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Glucose and insulin dynamics in horses and their association with endotoxemia and laminitis

Ferenc Tóth
University of Tennessee

To the Graduate Council:

I am submitting herewith a dissertation written by Ferenc Tóth entitled "Glucose and insulin dynamics in horses and their association with endotoxemia and laminitis." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

Nicholas Frank, Major Professor

We have read this dissertation and recommend its acceptance:

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Claudia Kirk

Jonathan Wall

James Schumacher

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Glucose and Insulin Dynamics in Horses and their Association with Endotoxemia and Laminitis

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Ferenc Tóth

May 2009

Abstract

The present studies were performed to investigate the effects of endotoxemia on glucose and insulin dynamics, and to examine their relationship to laminitis in horses. The frequently sampled intravenous glucose tolerance test was evaluated and refined for use in horses. A new FSIGTT was developed, consisting of 100 mg/kg dextrose followed by 20 mU/kg insulin 20 min later. This new test was used in subsequent experiments to assess glucose and insulin dynamics while minimizing urine glucose spillover. Serum C-peptide concentrations were measured in horses to investigate pancreatic function and this work revealed a decrease in C-peptide-to-insulin ratio after dextrose challenge, suggesting reduced hepatic insulin extraction. Administration of endotoxin both as an intravenous bolus and 8-hour continuous rate infusion significantly lowered insulin sensitivity and increased the acute insulin response to glucose. The effects of resting insulin sensitivity on the magnitude of insulin resistance induced by endotoxin administration were subsequently investigated. Resting insulin sensitivity was successfully reduced by oral administration of 20 mg dexamethasone, but levothyroxine sodium failed to improve insulin sensitivity. However, endotoxemia-induced insulin resistance was ameliorated by pretreatment with levothyroxine sodium. Administration of endotoxin alone, oligofructose alone, or endotoxin followed by oligofructose resulted in the development of Obel grade ≥ 2 laminitis in 0/8, 1/8 and 5/8 horses, respectively, indicating a significant association between endotoxemia and laminitis. In summary, results show that endotoxemia exerts an adverse effect on glucose and insulin dynamics and plays an important role in the development of laminitis in horses. Pretreatment with levothyroxine sodium prevented insulin resistance associated with endotoxemia, so this treatment warrants further investigation.

Keywords: horse, insulin resistance, endotoxemia, laminitis

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AIRg – acute insulin response to glucose

APS – adapter protein associated protein substrate

BM – basement membrane

BMI – body mass index

bwt – bodyweight

CAP – Cbl associated protein

CD14 – cluster-of-differentiation antigen 14

CGIT – combined glucose-insulin test

CHO – carbohydrate

COOH – carboxy

COX – cyclo-oxygenase-

C-peptide – connecting peptide

CRI – continuous rate infusion

DEX – dexamethasone

DI – disposition index

E – expected

EHC – euglycemic hyperinsulinemic clamp

EMS – Equine Metabolic Syndrome

ENaC – epithelial sodium channel

ET-1 – endothelin -1

FFA – free fatty acid

FSIGTT – frequently sampled intravenous glucose tolerance test

GLUT – glucose transporter

GSK3 – glycogen synthase kinase-3

HD – hemidesmosome

HE – human equivalent

I – steady state insulin concentration

IGF – insulin-like growth factor

IL – interleukin

ir – immunoreactive

IR – insulin resistance

IRS – insulin receptor substrate

JNK – C-Jun NH_2-terminal kinase

LBP – lipopolysaccharide-binding protein

LPL – lipoprotein lipase

LPS – lipopolysaccharide

L-T_4 – levothyroxine sodium

M – whole-body glucose uptake

M/I – whole body glucose uptake to steady state insulin concentration ratio

MAPK – mitogen activated protein kinase

MIRG – modified insulin-to-glucose ratio

MMP – matrix metalloproteinase

NDF – neutral detergent fiber

NF-κB – nuclear factor κB

NH2 – amino

NO – nitric oxide

NSC – non structural carbohydrate

O – observed

O/E – ratio of observed and expected

OF – oligofructose

PCV – packed cell volume

PDE3B – cyclic AMP phosphodiesterase-3-B

PI3 – phosphatidylinositol 3

PIP_3 – phosphatidylinositol (3,4,5) trisphosphate

PKA – protein kinase A

PKB – protein kinase B

PKC – protein kinase C

PMS – prelaminitic metabolic syndrome

PPID – pituitary pars intermedia dysfunction

PTP 1b – protein tyrosine phosphatase 1b

QUICKI – quantitative insulin sensitivity check index

RIA – radioimmunoassay

RISQI – reciprocal of the square root of insulin

Sg – glucose effectiveness

SGLT – sodium-glucose transporter

SI – insulin sensitivity

SLC2 – solute carrier family 2

TG – triglyceride

TLR - Toll-like receptors

TNFα – tumor necrosis factor α

Type 2 DM – type 2 diabetes mellitus

WBC – white blood cell count

CHAPTER 1

Literature review

1.1 Carbohydrate metabolism – the role of insulin

1.1.1 Carbohydrate digestion:

Carbohydrate (**CHO**) provides the majority of dietary energy for horses, with cereal grains, grain by products, and forage being the primary sources (Geor 2006). Generally, the carbohydrate content of the feed is divided into nonstructural carbohydrates (**NSC**) and neutral detergent fiber (**NDF**; Hoffman *et al.* 2001). The NSC content can be calculated using the following equation:

NSC = 100 - (water + ash + fat + crude protein + NDF)

Alternatively, NSC content can be divided into ethanol- and water-soluble CHOs, with the former containing mono- and di-saccharides and some low molecular weight oligosaccharides, and the latter comprising mostly fructans and some high molecular weight oligosaccharides (Virgona and Barlow 1991). Nevertheless, these groups relate better to ruminant than equine digestive physiology. Based on the primary site of digestion in the equine gastrointestinal tract Hoffman *et al.* (2001) divided the dietary CHO content into three groups: hydrolysable, rapidly fermented, and slowly fermented CHOs. Hydrolysable CHOs, including hexoses, disaccharides, oligosaccharides, and non-resistant starches are digested in the small intestine yielding glucose. If hydrolysable CHOs are delivered to the hindgut, they undergo rapid fermentation. Digestion of slowly and rapidly fermented CHOs occurs in the equine hind gut by enzymes secreted by resident microbes. Rapidly fermented CHOs, comprising resistant starches of galacto- and fructo-oligosaccharides, are readily fermented in the large intestine. Hemicellulose, cellulose and ligno-cellulose form the fraction of slowly fermented CHOs which primarily produce the volatile fatty acid acetate when fermented in the large intestine (Geor 2006; Hoffman *et al.* 2001).

Digestion of the hydrolysable CHO content of the feed is catalyzed by enzymes either acting within the lumen of the gastrointestinal tract or on the surface of mucosal epithelial cells (Herdt 2002). During the luminal phase of digestion, polysaccharides leaving the stomach are further metabolized by pancreatic α-amylase in the duodenum and proximal jejunum. Intraluminal digestion of carbohydrates yields polysaccharides of intermediate chain length, known as dextrines, which are then further broken down into maltose and maltotriose. Intraluminal digestion of amylopectin yields α-limit-dextrines containing α[1-6] linkages that are not hydrolyzed by α-amylase (Herdt 2002). During the membraneous phase of digestion, substrate-specific enzymes bound to the brush border of the enterocytes hydrolyze di- and tri-saccharides into monosaccharides (glucose, galactose, and fructose). Enteral absorption of monosaccharides occurs in two steps via secondary active transport. The first step involves the sodium-glucose transporter (SGLT) 1, which facilitates glucose uptake on the luminal side of the enterocyte (Bell et al. 1993). The second step occurs at the basolateral membrane where glucose exits the enterocyte through glucose transporter (GLUT) 2 proteins (Gould and Bell 1990). The abundance of SGLT1 in the equine small intestine is highest in the duodenum and then decreases towards the ileum, indicating that the majority of glucose absorption occurs in the proximal to mid small intestine (Shirazi-Beechey 2008). Horses on a concentrate diet have a 2 – 5 fold increase in SGLT1 protein expression in the mid and distal small intestine, compared to horses kept on pasture, which enhances their ability to absorb monosaccharides (Shirazi-Beechey 2008).

Fructans are a group of fructose oligosaccharides which are produced as storage CHO in plants (Bailey *et al.* 2007). Their general structure consists of a glucose molecule linked to varying numbers of fructose molecules (van Eps and Pollitt 2006). Degradation of fructans entering the equine gastrointestinal tract starts with microbial fermentation and acid hydrolysis within the stomach and small intestine, however, it is likely that the bulk of fructans reach the hindgut where they undergo microbial fermentation (Coenen *et al.* 2006; Longland and Byrd 2006). Absorption of fructose in the small intestine occurs via facilitated diffusion primarily through GLUT5 molecules expressed on the brush border membrane of the villus enterocytes (Merediz *et al.* 2004).

Transport of fructose out of the enterocytes across the basolateral membrane occurs via the facilitative transporter GLUT2 (Drozdowski *et al.* 2004). Absorbed monosaccharides then enter the portal system and are transported to the liver (Fonyo 2003).

Degradation of dietary pectin, cellulose, and hemicellulose is mediated by anaerobic bacteria, protozoa, and fungi present in the equine hindgut (Shirazi-Beechey 2008). Anaerobic metabolism of these fermented CHOs through the Embden-Meyerhoff pathway leads to the formation of pyruvate and subsequently the volatile fatty acids, acetate, propionate, and butyrate, as well as the gases carbon dioxide (CO_2), methane (CH_4), and hydrogen (Shirazi-Beechey 2008). Under normal circumstances only a small amount of lactate is formed. Volatile fatty acids are absorbed from the large intestine through a monocarboxylate/H^+ symporter, and supply 45 to 82 % of the absorbed energy depending on the composition of the diet (Shirazi-Beechey 2008; Vermorel *et al.* 1997).

