed as the ratio of Akt Ser^{473} over tubulin area density, before (Pre) and after (Post) a EHC in CON and DEX. Representative immunoblots (including tubulin as loading control) are shown. # indicates significant difference ($P<0.05$) from Pre within treatment. Total Akt and Akt Ser^{473} did not differ between treatments.

A



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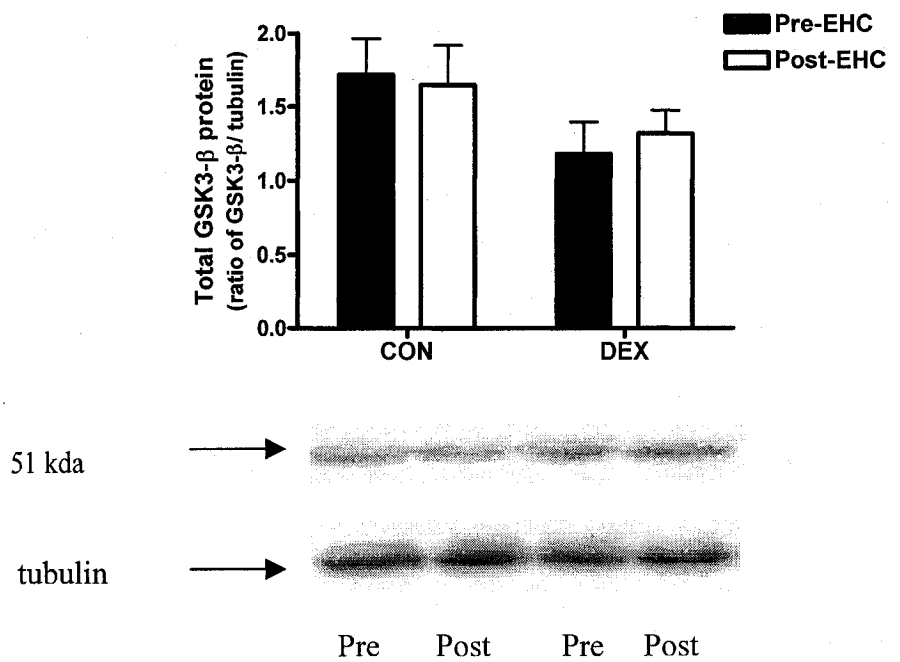
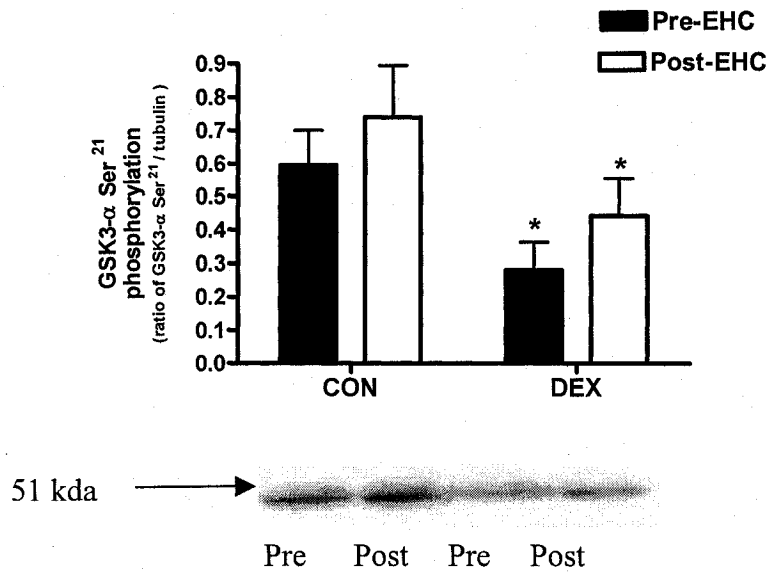


FIGURE 2.5: Muscle tissue protein lysates resolved by SDS-PAGE and immunoblotted with monoclonal anti-GSK-3α (A) and anti-GSK-3β (B) antibodies, expressed as the ratio of GSK-3α/β to tubulin area density, before (Pre) and after (Post) a EHC in CON and DEX. Representative immunoblots are shown below the graphs. There was no difference in total GSK3-α/β protein abundance between treatments or sample times (Pre vs. Post EHC).