Excessive intake of hydrolysable and/or rapidly fermentable CHO may result in their delivery to the hindgut where they undergo rapid bacterial fermentation (Longland and Byrd 2006). The presence of large amounts of starch and fructan in the hindgut facilitates the proliferation of streptococci and lactic acid-producing bacteria (Elliott and Bailey 2006; Milinovich *et al.* 2007). Decreased intraluminal pH, associated with increased lactic acid production, not only triggers the death of resident Gram-negative bacteria but also increases the permeability of the wall of the large intestine (Garner *et al.* 1978; Weiss *et al.* 2000). Due to this altered permeability of the large intestine, endotoxins released upon the death of Gram-negative bacteria, as well as biologically active amines present in the intestinal lumen, enter the systemic circulation and may contribute to the development of laminitis (Elliott and Bailey 2006).

1.1.2 Insulin and insulin secretion:

The role of the pancreas in the development of diabetes was first demonstrated by German scientists Minkowski and Von Mering in 1889 (Wilcox 2005). Three decades later in 1921, insulin was isolated and experiments were begun in dogs (Wilcox 2005). In their studies, Banting, Best, and McLeod successfully lowered blood glucose

concentrations in dogs that had been rendered diabetic by pancreatectomy by administering them chilled saline extracts of pancreas. Successful human experiments were completed in 1922, earning Banting and McLeod the Nobel Prize in 1923 (Wilcox 2005).

Insulin is a protein hormone coded on the short arm of chromosome 11 in humans, and is produced by the β-cells of the islets of Langerhans within the pancreas (Wilcox 2005). Insulin is produced as preproinsulin in the ribosomes of the rough endoplasmic reticulum. Proinsulin is formed following the cleavage of a signal sequence responsible for the intracellular transport of the molecule. Secretory vesicles transfer proinsulin into the Golgi apparatus. As immature vesicles form within the Golgi, a peptide chain referred to as connecting-peptide (**C-peptide**) is removed from the proinsulin molecule, transforming it into insulin. Thus, serum C-peptide concentrations reflect insulin secretion, and unlike insulin, C-peptide undergoes very little first-pass clearance by the liver after secretion from the pancreas (Johnson *et al.* 2005). The structure of insulin consists of two peptide chains connected by disulfide bonds. Six of these insulin molecules complex with zinc to form insoluble hexamers within mature granules (Wilcox 2005).

Insulin secretion is modulated by blood concentration of various nutrients, most importantly glucose (Wilcox 2005). Glucose enters the β-cells via GLUT2 proteins, which have a low glucose affinity at physiological blood glucose levels (Gould and Bell 1990). Once inside the cell, glucose is phosphorylated by the enzyme *glucokinase* to glucose-6-phosphate, which is subsequently oxidized to generate ATP. An increased intracellular ATP/ADP ratio then results in the closure of ATP-sensitive potassium channels, causing membrane depolarization, which in turn opens voltage-dependent calcium channels. Insulin release is stimulated by this increase in intracellular Ca^{2+} concentration (Bratanova-Tochkova *et al.* 2002; Wilcox 2005).

Glucose-mediated insulin secretion is augmented by both ATP-sensitive potassium channel-independent calcium-dependent and ATP-sensitive potassium channel-independent calcium-independent pathways. Enhanced insulin secretion also occurs in response to activation of phospholipases and protein kinase C (**PKC**), triggered

by acetylcholine. Other mediators that facilitate insulin release include vasoactive intestinal peptide and glucose-dependent insulinotropic polypeptide. These hormones act through the G-protein coupled mechanisms to activate adenyl cyclase and protein kinase A (**PKA**), and thereby potentiate insulin secretion (Bratanova-Tochkova *et al.* 2002). Glucagon-like peptide 1 exerts its effect on insulin secretion by inhibiting ATP-sensitive potassium channels and enhancing currents through voltage-dependent calcium channels via cAMP/PKA dependent pathways, as well as by inhibiting currents through voltage dependent K^+ channels (MacDonald *et al.* 2002). Arginine, a positively charged amino acid that enters the β-cells via cationic amino acid transporters, triggers depolarization of cell membrane and gating of voltage-dependent calcium channels, causing insulin excretion (Bratanova-Tochkova *et al.* 2002; Soria *et al.* 2004). In contrast, somatostatin produced by the δ-cells of the islets of Langerhans and catecholamines acting through α2-adrenoreceptors inhibit insulin release (Wilcox 2005).

In humans, basal insulin secretion accounts for 50% of total daily insulin secretion, and 60% of insulin secreted into the portal vein is subsequently removed by the liver (Wilcox 2005). In the basal state, insulin secretion into the portal vein is characteristically pulsatile due to coordinated secretory bursts from islet cells (Porksen *et al.* 2002). In cases of type 2 diabetes mellitus (**type 2 DM**), pulsatile insulin secretion may be impaired (Porksen *et al.* 2002). Restoration of oscillatory insulin secretion in type 2 DM improves insulin action in liver, muscle, and adipose tissues (Porksen *et al.* 2002). Insulin secretion from the β-cells in response to sustained glucose stimulation is characteristically biphasic, with the first phase occurring promptly after exposure to glucose, followed by a decrease to a nadir, and then a prolonged second phase (Bratanova-Tochkova *et al.* 2002; Wilcox 2005). The first rapid phase of insulin release is attributed to the secretion of readily releasable pool of secretory granules (Bratanova-Tochkova *et al.* 2002; Soria *et al.* 2004). The second phase requires the amplifying action of glucose, increasing the efficacy of Ca^{2+} on exocytosis of insulin granules (Henquin *et al.* 2002), and is preceded by the mobilization of granules from a reserve pool to the readily releasable pool (Bratanova-Tochkova *et al.* 2002).

1.1.3 Insulin signal transduction:

Effects of insulin are mediated by membrane bound insulin receptors. The insulin receptor is a heterotetrameric membrane glycoprotein containing 2 α and 2 β subunits linked together by disulfide bonds (Kido *et al.* 2001). Insulin binding to the extracellular α subunits results in a conformational change that enables ATP binding to the intracellular domain of the β subunits, resulting in autophosphorylation, which in turn triggers its tyrosine kinase activity. In addition to binding insulin, the insulin receptor also binds insulin-like growth factor (**IGF**; Kido *et al.* 2001).

Signal transduction through the insulin receptor can be divided into phosphatidylinositol 3 (**PI3**)-kinase dependent and independent pathways (Chang *et al.* 2004; Kido *et al.* 2001; Watson and Pessin 2001). In the PI3-kinase dependent pathway, the autophosphorylated insulin receptor phosphorylates intracellular substrate proteins known as insulin receptor substrates (**IRS**). There are four closely related members of the IRS family and a more distantly related homolog, Gab-1 (Kido *et al.* 2001). Tyrosine phosphorylation of IRS-1 can be mediated by the receptors of insulin and IGF-1 (Withers and White 2000). Insulin receptor substrate-1 is most abundantly expressed in skeletal muscle and primarily mediates mitogenic effects and insulin-stimulated peripheral glucose uptake (Kido *et al.* 2001; Withers and White 2000). Deletion of IRS-1 in mice leads to significant growth retardation, a twofold increase in β-cell mass, and an 80 percent decrease in the response to insulin in muscle (Burks and White 2001). However, only mild insulin resistance is detected and insulin signaling within the liver remains close to normal (Burks and White 2001). The relatively normal glucose homeostasis exhibited by IRS-1 knockout mice may be due to compensatory hyperinsulinemia and to the near normal response to insulin in the liver, primarily mediated by IRS-2 (Burks and White 2001).

In addition to its important role in the liver, IRS-2 is also important for the normal growth of pancreatic β-cells (Kido *et al.* 2001). Insulin receptor substrate-2 knockout mice show profound insulin resistance primarily affecting the liver, and their β-cell mass is approximately 50% lower compared to healthy controls (Burks and White 2001).

Carbohydrate metabolism is significantly altered in these animals. In IRS-2 knockout mice, the inhibitory effect of insulin on hepatic gluconeogenesis is weak and the glycogen content of the liver is low, but insulin action in skeletal muscle and adipose tissue remains close to normal (Burks and White 2001). Mice deficient in IRS-2 become overtly diabetic by 12 weeks of age, and if untreated, die from dehydration and hyperosmolar coma (Burks and White 2001).

Insulin receptor substrate-3 is located within adipose tissue, β-cells, and the brain, whereas IRS-4 is found in thymus, brain, and kidney (Withers and White 2000). Insulin receptor substrates 3 and 4 appear to play redundant roles in the IRS signaling system (Sesti *et al.* 2001). In adipocytes obtained from IRS-1 knockout mice, approximately 50% of GLUT4 translocation was preserved by a signaling pathway involving IRS-3 (Sesti *et al.* 2001). *In vivo* studies found no major physiologic role of IRS-4 in controlling growth or metabolism in mice (Sesti *et al.* 2001).

The carboxy (**COOH**) terminus of each IRS protein contains a set of tyrosine phosphorylation sites that regulate different downstream signaling proteins. Each site binds and activates specific effector proteins including adapter proteins and enzymes, such as PI3-kinase (Withers and White 2000). The p85 regulatory subunit of PI3-kinase recognizes the phosphorylated insulin receptor substrate molecules and brings the p110 catalytic subunit of the heterodimer enzyme to the vicinity of its physiological substrate, phosphatidylinositol (4,5) bisphosphate (Lizcano and Alessi 2002; Shepherd and Kahn 1999). Phosphatidylinositol (4,5) bisphosphate is subsequently phosphorylated in the D3 position generating phosphatidylinositol (3,4,5) trisphosphate (**PIP_3**; Lizcano and Alessi 2002). A key effector of PIP_3 is protein kinase B (**PKB**), which is also known as Akt (Lizcano and Alessi 2002). PIP_3 mediated recruitment of PKB from the cytosol brings it closer to another protein kinase termed phosphoinositide-dependent protein kinase-1 which contributes to the activation of PKB (Lizcano and Alessi 2002; Watson and Pessin 2001). Protein kinase B phosphorylates and thereby inactivates *glycogen synthase kinase-3* (**GSK3**; Lizcano and Alessi 2002). Inactivation of GSK3 leads to disinhibition of *glycogen synthase*. Insulin-mediated glycogen synthesis is therefore facilitated by the activation of PKB. Protein kinase B also phosphorylates *cyclic AMP phosphodiesterase-*

3-B (**PDE3B**) and the increased activity of this enzyme lowers intracellular cAMP concentrations within adipocytes, which prevents the activation of PKA. In the absence of active PKA, *hormone sensitive lipase* remains inactive, which therefore explains the inhibitory effect of insulin on lipolysis (Salway 2004). Atypical protein kinase C (PKCζ/λ) is also activated by phosphoinositide-dependent protein kinase-1, downstream of PI 3-kinase (Chang *et al.* 2004).

A PI3-kinase independent insulin signaling pathway also exists within cells. This pathway is localized in lipid raft microdomains, which are specialized regions of the plasma membrane (Chang *et al.* 2004). Activation of the insulin receptor in these plasma membrane subdomains leads to the recruitment and phosphorylation of the adaptor protein called associated protein substrate (**APS**), which in turn facilitates the phosphorylation of the proto-oncogene Cbl (Chang *et al.* 2004). Next, the Cbl associated protein (**CAP**) is recruited with Cbl to the insulin receptor:APS complex (Chang *et al.* 2004). At this point, the CAP-Cbl complex dissociates from the insulin receptor:APS complex and interacts with the lipid raft domain protein, flotillin (Chang *et al.* 2004). Tyrosine-phosphorylated Cbl then interacts with CrkII, a protein constitutively associated with C3G, resulting in their translocation to the lipid raft (Chang *et al.* 2004). Upon its translocation to lipid raft, C3G can catalyze the activation of the small G-proteins TC10α and TC10β, which triggers GLUT4 translocation through interaction with a number of potential effector molecules (Chang *et al.* 2004).

Recruitment of PKCζ/λ, an enzyme downstream of PI3-kinase, to lipid rafts in a TC10-dependent manner, represents a point of convergence for the PI3-kinase dependent and PI3-kinase independent pathways (Chang *et al.* 2004).

The mitogen activated protein kinase (**MAPK**; also known as extracellular signal-regulated kinase) cascade is also involved in the insulin signal transduction (Saltiel and Kahn 2001). This pathway involves the tyrosine phosphorylation of IRS proteins which subsequently interact with the adapter protein Grb2, recruiting the Son-of-sevenless exchange protein to the plasma membrane for activation of Ras. After its Son-of-sevenless-mediated activation, Ras operates as a molecular switch stimulating stepwise activation of Raf, mitogen activated protein kinase kinase, and MAPK. Activated MAPK

translocates to the nucleus, where it catalyses the phosphorylation of transcription factors initiating cellular differentiation or proliferation (Saltiel and Kahn 2001). A src-homology-2 domain containing protein called SHC, which is another substrate of the insulin receptor, is also capable of activating the Ras/MAPK pathway through the adapter protein Grb2, independent of IRS-1 (**Figure 1.1;** Bevan 2001).

Shortly after insulin binding, the insulin receptor-insulin complex is internalized into endosomes (Bertacca *et al.* 2007; Bevan 2001). The endocytosis of the activated receptor-insulin complex enables phosphorylation of substrate molecules that are spatially distinct from those accessible at the plasma membrane (Bevan 2001). Acidification of the endosomal lumen results in the dissociation and degradation of insulin from the insulin receptor (Bevan 2001). This loss of the ligand receptor complex attenuates any further insulin receptor rephosphorylation (Bevan 2001). The receptor is recycled back to the plasma membrane (Bertacca *et al.* 2007), while the insulin is degraded by the endosomal acidic insulinase (Bevan 2001). Tyrosine phosphatases also play an important role in termination of the signal generated by insulin by dephosphorylating the insulin receptor and its targets (Kido *et al.* 2001). Mice lacking protein tyrosine phosphatase 1b have significantly enhanced insulin-stimulated whole body glucose disposal. Interestingly, increased insulin sensitivity in these mice was found to be tissue specific, occurring mainly in skeletal muscle (Klaman *et al.* 2000).

The insulin signaling pathway has a negative feed-back loop, which is activated by insulin and involved in the inhibition or termination of the metabolic branch of the insulin receptor pathway (Ye 2007). Inducible Ser/Thr phosphorylation of IRS-1 leads to the inhibition of IRS-1 function and also contributes to the development of insulin resistance (Ye 2007). Several of the serine kinases capable of inhibiting IRS-1 are activated by insulin, including PI3-kinase, MAPK, PKB, and PKC, forming the negative feedback loop in the insulin receptor signaling pathway (Ye 2007).

Insulin-stimulated glucose uptake is mediated by translocation of GLUT4 from the intracellular pool to the plasma membrane (Kido *et al.* 2001). A synaptic vesicle model has been proposed to explain how GLUT4 molecules dock and fuse with the plasma membrane (Kido *et al.* 2001).

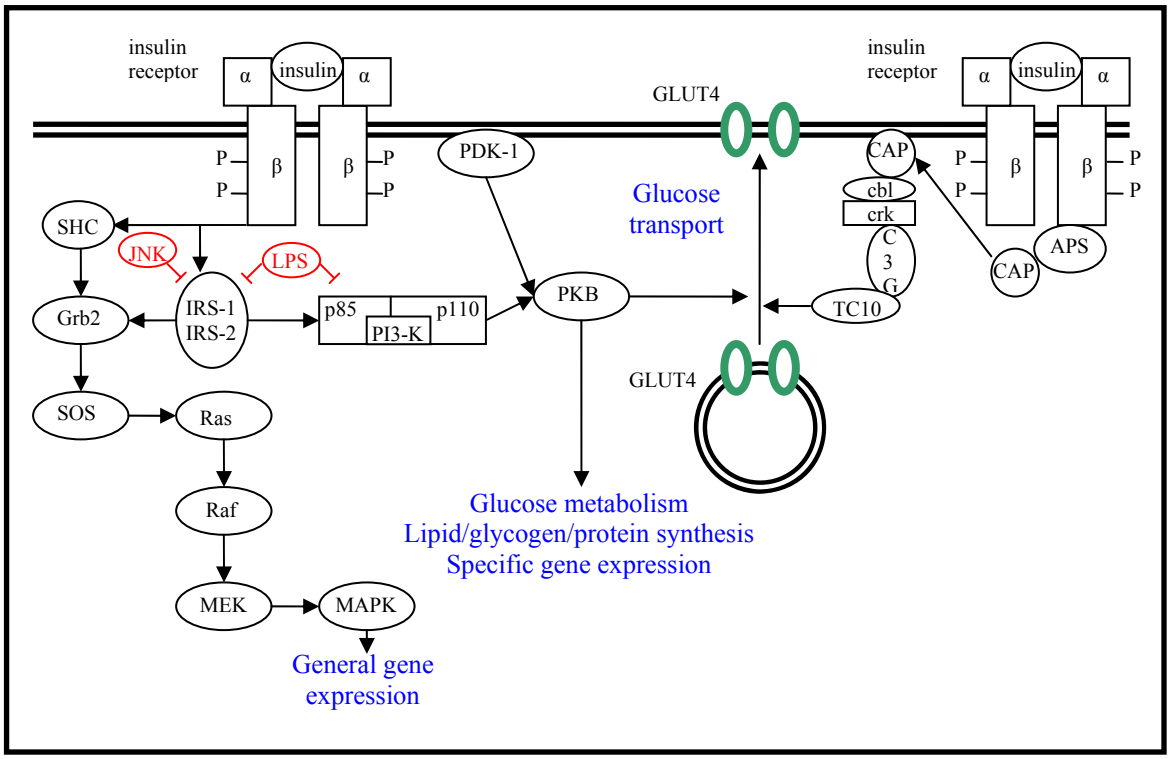

Figure 1.1 – Signal transduction in insulin action. The insulin receptor is a tyrosine kinase which undergoes autophosphorylation after ligand binding and catalyzes phosphorylation of cellular proteins eventually resulting in alterations in glucose transport, gene expression and metabolic pathways.

1.1.4 Glucose transporters:

Since glucose does not diffuse readily through plasma membrane, its entry into cells is carried out by proteins belonging to two distinct molecular families (Shepherd and Kahn 1999). Energy independent transport of glucose is mediated by members of facilitative glucose transporter family of glucose carriers (Gould and Holman 1993). This type of glucose transport is saturable, bidirectional, and stereoselective (Bell *et al.* 1993). The second family is represented by the sodium-linked glucose transporters, which are largely restricted to the kidneys and intestines (Shepherd and Kahn 1999). They utilize the electromechanical Na^{2+} gradient to actively transport glucose and galactose against their concentration gradient (Bell *et al.* 1993).