A



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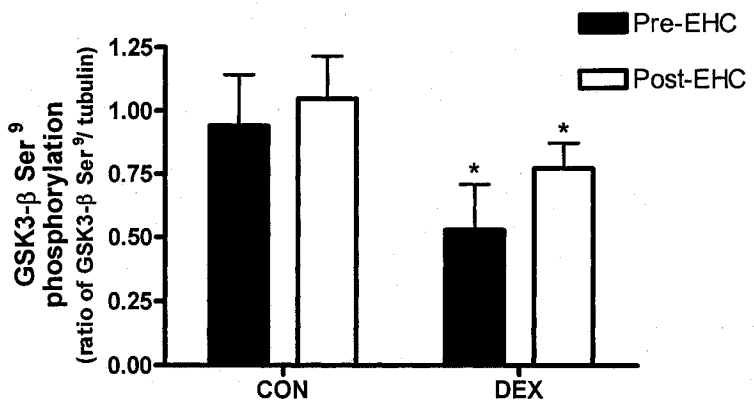


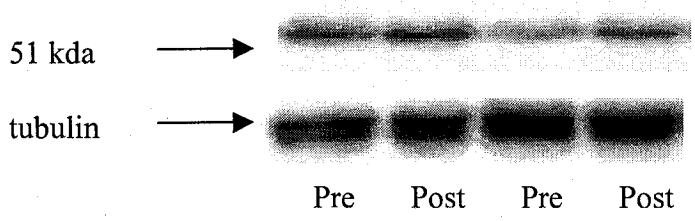
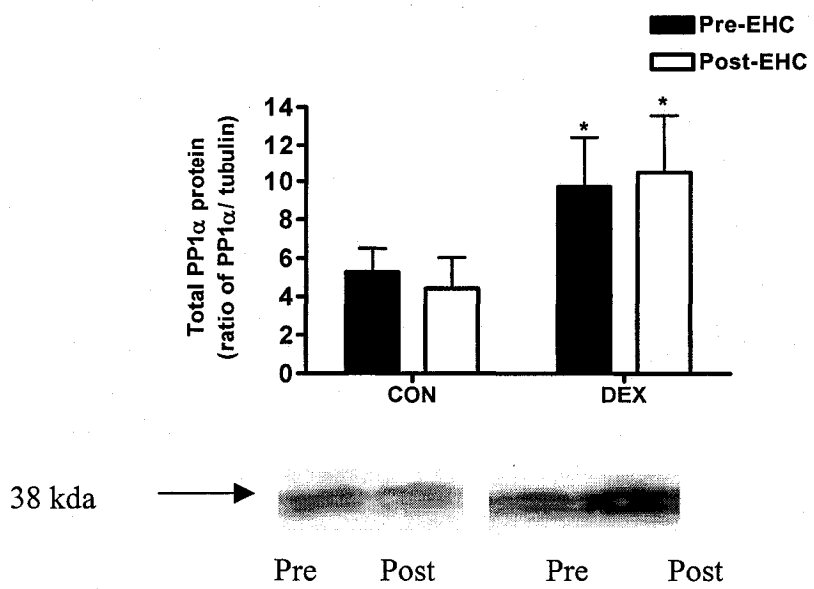


FIGURE 2.6: Muscle tissue protein lysates resolved by SDS-PAGE and immunoblotted with anti-phospho GSK-3α Ser^{21} antibodies (**A**) and anti-phospho GSK-3β Ser^{9} antibodies (**B**) and expressed as the ratio of GSK-3α/β $^{21/9}$ over tubulin area density, before (Pre) and after (Post) a EHC in CON and DEX. Representative immunoblots are shown in the lower panels. * indicates significant difference ($P<0.05$) from CON.