The current model of GLUT proteins includes 12 membrane-spanning α-helical segments, connected by short, hydrophilic chains of 7 to 14 amino acids, with a large intracellular loop connecting transmembrane segments 6 and 7 (Bell *et al.* 1993). The 12 α-helices are combined to form a central aqueous pore, with up to 8 of the 12 helices contributing to parts of the surface lining of the pore (Manolescu *et al.* 2007). Both the COOH and amino (**NH_2**) terminals of the molecule are located on the intracellular side of the plasma membrane. The NH_2 half of the protein appears to have a role in correctly inserting the molecule into the plasma membrane, while the COOH terminus is involved in sugar transport itself (Bell *et al.* 1993). It is currently thought that glucose transport occurs by a mechanism in which the transporter alternates between two conformational states, with the substrate-binding site facing in either an extracellular or intracellular direction (Bell *et al.* 1993). It is believed that GLUT1 exists in tetramers increasing its catalytic activity 2-8 fold (Bell *et al.* 1993). Recent sequencing of the human genome identified 14 members of the solute carrier family 2 (**SLC2**) genes, which are known to encode GLUT proteins (Manolescu *et al.* 2007). Sequence alignments indicate that the 14 GLUT proteins encoded by these genes can be clustered into three families or subclasses I, II, and III (Manolescu *et al.* 2007).

Glucose transporter 1 is the erythrocyte type glucose transporter. It is an integral membrane protein that can be found in erythrocytes, fetal tissues, kidney, liver, colon,

and brain (Fonyo 2003). This transporter is also expressed in most cultured cells (Gould and Bell 1990; Gould and Holman 1993).

Glucose transporter 2 is preferentially expressed in the liver, on the basolateral membrane surface of kidneys and small intestinal epithelia, and in the insulin-producing β-cells of the pancreas (Bell *et al.* 1993; Gould and Bell 1990). This glucose transporter has a high K_{max} value of 42 mmol/L for glucose (Shepherd and Kahn 1999), providing a rationale for its localization to those tissues that are involved in the net release of glucose during fasting (liver), glucose sensing (β-cells of the pancreas), and transepithelial transport of glucose (kidney and small intestine; Gould and Holman 1993). The high K_{max} of GLUT2 prevents its saturation at physiological glucose concentrations, therefore glucose flux through GLUT2 changes in an almost linear fashion with intracellular/extracellular glucose concentrations (Gould and Holman 1993).

Messenger RNA coding the glucose transporter 3 has been found in most tissues, but is most abundant in kidney, brain, placenta, and platelets (Gould and Bell 1990; Heijnen *et al.* 1997). Using immunocytochemical methods, GLUT3 has been localized to the α-granule membrane in resting platelets, and it is from this location that it moves to the cell surface following thrombin stimulation (Heijnen *et al.* 1997). Elevated GLUT3 mRNA levels have also been detected in the hippocampus of diabetic rats, likely representing a compensatory mechanism to increase glucose utilization during diabetes (Reagan *et al.* 1999).

Adipose tissue and muscle possess a unique, insulin-sensitive glucose transporter designated as GLUT4 (Gould and Holman 1993). Immunoblot analysis has been used to characterize GLUT4 protein in equine skeletal and cardiac muscle suggesting a mean theoretical value of 53.3 ± 0.5 kDa for equine GLUT4 (Lacombe *et al.* 2003). Further investigation revealed that the molecule contains 509 amino acids, which are coded by a DNA segment measuring 1,623 base pairs in length, including 1530 base pairs of open reading frame (Jose-Cunilleras *et al.* 2005). Glucose transporter 4 protein expression was demonstrated to be higher in equine skeletal muscle fiber type 2B than in types 1 or 2A using indirect immunofluorescence assays (van Dam *et al.* 2004). Insulin facilitates translocation of GLUT4 from the intracellular pools to the cell membrane in adipose and

muscle tissue, thereby increasing glucose uptake across the plasma membrane within minutes (Gould and Bell 1990). Interestingly, insulin also causes a 3-fold increase in the number of GLUT1 molecules on the surface of adipocytes (Gould and Bell 1990). Studies examining rats made diabetic by streptozocin treatment have shown significant reduction in GLUT4 mRNA within muscle tissue and adipocytes, suggesting that insulin not only plays a role in the translocation of GLUT4 from the intracellular pools to the cell membrane, but also influences GLUT4 synthesis (Garvey *et al.* 1989).

In skeletal muscle, both insulin stimulation and increased muscle activity cause translocation of GLUT4 from the intracellular pool to the cell surface, with the exercise-sensitive pool associated with an endosomal transferrin receptor (van Dam *et al.* 2004). Indeed, two distinct pools of GLUT4 molecules have been identified in rat skeletal muscle (Aledo *et al.* 1997). One fraction is highly enriched with transferrin receptor and annexin II, characteristic for the early endosome compartment, whereas the other fraction is proven to be insulin sensitive (Aledo *et al.* 1997). In rats, exercise not only enhances GLUT4 translocation but also influences GLUT4 expression at both the transcriptional and translational level (Dohm 2002). Skeletal muscle GLUT4 gene expression was also found to be increased in horses following exercise (Jose-Cunilleras *et al.* 2005; Lacombe *et al.* 2003). Conversely, McCutcheon *et al*. (2002) demonstrated no significant change in equine skeletal muscle GLUT4 content in samples collected from horses within 2 minutes after cessation of exercise. Ingestion of starch-rich meals did not influence GLUT4 gene expression in horses compared with the consumption of isocaloric fiber-rich meals or feed withdrawal following exercise (Jose-Cunilleras *et al.* 2005). Administration of dexamethasone also failed to induce a significant change in equine skeletal muscle GLUT4 protein content (Tiley *et al.* 2008).

Glucose transporter 5 is expressed at high levels in human small intestine(Gould and Bell 1990), and plays an important role in fructose transport (Manolescu *et al.* 2007; Merediz *et al.* 2004). This protein has a 42%, 40%, 39% and 42% homology with glucose transporters 1, 2, 3, and 4, respectively (Gould and Bell 1990). In the equine small intestine, GLUT5 is expressed on the brush-border membrane of the villous enterocytes with its levels decreasing from the duodenum towards the ileum (Merediz *et*

al. 2004). The presence of GLUT5 in the equine small intestine suggests the capacity of equine small intestine to absorb fructose. Kinetic studies suggest that the GLUT5 molecules are low affinity and high capacity transporters (Merediz *et al.* 2004).

Glucose transporter 6 mRNA is principally expressed in the spleen, brain, and peripheral leukocytes and appears to be a low affinity facilitator (Joost and Thorens 2001). Both glucose and fructose are proposed substrates for glucose transporter 7, which is primarily expressed in the apical membrane of the enterocytes of the small intestine and colon, although its mRNA has been detected in the testes and prostate as well (Cheeseman 2008).

Glucose transporter 8 (formerly designated as GLUTX1), like GLUT6, belongs to the class III sugar transport facilitators because it contains the characteristic glycosylation motif in loop 9 (Joost and Thorens 2001). It is expressed in the heart, skeletal muscle, brain, spleen, prostate and intestine, with the highest level of expression in the testis (Kim and Moley 2007). Expression of GLUT8 has also been demonstrated to play an important role in blastocyst survival (Kim and Moley 2007).

Glucose transporter 9 is a high affinity glucose transporter which also transports fructose (Kim and Moley 2007). Human GLUT9 is expressed in the kidney, liver, heart and adrenal gland. In mice, GLUT9 has two splice variants: GLUT9a, which is a long form that has 12 transmembrane segments, and GLUT9b that has a deletion resulting in the loss of 2 transmembrane spanning domains (Kim and Moley 2007).

Both GLUT10 and GLUT11 were identified using homology-based searches of expressed sequence tag databases (Doege *et al.* 2001; McVie-Wylie *et al.* 2001). Northern blot analysis revealed that GLUT10 is expressed at the highest levels in the liver and pancreas, and a lower level of expression was detected in heart, brain, placenta, lung, skeletal muscle and kidneys (McVie-Wylie *et al.* 2001). Glucose transporter 11 has also been detected exclusively within heart and skeletal muscle in humans. It appears to transfer fructose with comparable affinity for glucose, which is consistent with its sequence similarity to GLUT5 (Doege *et al.* 2001).

A novel glucose transporter-like protein, GLUT12, was found in MCF-7 breast cancer cells (Rogers *et al.* 2002). Immunoblotting also demonstrated its presence in

adipose tissue, skeletal muscle, and small intestine (Rogers *et al.* 2002). It was located inside the cell in the perinuclear region in the absence of insulin, suggesting that it may play a role in insulin-mediated glucose uptake (Rogers *et al.* 2002).

The only glucose transporter demonstrated to use a proton gradient to energize the movement of substrate is the GLUT13 (H+/myoinositol transporter), which is a proton coupled myo-inositol transporter (Manolescu *et al.* 2007). Glucose transporter 14 appears to have resulted from a gene duplication of GLUT3 (Manolescu *et al.* 2007; Wu and Freeze 2002). It has two alternatively spliced forms, which are both specifically expressed in the testes. The mRNA level of GLUT14 in testicular tissue is approximately four times higher than that of GLUT3 (Wu and Freeze 2002).

1.1.5 Physiologic role of insulin:

Insulin is of paramount importance in controlling metabolism by regulating cellular energy supply, macronutrient homeostasis, and anabolic processes (Wilcox 2005). At the cellular level, insulin influences carbohydrate, lipid, and amino acid metabolism as well as mRNA transcription and translation. Insulin exerts its action in concert with other counter regulatory hormones that influence metabolism, including glucagon, glucocorticoids, and catecholamines. Insulin-mediated growth hormone secretion also helps to prevent insulin-induced hypoglycemia (Wilcox 2005).

Insulin profoundly influences glucose uptake into skeletal muscle by facilitating GLUT4 translocation to the sarcolemma from intracellular pools (Shepherd and Kahn 1999). Muscle tissue accounts for 60 to 70 % of insulin-mediated whole body glucose disposal (Fonyo 2003). In the fed state, insulin activates *glycogen synthase* and promotes glycogen synthesis, while also suppressing catabolic processes and maintaining muscle mass (Fonyo 2003; Wilcox 2005). In skeletal muscle, GLUT4 translocation into the plasmalemma is also facilitated by exercise (van Dam *et al.* 2004).