A



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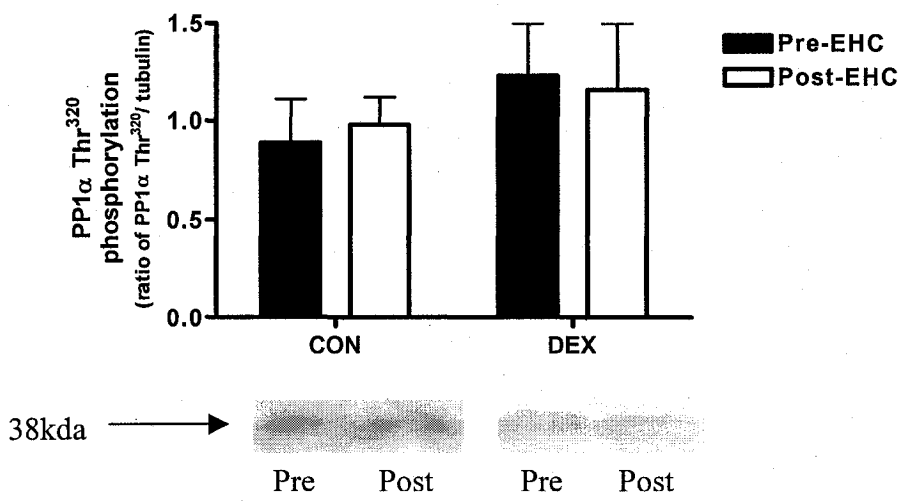

FIGURE 2.7: Muscle tissue protein lysates resolved by SDS-PAGE and immunoblotted with anti-PP-1α total protein antibodies (**A**) and anti-phospho PP-1α-Thr^{320} antibodies (**B**) and expressed as ratio of PP-1α and PP-1α-Thr^{320} over tubulin area density, before (Pre) and after (Post) a EHC in CON and DEX. Representative immunoblots are shown for each graph. * indicates significant difference ($P<0.05$) from CON. Phospho-PP-1α did not differ between treatments or sample times (Pre vs. Post EHC).

SUMMARY & GENERAL DISCUSSION

This thesis comprised two separate studies investigating the effects of dexamethasone treatment on insulin sensitivity and glucose dynamics, and selected aspects of skeletal muscle insulin signalling and glucose metabolism in Standardbred horses. The first study examined the effects of a 21-day period of dexamethasone treatment on whole body insulin sensitivity and glucose homeostasis, using basal plasma proxy analysis and minimal model analysis of a frequent-sampled, intravenous glucose tolerance test (FSIGT). The second study investigated the effects of the same treatment regimen on GLUT-4 protein abundance, muscle glycogen content, glycogen synthase (GS) and hexokinase (HK) enzyme activities, and the abundance and phosphorylation of Akt, GSK-3α/β and PP-1α in samples of skeletal muscle obtained before and after a 2-h euglycemic-hyperinsulinemic clamp (EHC).

In the first study, dexamethasone treatment resulted in 1) an approximately 2-fold increase in basal serum insulin concentration on days 7, 14 and 21 of treatment, 2) a 4-fold decrease in whole-body insulin sensitivity (Si) and 2-fold increase in acute insulin response to glucose (AIRg) measured by minimal-model analysis of FSIGT, and 3) an approximately 50% decrease in RISQI (a proxy measure of insulin sensitivity) and increase in MIRG (a proxy measure of pancreatic beta cell responsiveness), measured on days 7, 14 and 21. In the second study, dexamethasone treatment resulted in 1) development of marked insulin resistance evidenced by a more than threefold decrease in glucose infusion rate during the EHC and increased basal serum insulin concentration in samples collected before the EHC, 2) abrogation of the insulin-stimulated increase in glycogen synthase fractional velocity and a 30-40% increase in hexokinase activity, 3) an approximately 40-50% decrease in GSK-

3α/β $Ser^{21/9}$ phosphorylation, 4) a two-fold increase in total PP-1α protein abundance, and 5) no change in GLUT-4 protein abundance, muscle glycogen content, or insulin-stimulated Akt Ser^{473}.