Basal glucose uptake of adipose tissue is mediated via GLUT1, whereas insulin-mediated glucose transport occurs via both GLUT1 and GLUT4 molecules (Tafuri 1996). In rat adipose tissue, insulin produces an approximately 20- to 30-fold increase in glucose

uptake, whereas in human adipocytes only a 2- to 4-fold increase is demonstrated (Gould and Holman 1993). It has been estimated that adipose tissue accounts for 10 % of insulin-stimulated whole body glucose disposal (Wilcox 2005). Insulin also increases fat stores by promoting lipid synthesis and inhibiting lipolysis (Saltiel and Kahn 2001). Insulin-mediated glucose uptake and the presence of free fatty acids (**FFA**) are both necessary for triglyceride (**TG**) synthesis in adipose tissue. The most available source of FFAs is triglyceride carried within lipoproteins that is metabolized by *lipoprotein lipase* (**LPL**) bound to the endothelial surface of capillaries (Adam 2004). Insulin stimulates gene transcription and synthesis of LPL in adipocytes (Salway 2004). Insulin also enhances the activity of enzymes involved in lipogenesis within adipose tissues, including *pyruvate dehydrogenase*, *fatty acid synthase*, and *acetyl-CoA-carboxylase* (Saltiel and Kahn 2001). Protein kinase A-mediated activation of *hormone sensitive lipase,* and therefore lipolysis, is inhibited by insulin (Fonyo 2003; Saltiel and Kahn 2001; Wilcox 2005). This occurs through insulin-mediated activation of PDE3B and lowers intracellular cAMP levels, which prevents the activation of PKA (Salway 2004).

One of the most important effects of insulin is to control glucose homeostasis by regulating metabolic processes in the liver (Guyton and Hall 2006). Although hepatocellular glucose transport occurs via insulin-independent GLUT2 molecules (Wilcox 2005) insulin still increases hepatic glucose uptake by facilitating intracellular glucose trapping through *glucokinase*-mediated phosphorylation (Guyton and Hall 2006). Insulin inhibits the production and release of glucose by blocking gluconeogenesis and glycogenolysis within the liver (Saltiel and Kahn 2001). Transcription of gluconeogenic genes encoding *phosphoenolpyruvate-carboxykinase*, *fructose 1,6-bisphosphatase,* and *glucose 6-phosphatase* are reduced under the influence of insulin (Salway 2004). Insulin also inhibits glycogenolysis by interfering with the PKA-mediated activation of *glycogen phosphorylase* (Salway 2004). Insulin generated signals are also important in the activation of the enzyme *protein phosphatase-1G*, which is capable of dephosphorylating, and thereby activating, the enzyme *glycogen synthase*. This facilitates glycogen synthesis (Salway 2004).

Insulin administration has also been shown to stimulate glycolysis by increasing the rate of *pyruvate kinase* synthesis in the liver (Miyanaga *et al.* 1982). The ketone bodies β-hydroxy-butyrate and acetic acid are synthesized from FFAs in the liver. Insulin prevents ketogenesis by lowering the amount of available FFAs (Fonyo 2003). Mitogenic effects of insulin and growth hormone are mediated by hepatic production of IGFs (Wilcox 2005).

Insulin deficiency is permissive of protein catabolism, releasing amino acids which are subsequently utilized for gluconeogenesis (Wilcox 2005). Insulin is anabolic in conjunction with IGF-1 in the presence of amino acids by modulating the phosphorylation status of intermediates in the protein synthetic pathway (Wilcox 2005).

In addition to its essential metabolic actions, insulin has important vascular actions as well. It stimulates the production of nitric oxide (**NO**) via the PI3-kinase-dependent pathway in endothelial cells leading to vasodilatation and increased blood flow (Kim *et al.* 2006). Insulin also facilitates the secretion of the vasoconstrictor endothelin-1 (**ET-1**) from endothelia (Kim *et al.* 2006). Effects of insulin on the vascular system are discussed in greater detail below.

Although neuronal glucose uptake is not dependent upon insulin, the hormone acts as a neuropeptide, and insulin is involved in satiety and the regulation of appetite (Wilcox 2005). In the anterior pituitary gland, insulin stimulates growth hormone secretion, which in turn promotes IGF-1 production by the liver (Wilcox 2005).

1.1.6 Insulin resistance:

Insulin resistance (**IR**) is defined as a state in which normal concentrations of insulin fail to elicit the expected physiological response (Hoffman *et al.* 2003; Kahn 1978). Insulin sensitivity is influenced by certain physiological and pathological factors such as diet. Consumption of a diet rich in sugar and starch lowered insulin sensitivity in Thoroughbred geldings, compared to a diet containing primarily fat and fiber (Hoffman *et al.* 2003). In men, glucose, fatty acids, and dietary fiber are all known to upregulate

glucagon-like peptide 1 and by doing so improve insulin sensitivity (MacDonald *et al.* 2002).

Pregnancy is another physiologic factor associated with insulin resistance (Butte 2000). In women, insulin action is 50-70% lower than normal during late pregnancy, and both basal and 24h mean insulin concentrations show a two-fold increase (Butte 2000). Postprandial glucose concentration, basal endogenous hepatic glucose production, and total gluconeogenesis are also increased during late pregnancy. These alterations are most likely due to the rising concentrations of prolactin, cortisol, and glucagon that serve to ensure continuous delivery of nutrients to the growing fetus (Butte 2000). Hyperinsulinemia, enhanced β-cell sensitivity to endogenous and exogenous glucose, increased degradation of insulin and decreased insulin sensitivity have also been demonstrated in pregnant mares (Fowden *et al.* 1984).

Stressors activating the hypothalamus-pituitary-adrenal axis cause increased secretion of counter-regulatory hormones such as catecholamines and glucocorticoids, resulting in decreased insulin sensitivity (Rosmond 2005). Conditions associated with elevated glucocorticoid levels are characterized by increased glucose production in the liver and decreased peripheral glucose transport and utilization, leading to IR (Ruzzin *et al.* 2005; Saad *et al.* 1993). A study performed on rat skeletal muscle revealed that dexamethasone treatment lowered insulin-stimulated glucose uptake, glycogen synthesis, and glycogen synthase fractional activity (Ruzzin *et al.* 2005). It was also demonstrated that chronic dexamethasone treatment impairs insulin-mediated PKB activation, so the enzyme is unable to inactivate GSK3 via phosphorylation. *Glycogen synthase kinase-3*, in its active form, phosphorylates and thereby inactivates *glycogen synthase*, interfering with glycogen synthesis (Ruzzin *et al.* 2005). The authors speculated that the decreased PKB activity is due to a dexamethasone-mediated increase in the expression of the p85α subunit of the upstream enzyme PI3-kinase, which may act as a competitive antagonist of the p85α-p110 heterodimer in IRS-1 binding (Ruzzin *et al.* 2005).

Another study identified lower activity of PI3-kinase in rat liver and skeletal muscle following dexamethasone administration (Saad *et al.* 1993). Pretreatment with glucocorticoids decreased insulin-stimulated glucose uptake in rat adipocytes, but uptake

was restored by preincubation with conventional PKC inhibitors (Kawai *et al.* 2002). These results indicate that PKC plays an important role in glucocorticoid-induced IR. Using the minimal model analysis of the frequently sampled intravenous glucose tolerance test (**FSIGTT**), Tiley *et al.* (2007) established that insulin sensitivity decreases in horses receiving 0.08 mg/kg bodyweight (**bwt**) dexamethasone intravenously every other day for 3 weeks.

Epinephrine was demonstrated to raise plasma glucose concentrations in humans by reducing peripheral glucose clearance and increasing hepatic glucose production via gluconeogenesis or glycogenolysis (Nonogaki 2000; Vicini *et al.* 2002). Epinephrine, but not norepinephrine, also inhibits insulin-stimulated glucose uptake in skeletal muscle (Nonogaki 2000). An *in vivo* study of human subjects revealed that elevated epinephrine concentrations significantly delay the inhibitory action of insulin on hepatic glucose production without influencing the maximal inhibitory effect of insulin (Vicini *et al.* 2002). Similarly, physiologic epinephrine concentrations were demonstrated to reduce muscle glucose uptake activated by a moderate, but not high, physiological insulin concentration through the suppression of IRS-1 associated PI3-kinase activation (Hunt and Ivy 2002). Endocrinopathies, such as pheochromocytomas and pituitary pars intermedia dysfunction (**PPID**) have also been associated with IR in horses (Cefalu 2001; Garcia and Beech 1986).

There are several pathologic processes associated with reduced insulin sensitivity. In humans, obesity and IR are significantly related (Garca-Estevez *et al.* 2004). The degree of IR increases along with increasing body mass index (**BMI**; Garca-Estevez *et al.* 2004). However, the decrease in insulin sensitivity was blunted above a BMI value of 30 $kg \times m^{-2}$ (Garca-Estevez *et al.* 2004; Kern *et al.* 2001). Chronic, low grade inflammation associated with obesity is an important factor in the development of decreased insulin sensitivity (de Luca and Olefsky 2008). Increased tumor necrosis factor α (**TNFα**) secretion by adipocytes and higher circulating plasma interleukin (**IL**)-6 levels were demonstrated in obese subjects (Kern *et al.* 2001). Both of these factors were significantly associated with IR, and therefore implicated in its development (Kern *et al.* 2001). Tumor necrosis factor α lowers insulin sensitivity by enhancing lipolysis

(Trayhurn and Wood 2005), by decreasing LPL activity (Kern *et al.* 1995), and by inhibiting insulin receptor signaling within adipocytes (Borst 2007). Impaired insulin signaling and increased hepatic glucose production have been demonstrated in association with IL-6 (Borst 2007). Resistin, an adipokine expressed predominantly in adipose tissue, provides another link between obesity and IR (Shuldiner *et al.* 2001). In obese, insulin-resistant mice, injection of antibodies raised against resistin decreased blood glucose concentrations and improved insulin sensitivity (Shuldiner *et al.* 2001). Leptin, a different adipokine secreted by adipocytes, exerts its effect through activation of leptin receptors in the hypothalamus (Borst 2007). The primary role of leptin is to decrease food intake and increase energy expenditure through uncoupling proteins, present in mitochondria. Leptin is also an insulin sensitizing hormone, therefore reduced leptin responsiveness associated with obesity is likely to contribute to the development of insulin resistance (Borst 2007). Adiponectin, a hormone produced exclusively by adipocytes also enhances insulin sensitivity, but in contrast to leptin, its concentration is low in obese people and increases after weight loss (Borst 2007).

Elevated FFA concentrations contribute to IR in people by hampering glycogen synthesis in skeletal muscle via inhibition of transmembrane glucose transport (Shulman 2004). It was demonstrated that the FFA-mediated decrease in transmembrane glucose transport is associated with abolished insulin-stimulated, IRS-1-associated PI3-kinase activity (Shulman 2004). It is hypothesized that increased amounts of fatty acid-derived metabolites (e.g. fatty acyl-CoA, diacylglycerol) activate PKC leading to inhibition of IRS-1 by Ser/Thr phosphorylation (Shulman 2004). Additionally, elevated plasma FFA concentrations have also been shown to cause IR by increasing insulin concentrations within the blood (Ye 2007). There are three proposed mechanisms explaining FFA-mediated beta cell activation. The G-protein coupled receptor 40 is a molecule that is abundantly expressed on the surface of pancreatic beta cells and is capable of amplifying the glucose signal, which enhances insulin secretion. This occurs when G-protein coupled receptor 40 is activated by its ligand, long-chain fatty acids. Derivates of FFAs such as long chain acyl-CoA and malonyl CoA may directly promote the beta cell response as well. Finally, FFA activates G-protein coupled receptor 120 in the intestinal

tract, stimulating glucagon-like peptide 1 secretion which in turn enhances insulin release (Ye 2007). Chronic hyperinsulinemia induces IR primarily involving the glycogen synthetic pathway and by increasing lipogenesis (Koopmans *et al.* 1999). Indeed, induction of chronic, physiologic hyperinsulinemia in rats resulted in an impairment of insulin's ability to suppress plasma FFA levels, as well as reduced whole body insulin mediated glucose uptake by 38% (Koopmans *et al.* 1999).

Defective muscle glycogen synthesis is also important in the development of IR (Shulman 2004). In skeletal muscle, control of glucose uptake is distributed among three steps: delivery, transmembrane transport, and phosphorylation of glucose (Wasserman and Ayala 2005). Exercise facilitates muscle glucose uptake by modulating all three steps of the control mechanisms. Exercise and insulin not only stimulate GLUT4 translocation to the sarcolemma, but also cause marked hyperemia and capillary recruitment in muscle, thereby facilitating glucose delivery. Additionally, both insulin and exercise have been suggested to increase the rate of intracellular glucose phosphorylation by causing *hexokinase* to bind to mitochondria, providing it easy access to ATP, a substrate necessary for the glucose-6-phosphate synthesis. Disturbances affecting any of these three steps of muscle glucose uptake could result in the development of IR (Wasserman and Ayala 2005). Using magnetic resonance spectroscopy, Shulman *et al*. (2004), demonstrated that the insulin-stimulated change in intracellular concentration of glucose-6-phosphate, an intermediate metabolite between glucose transport and glycogen synthesis, is blunted in human patients with type 2 DM. This observation suggests that either decreased glucose transport or decreased *hexokinase* activity is the cause of IR within the skeletal muscle of these patients. Further experiments performed in humans with type 2 DM have revealed that intracellular glucose concentrations are far lower than would be expected if *hexokinase* were the primary rate-limiting enzymatic step for insulin-mediated glycogen synthesis (Shulman 2004). Therefore, it appears that impaired insulin-mediated glucose uptake plays the central role in the defective muscle glycogen synthesis in cases of type 2 DM.

1.1.7 IR in Horses

Horses and ponies are affected by IR with obesity being one of the most important predisposing factors for this condition (Frank *et al.* 2006; Hoffman *et al.* 2003; Jeffcott *et al.* 1986). Oral glucose tolerance tests revealed impaired glucose tolerance and hyperinsulinemia in ponies that were obese or had previously suffered from laminitis (Jeffcott *et al.* 1986). The presence of IR was confirmed by the failure of exogenous insulin to lower serum glucose concentrations when these animals were compared with healthy nonobese ponies and Standardbred horses (Jeffcott *et al.* 1986). Similarly, a study by Powell *et al.* (2002) demonstrated that horses with a body condition score <5 were less insulin resistant than horses with a body condition score >8. Decreased insulin sensitivity was also detected in obese Thoroughbred geldings when compared to their nonobese counterparts (Hoffman *et al.* 2003). Resting insulin concentrations and leptin levels, along with plasma VLDL, HDL-cholesterol, and nonesterified fatty acid concentrations were significantly elevated in obese horses with IR compared to their healthy counterparts (Frank *et al.* 2006). In this study, insulin resistance was defined as maintenance of plasma glucose concentrations above the pre-injection concentration for ≥ 45 minutes during a combined glucose-insulin test (Frank *et al.* 2006). The same study revealed that neck circumference, as well as resting insulin and leptin concentrations, were helpful in identifying horses with IR (Frank *et al.* 2006). In another study of 60 horses, increased obesity was associated with elevated plasma concentrations of TNFα protein and enhanced blood mRNA expression of IL-1 and TNFα (Vick *et al.* 2007). Furthermore, body condition score and plasma TNFα concentrations were inversely related to insulin sensitivity. These findings suggest that increased concentrations of inflammatory cytokines contribute to the development of IR in obese horses, which is consistent with findings in other species (Kern *et al.* 1995; Trayhurn and Wood 2005; Vick *et al.* 2007). Negative correlation between percent body fat and plasma adiponectin concentrations, demonstrated in mature mares and weanling fillies (Kearns *et al.* 2006) may provide further explanation for the decreased insulin sensitivity registered in obese horses.

Insulin sensitivity in horses is profoundly influenced by diet, in part by affecting body weight and body condition score (Vick *et al.* 2006). In obese mares, feed restriction resulted in a significant decrease in body weight, percentage body fat and circulating concentrations of leptin and insulin, accompanied by a significantly increased insulin sensitivity, relative to obese mares fed *ad libitum* (Vick *et al.* 2006). Insulin sensitivity was also found to be significantly lower in Thoroughbred geldings fed a diet rich in sugar and starch when compared to geldings eating forage and a supplement rich in fat and fiber (Hoffman *et al.* 2003). Consumption of pasture grass containing large amounts of NSC is associated with high glycemic and insulinemic responses, likely contributing to the exacerbation of IR (Longland and Byrd 2006). In humans, increased consumption of fructans has been associated with enhanced *de novo* lipogenesis and triglyceride synthesis in the liver, which contributes to the development of IR (Basciano *et al.* 2005). Serum insulin concentration measurements were used to investigate the effects of fructan supplementation on insulin sensitivity in ponies (Bailey *et al.* 2007). After the addition of inulin to a basal hay diet, serum insulin concentrations were significantly increased in laminitis-predisposed ponies, and a similar, albeit not significant, increases in insulin levels were also demonstrated in healthy ponies, suggesting exacerbation of IR. Plasma TG concentrations were higher, but not significantly increased, in laminitis-predisposed ponies when consuming basal hay diet supplemented with fructans relative to basal hay diet alone (Bailey *et al.* 2007).

Genetic factors also seem to play a role in the development of IR in horses (Johnson *et al.* 2004). Specific breeds that have been associated with the occurrence of IR include Morgan horses, domesticated Spanish mustangs, European Warmbloods, American Saddlebreds, and different pony breeds (Johnson 2002; Johnson *et al.* 2004). A higher degree of IR was also noted in healthy ponies relative to Dutch Warmblood horses (Rijnen and van der Kolk 2003). The presence of a certain level of insulin insensitivity in ponies has been suggested to originate from natural selection, facilitating survival under harsh circumstances (Jeffcott *et al.* 1986). Thus, ponies may have developed a highly efficient metabolism, with a degree of IR in the muscle, enhancing CHO delivery to the liver where coexistent hyperinsulinemia promotes lipid synthesis

(Jeffcott *et al.* 1986). Genetic variability with respect to insulin action has been implicated in the development of equine Cushing's syndrome as well (Johnson *et al.* 2004).

Equine Cushing's disease and IR are commonly registered as comorbid conditions in horses, suggesting associations between these conditions (Johnson *et al.* 2004). Resting hyperinsulinemia, a nonspecific indicator of IR (Kronfeld *et al.* 2005a), was detected in horses with pituitary adenoma (Garcia and Beech 1986). Insulin resistance was confirmed in these horses by a subsequent intravenous glucose tolerance test demonstrating prolonged hyperglycemia (Garcia and Beech 1986). However, the lack of correlation between plasma cortisol and glucose concentrations in horses suffering from hyperadrenocorticism suggests that cortisol is not the only factor influencing the development of IR in these cases (van der Kolk *et al.* 1995). Additionally, serum insulin concentrations have been successfully used as a prognostic indicator for survival in horses with equine Cushing's disease (McGowan *et al.* 2004). Insulin resistance is also a hallmark feature of equine metabolic syndrome (**EMS**), or "peripheral Cushing's syndrome" (Johnson 2002). Horses suffering from EMS are characteristically obese, prone to develop laminitis, aged between 8-18 years, often have a cresty neck, and are described by their owners as "easy keepers", implying that it is difficult to reduce the weight of these horses (Johnson 2002).