Excess glucocorticoid has been implicated as a cause of insulin resistance in horses with Cushing's disease and the "equine metabolic syndrome." There also is limited clinical evidence linking the administration of glucocorticoids with increased risk of laminitis in horses and ponies, perhaps via exacerbation of insulin resistance (Love, 1993; Pass *et al*, 1998; Johnson, 2002b; French *et al*, 2000; Johnson, 2004a&b). However, the specific effect of glucocorticoids on insulin sensitivity and glucose dynamics has been little studied in equids. One previous study of Quarter horses with polysaccharide storage myopathy (PSSM) demonstrated marked insulin resistance, evidenced by a decrease in whole-body insulin stimulated glucose uptake, after dexamethasone treatment (Firshman *et al*, 2005). Non-specific evidence of insulin resistance (increased circulating glucose and insulin) was also demonstrated in healthy horses after single or multiple doses of triamcinolone acetonide (French *et al*, 2000). The present studies have extended this work by first applying two quantitative methods (the EHC and minimal model) for characterization of the effects of dexamethasone on insulin sensitivity, glucose dynamics and insulin secretory response. Second, this study is the first in horses to examine the effects of chronic dexamethasone on mechanisms of insulin signalling and glucose metabolism in skeletal muscle.

The observed decreases in GSK-3α/β $Ser^{21/9}$ phosphorylation and insulin-stimulated GS activity were consistent with findings in rats (Ruzzin and Jensen, 2005;

Ruzzin *et al*, 2005). In rats, a decreased rate of glycogen synthesis in muscle accompanies the dexamethasone-induced abrogation of GS activation. In the present study, we did not detect a significant effect of dexamethasone on muscle glycogen content when comparing pre- and post-EHC samples. However, this finding was not surprising given the small quantity of glucose infused during the 2-h EHC and the relative insensitivity of the glycogen measurement method for detection of small changes in muscle glycogen content. Studies involving the administration of labelled glucose with subsequent measurement of label incorporation into muscle glycogen might provide a more sensitive means for assessment of the effects of dexamethasone on glycogen synthetic rate.

An interesting finding was the increase in skeletal muscle HK activity after dexamethasone treatment. To the author's knowledge, there have been no reports of the effects of dexamethasone treatment on muscle HK activity in any species. In healthy humans, the infusion of insulin results in an increase in HK mRNA, protein abundance and activity in skeletal muscle (Vogt *et al*, 2000), suggesting that insulin might be a physiological regulator of HK expression in muscle. Perhaps the chronic state of hyperinsulinemia invoked by dexamethasone (Chapter 1) contributed to the increased HK activity in the horses of the present study. In insulin-resistant Zucker rat models and humans with non-insulin dependent diabetes mellitus, both unchanged and increased insulin-stimulated HK activity has been observed (Rothman *et al*, 1992; Sanderson *et al*, 1996). In these studies, an increase in muscle glucose-6-phosphate (G-6-P) content was also found, perhaps reflecting an enhanced rate of glucose phosphorylation in association with increased HK activity. In the present study, G-6-P

content was not measured, thereby limiting the interpretation of the HK activity results. However, it is possible that the increase in HK activity was, in part, a compensatory response to maintain glucose phosphorylation in the face of decreased glucose uptake into the muscle cell.

It should be taken into consideration that the assessments of skeletal muscle glucose metabolism were based on the evaluation of a single muscle (the middle gluteal), and therefore it is not possible to generalize the findings to all equine skeletal muscle. Interpretation of the muscle findings was further limited by lack of knowledge with regard to fiber-type distribution in the biopsy samples. However, as the muscle biopsies were taken at a standardized site and depth, this should have limited the variability in muscle composition. Furthermore, in addition to myocytes, there is the possibility that other cells, such as adipocytes and erythrocytes, were removed with the muscle biopsy. These tissues could have been a confounding factor in the assessment of certain signalling proteins. This should be taken into consideration for the assessment of Akt specifically, as it plays a number of regulatory roles in both of the cell types mentioned above.

A limitation in this study was the absence of direct measures of glucose transport into skeletal muscle. In several studies of rats, direct measures of GLUT-4 translocation (membrane fractionation techniques) and glucose uptake (as 2-deoxyglucose) have been used to document the effects of dexamethasone on muscle glucose transport (Weinstein *et al*, 1995; Weinstein *et al*, 1998). These studies have shown that dexamethasone treatment in rodents decreases insulin-stimulated glucose uptake by 48%, with a similar decrease in GLUT-4 recruitment to the cell surface.

Future studies in horses should incorporate more direct measures of the cellular distribution GLUT-4 and glucose transport in skeletal muscle. Application of membrane fractionation techniques or immunohistochemistry with confocal microscopy might be useful for examination of the cellular distribution (cytoplasm vs. sarcolemma) of GLUT-4 in muscle. For glucose transport studies, use of plasma membrane vesicles preparations or freshly harvested muscle strips (e.g. taken from the semimembranosus muscle) would be valuable. The ability to analyse GLUT-4 recruitment at the cell surface and rates of glucose transport into muscle would yield important information regarding the effects of glucocorticoid treatment on glucose transport, and the relationship of these variables to whole-body measures of insulin sensitivity.

The study presented in Chapter 2 would have been further strengthened by: a) use of a more accurate analysis of PP-1α phosphorylation, specifically the assessment of amino acid residues known to be involved in the regulation of GS activity, and b) the direct measurement of PP-1α activity (indeed, use of direct activity assays would also be useful for assessment of insulin signalling via Akt and GSK-3). The phosphorylation of PP-1α by insulin activates the phosphatase, allowing it to interact with GS and glycogen and initiating the dephosphorylation and activation of GS (Ragolia *et al*, 1998; Brady and Saltiel, 2001). In the present study, the phosphorylation of PP-1α was measured at threonine residue 320 (Thr ^{320}). However, after further investigation it was determined that phosphorylation of the threonine^{320} residue on PP-1α actually plays a role in regulating the cell cycle (Liu *et al*, 1999) and not glycogen synthase regulation. Rodent studies investigating the role of PP-1α

in the regulation of GS activation have yielded somewhat conflicting results. For example in a study by Dent *et al.* (1990), phosphorylation of Ser^{48} residue increased the rate at which the glycogen associated PP1-G_M complex dephosphorylated and activated GS. In contrast, Walker *et al.* (2000) reported that the Ser^{48} residue is not phosphorylated by insulin, nor is this site involved in the dephosphorylation of GS by PP-1α. Nonetheless, studies in which the activity of PP-1α has been directly measured lend support to the idea that PP-1α is important in the dephosphorylation of GS. In one study, insulin-resistant Zucker rats had significantly decreased PP-1α activity and GS fractional velocity when compared to the control animals (Semiz and McNeill, 2002). Future studies in horses should employ direct measures of PP-1α activity in skeletal muscle.

The small sample size used in the present study may have also been a limiting factor, specifically by increasing the risk of type II statistical error. For example, had there been a larger sample size, a significant treatment effect may have been detected in the minimal-model parameter disposition index (DI), in which the value for DEX tended (P=0.08) to be lower when compared to CON. Unfortunately, sample size for equine studies is often constrained by economics and the availability of healthy, sound animals that fit the criteria for inclusion in the study cohort.

Future directions in this field should include a more complete examination of the mechanisms of insulin signalling and glucose utilization in equine skeletal muscle, and alterations in the processes in insulin resistance. Measurement of some of the upstream regulators in glucose metabolism, such as the phosphorylation and abundance of insulin receptor and insulin receptor substrate, might provide further

insight into the effects of dexamethasone on insulin signalling in skeletal muscle. As mentioned, the combined measurement of protein activity and phosphorylation (western blot) would also allow for a more comprehensive assessment of insulin signalling. Application of techniques for measurement of GLUT-4 translocation and glucose transport activity are also needed for advancements in this field. Finally, given that increased risk of laminitis has been associated with the administration of glucocorticoids in horses, studies are warranted to examine the effects of dexamethasone on insulin signalling and glucose metabolism in tissues of the hoof (e.g. digital blood vessels, laminar epithelium).

In conclusion, the two studies described in this thesis have provided some of the initial information regarding the effects of dexamethasone treatment in horses on whole-body insulin sensitivity and glucose dynamics, as well as components of insulin signalling and glucose metabolism in skeletal muscle. The dexamethasone model of insulin resistance may be useful for future studies that seek to examine the health and performance effects of insulin resistance in horses.

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