Lower insulin sensitivity has been described in pregnant mares, likely representing a physiologic adaptation process aimed at enhancing nutrient delivery to the developing fetus (Fowden *et al.* 1984). Reduced insulin sensitivity was implied by the significantly higher insulin concentrations seen in mares less than 270 days pregnant than in non-pregnant animals or in mares nearer to term. Increased insulin concentrations were attributed to the enhanced responsiveness of pancreatic β-cells to glucose. Additionally, exogenous insulin infusion decreased plasma glucose concentrations to a lesser degree in mares more than 270 days pregnant than in non-pregnant animals, providing corroborative evidence of IR in pregnant mares (Fowden *et al.* 1984).

In weanlings with osteochondritis dissecans, plasma insulin and glucose concentrations were significantly greater 2 hours after feeding a concentrate meal,

relative to unaffected foals, indicating insulin resistance, although insulin/glucose ratios were not significantly different between groups (Pagan 2001). Higher basal IGF-I concentrations were also observed in Thoroughbred weanlings rendered insulin resistant by feeding them a diet rich in sugar and starch (Treiber *et al.* 2005a). Increased IGF-I may be a risk factor for osteochondrosis dissecans, explaining the association between high CHO diets, IR and osteochondrosis dissecans (Treiber *et al.* 2005a). Similar to humans (Reaven 2003), IR has been associated with elevated blood pressure in a group of laminitis prone ponies (Bailey *et al.* 2008).

1.1.8 Factors enhancing insulin sensitivity in horses:

There are a number of factors known to enhance insulin sensitivity in humans including exercise, weight reduction, a calorie restricted diet and pharmacologic interventions (Cefalu 2001). Exercise has been demonstrated to enhance GLUT4 gene transcription and translation in rats, as well as translocation of GLUT4 molecules from the intracellular pools to the sarcolemma (Dohm 2002). The effect of submaximal treadmill exercise prior to and following 6 weeks of exercise training was examined on equine skeletal muscle GLUT4 protein content and on glucose transport in muscle membrane vesicles (McCutcheon *et al.* 2002). Middle gluteal muscle GLUT4 protein content increased 2- to 3-fold by the end of the training program relative to values measured at the initiation of the study. Conversely, GLUT4 content was not different at the end of the 60 min submaximal exercise period from values measured preceding it. Following treadmill exercise, increased glucose transport was demonstrated in the muscle membrane vesicles; however, this response was blunted by 6 weeks of training, suggesting a reduction in the extent of translocation of GLUT4 to the sarcolemma in trained muscle (McCutcheon *et al.* 2002). Another study in horses has demonstrated that 6 weeks of exercise training lowers glucose production and whole body glucose disposal by approximately 22%, relative to pre-training values, when the same absolute workload is used (Geor 2006). No significant change was registered when the same relative workload was applied after completion of training. During submaximal exercise, insulin

concentrations decreased whereas glucagon concentrations increased. Training blunted these alterations during submaximal exercise at the same absolute, but not relative, workload (Geor *et al.* 2002). In another study, euglycemic hyperinsulinemic clamp (**EHC**) procedures performed 24 hours after the completion of 7 consecutive days of light exercise revealed 60 and 48% increases in insulin sensitivity in obese and lean horses, respectively, relative to sedentary controls (Powell *et al.* 2002). Insulin sensitivity returned to pre-exercise values 9 days following the cessation of the exercise program (Powell *et al.* 2002).

A study investigating the role of obesity and diet on glucose and insulin dynamics in horses revealed that obese Thoroughbred geldings were significantly less insulin sensitive than nonobese ones, and a diet rich in fat and fiber enhanced insulin sensitivity relative to a feed rich in starch and sugar (Hoffman *et al.* 2003). A study by Vick *et al*. (2006) has confirmed that feed restriction in obese mares significantly decreases body weight and improves IR compared to results obtained from obese mares fed *ad libitum*.

Short and long term oral administration of levothyroxine sodium has been demonstrated to increase insulin sensitivity in horses (Frank *et al.* 2008; Frank *et al.* 2005b). Levothyroxine sodium was speculated to enhance insulin sensitivity by increasing abundance of glucose transporter proteins in adipose tissue and skeletal muscle (Casla *et al.* 1990; Matthaei *et al.* 1995; Romero *et al.* 2000; Weinstein *et al.* 1994), as well as by increasing GLUT1 gene expression and the partitioning of glucose transporters into the cell membrane within hepatocytes (Haber *et al.* 1995). From the variety of medications available for the management of IR in humans, only the biguanide metformin and the sulfonylurea glyburide have been evaluated for use in horses (Durham *et al.* 2008; Johnson *et al.* 2005; Vick *et al.* 2006). In a Spanish Mustang suffering from diabetes mellitus, with the diagnosis based on the concurrent presence of hypoinsulinemia and hyperglycemia, treatment was attempted using glyburide and metformin (Johnson *et al.* 2005). This treatment successfully lowered the interstitial glucose concentration to physiologic levels within 4.5 h (Johnson *et al.* 2005). Oral metformin treatment at a dosage of 15 mg/kg twice daily significantly improved insulin sensitivity in horses and ponies with a history of laminitis, with the magnitude of effect

greatest early in the course of treatment (Durham *et al.* 2008). Similarly, insulin sensitivity increased significantly only at the end of the first 30 day treatment period when mares were treated with 1.5, 3, and 4.5 g metformin twice daily for successive periods of 30 days (Vick *et al.* 2006).

Oral administration of chromium L-methionine has been shown to decrease postprandial insulin secretion without affecting plasma glucose concentrations in aged mares, suggesting enhanced insulin sensitivity (Ralston 2002). These findings, however, were not substantiated by performing an intravenous glucose tolerance test (Ralston 2002). Studies performed in mice demonstrated that chromium supplementation enhances insulin sensitivity by facilitating tyrosine-phosphorylation and inhibiting serine-phosphorylation of IRS-1, the latter occuring through the downregulation of C-Jun NH_2-terminal kinase (**JNK**; Chen *et al.* 2008). Furthermore, addition of chromium to the diet also significantly reduced TNFα and IL-6 in the serum of diabetic mice (Chen *et al.* 2008).

1.1.9 IR and laminitis:

Laminitis refers to inflammation of the lamellae within the hoof capsule that can result in the failure of the attachment between the distal phalanx (coffin bone) and the inner hoof wall (Fagliari *et al.* 1998). The distal phalanx is suspended within the hoof capsule by a resilient lamellar apparatus containing the primary epidermal lamellae, which form the hard keratinized backbone to which the semi-keratinized secondary epidermal lamellae attach. The primary and secondary epidermal lamellae interdigitate with the primary and secondary dermal lamellae that are attached to the periosteum of the third phalanx. A basement membrane (**BM**), found at the basal side of the epidermal basal cells separates the epidermal compartment of the hoof from the dermal compartment (French and Pollitt 2004a; Johnson *et al.* 1998; Mungall and Pollitt 2001; Pollitt 1994).

When laminitis occurs, attachment of the BM at the dermo-epidermal junction fails, and the distal phalanx separates from the hoof capsule (French and Pollitt 2004a).

Epidermal basal cells are attached to the BM by electron dense plaques called hemidesmosomes (**HD**). The major components of the HD intracellular plaque are plectin, BP230, BP180, and integrin $\alpha_6\beta_4$. Anchoring filaments connect the HD to the lamina densa of the basement membrane. Intermediate filaments of the basal cell cytoskeleton insert into the HD connecting the cytoskeleton to the plasmalemma (French and Pollitt 2004a).

There are at least two mechanisms proposed to explain why separation occurs at the dermo-epidermal junction during laminitis (French and Pollitt 2004a). In the absence of glucose, the number of HDs decline and the intermediate filaments of the cytoskeleton lose their attachment to the intracellular dense plaque of the HD. This results in weakening of the connection between the germinal cells and basement membrane. Failure of the cytoskeleton also causes basal cell nuclei to become rounded and then more spindle shaped; they are positioned more basally instead of apically (French and Pollitt 2004a). The second mechanism involves matrix metalloproteinases (**MMP**). Cultured equine hoof explants secrete the inactive pro-enzymes MMP-2 and MMP-9 which are activated by a multistep process when they are needed (Mungall and Pollitt 2002; Pollitt 1996). Mononuclear phagocytes present in higher concentration within laminitic hooves are also a potential source of MMPs (French and Pollitt 2004a; Pollitt and Daradka 1998). Activation of MMPs results in the destruction of the anchoring filaments, but HDs remain unaffected (French and Pollitt 2004a). The importance of MMPs in the development of laminitis is emphasized by the fact the connective tissue samples obtained from laminitic horses have significantly greater MMP activity (Johnson *et al.* 1998). Bacterial proteases such as thermolysin are also capable of activating MMPs (Mungall and Pollitt 2001).

Impaired glucose delivery to the laminar keratinocytes in horses suffering from IR may contribute to the development of laminitis. Healthy laminar keratinocytes express GLUT1, GLUT4 and epithelial sodium channel (**ENaC**; Mobasheri *et al.* 2004). The presence of GLUT4 suggests that these cells are metabolically active and their glucose uptake is stimulated by insulin. In severe cases of laminitis, immunostaining of GLUT1, GLUT4, and the α-subunit of ENaC markedly decrease, suggesting necrotic death of

resident laminar keratinocytes (Mobasheri *et al.* 2004). However, insulin mediated glucose uptake into equine lamellar tissues has been questioned by Asplin *et al*. (2007a), who failed to demonstrate increased glucose uptake in lamellar explants after insulin stimulation. Furthermore, the same study demonstrated no evidence of GLUT4 gene expression in laminar tissues, suggesting that glucose uptake in the hoof occurs thorough the insulin independent GLUT1 molecules which are expressed in the coronary band and lamellar tissues (Asplin *et al.* 2007b).

The importance of IR in the development of pasture associated laminitis has been emphasized by Treiber *et al.* (2006b), because this group was able to identify ponies suffering from prelaminitic metabolic syndrome (**PLMS**) with 74% sensitivity and 79% specificity using three or more of the following characteristics: IR, compensatory β-cell response, hypertriglyceridemia, or obesity. The same study revealed that ponies with PLMS have an odds ratio of 10 for the development of pasture associated laminitis when compared to ponies without PLMS (Treiber *et al.* 2006b). Further evidence for the role of IR in the development of laminitis is provided by results of a study in which prolonged hyperinsulinemia was induced by application of an EHC for up to 72 h, which led to the development of Obel grade 2 laminitis in clinically normal ponies (Asplin *et al.* 2007b).

Changes in the digital vasculature are also proposed to occur during the acute stage of equine laminitis, with shunting of blood away from lamellar capillaries via arteriovenous anastomoses (Pollitt and Molyneux 1990). If this phenomenon lasts for long enough, lamellar ischemia is thought to develop, which results in ischemic necrosis of digital lamellae (Pollitt and Molyneux 1990). During the prodromal stages of laminitis; lamellar microvascular blood flow fluctuates. The blood flow decreases in the first 2 hours and then returns to near baseline value before finally decreasing again 8 hours after administration of black walnut extract (Adair *et al.* 2000). Insulin resistance may also contribute to the vascular changes preceding laminitis. Inhibited insulin signaling via the PI3-kinase pathway, involved in NO synthesis, was demonstrated during IR, whereas the Ras/MAPK pathway mediated production of ET-1 remained undisturbed (Jiang *et al.* 1999; Kim *et al.* 2006). The additive effect of these mediators likely leads to pathologic vasoconstriction (Kim *et al.* 2006). The dyslipidaemia that develops in insulin

resistant patients plays a central role in diabetic microangiopathy (Jenkins *et al.* 2004a; Jenkins *et al.* 2004b). Microvascular changes demonstrated in diabetic patients may occur in the laminar vessels of horses with chronic IR and contribute to the development of laminitis.

Accumulation of platelets in the equine hoof wall distal to the coronary band occurs at the onset of laminitis, implicating microvascular thrombosis and systemic coagulopathy in the development of CHO induced laminitis (Weiss *et al.* 1994). Elevated fasting insulin levels, a characteristic feature of IR (Cefalu 2001), have been associated with impaired fibrinolysis and hypercoagulability in people (Meigs *et al.* 2000). In human subjects, a strong positive association was demonstrated between fasting insulin concentrations and levels of plasminogen activator inhibitor 1, fibrinogen, von Willebrand factor, and factor VII (Meigs *et al.* 2000).

Destruction of the basement membrane between the dermal and epidermal compartment of the hoof by MMPs is often implicated in the development of laminitis (Kyaw-Tanner *et al.* 2008; Souza *et al.* 2006). Increased MMP-1 and MMP-2 protein levels, as well as increased MMP-2 activity, were registered in human umbilical vein endothelial cell culture media following exposure to high (25 nM) glucose concentrations for 48 h (Death *et al.* 2003). Furthermore, the mRNA, protein, and activity levels of MMP-9 were all significantly higher in human monocyte-derived macrophages exposed to a high glucose concentration, compared with cells exposed to either a normal glucose concentration or a high concentration of mannitol (Death *et al.* 2003). Thus, IR and accompanying hyperglycemia likely plays an integral role in the development of laminitis by activating MMPs (Treiber 2005).

1.1.10 Nonspecific indicators of IR in horses:

Measurement of basal hyperglycemia is one of the most common screening tests for diabetes mellitus in human beings, but it does not differentiate between inadequate insulin secretion by pancreatic β-cells and the reduced ability of peripheral tissues to take up glucose (Firshman and Valberg 2007; Kronfeld *et al.* 2005a). It has been applied to

horses as well, albeit in conjunction with intravenous glucose tolerance testing (Jeffcott *et al.* 1986). The primary advantage of using basal hyperglycemia as an indicator of IR is the ease of sample collection and measurement of plasma glucose concentrations. On the other hand, the nonspecific nature of this test as an indicator for IR is an important drawback. Glucose responses vary significantly even when measured 5-6 h after feeding and are influenced by the type of feed provided (Ralston 2002). If basal glucose measurements are taken, samples should be chilled after collection, and plasma glucose concentrations should be determined promptly, to prevent registering false-low results due to glucose consumption by red blood cells (Christopher and O'Neill 2000). Stress-induced variations in cortisol may also influence findings, so stress should be minimized prior to sample collections (Ralston 2002).

Fasting hyperinsulinemia has also been used as an indicator of IR (Kronfeld *et al.* 2005b). Hyperinsulinemia develops to compensate for IR but declines if compensation becomes inadequate (Firshman and Valberg 2007; Kronfeld *et al.* 2005a). False negative responses may therefore occur during impending type 2 DM, a decompensation that is common in human beings but rare in horses (Kronfeld *et al.* 2005a). Enhanced β-cell sensitivity to glucose has been implicated in the development of basal hyperinsulinemia during pregnancy in mares (Fowden *et al.* 1984). Advantages of measuring fasting insulin concentrations to establish IR include uncomplicated sample collection and inexpensive sample processing. The primary disadvantage of this method is its low accuracy, because insulin secretion and clearance are profoundly influenced by a multitude of factors including diet, duration of fasting, diurnal variations, and stress-induced alterations in cortisol concentrations (Ralston 2002). The nonspecific character of this test has been demonstrated in horses, and only a trend ($P = 0.065$) for higher basal insulin concentrations was registered in geldings fed diets high in sugar and starch relative to geldings consuming feed rich in fat and fiber (Treiber *et al.* 2005a). On the other hand, the minimal model analysis of FSIGTT demonstrated a ($P = 0.007$) significant difference in insulin sensitivity for the same groups of horses (Treiber *et al.* 2005a).

Glucose tolerance is assessed by the plasma glucose response to oral or intravenous glucose administration (Firshman and Valberg 2007; Kronfeld *et al.* 2005a). The oral glucose tolerance test is used to evaluate small intestinal absorption, hepatic glucose uptake, and the endocrine function of the pancreas (Firshman and Valberg 2007). The intravenous glucose tolerance test on the other hand, provides information about the functional turnover rate of glucose, a measure of insulin sensitivity (Firshman and Valberg 2007). Marked or prolonged elevation of plasma glucose levels may be attributable to impaired insulin secretion or decreased insulin sensitivity (Kronfeld *et al.* 2005a). The intravenous glucose tolerance test has been used in horses to evaluate the effects of diet and pituitary pars intermedia dysfunction on glucose and insulin responses (Garcia and Beech 1986).

The insulin tolerance test measures the blood glucose response to an exogenous insulin challenge (Firshman and Valberg 2007). The inability of exogenous insulin to elicit a decrease in blood glucose levels is suggestive of insulin resistance. It has been successfully applied to horses and ponies to assess insulin sensitivity (Jeffcott *et al.* 1986). However, glycemic responses to exogenous insulin are confounded by endogenous insulin secretion (Kronfeld *et al.* 2005a). In healthy horses, completion of an insulin tolerance test, comprising the administration of 0.1 U/kg insulin intravenously, resulted in hypoglycemia that reached a nadir at approximately 40 min with blood glucose concentrations then returning to baseline values by 130 min (Eiler *et al.* 2005).

The combined glucose-insulin test (**CGIT**) provides more information than either the glucose tolerance test or insulin tolerance test alone (Eiler *et al.* 2005). During the test, insulin and glucose are administered concurrently as a bolus at the initiation of the study, then successive blood samples are collected for 150 minutes. For the CGIT, insulin resistance is defined as maintenance of blood glucose concentrations above the baseline level for ≥ 45 min (Frank *et al.* 2006). Frank *et al.* (2006) applied the CGIT method to define insulin resistance in obese horses. The advantages of this procedure include its simplicity to perform and the low number (n=14) of blood samples collected during the test, which makes it a useful clinical diagnostic test (Eiler *et al.* 2005). Although in its current form no quantitative measures of glucose and insulin dynamics

are obtained from this procedure, calculations of area under the glucose (**AUCg**) and insulin (**AUCi**) curves may remedy this deficiency. Nevertheless, administration of exogenous insulin at the initiation of the procedure interferes with the evaluation of endogenous insulin response.

In human subjects, glucose-to-insulin ratios correlate positively with insulin sensitivity, whereas the insulin-to-glucose ratio correlates with insulin secretion (Kronfeld *et al.* 2005a). An initial decrease in the insulin-to-glucose ratio during intravenous glucose tolerance test in horses with pituitary adenoma indicated an impaired insulin response to acute hyperglycemia (Garcia and Beech 1986).

Surrogates for quantitative methods have been developed for screening IR in people and horses as well (Kronfeld *et al.* 2005a). The quantitative insulin sensitivity check index (**QUICKI**) estimates insulin sensitivity in human beings from fasting insulin (I_0) and glucose (G_0) concentrations using the following formula: $QUICKI = 1/[\log(I_0) + \log(G_0)]$ (Katz *et al.* 2000). In people, the correlation between QUICKI and EHC was stronger than the correlation between EHC and minimal model analysis of glucose and insulin dynamics (Katz *et al.* 2000).

Two proxies, the reciprocal of the square root of insulin (**RISQI**) and the modified insulin-to-glucose ratio (**MIRG**), were developed by Treiber *et al*. (2005b) for the prediction of insulin sensitivity and beta cell responsiveness, respectively. Beta cell responsiveness represented by the MIRG is similar to the acute insulin response to glucose [**AIRg**], obtained via minimal model analysis of frequently sampled intravenous glucose tolerance test (**FSIGTT**) data (Treiber *et al.* 2005b). Resting glucose and insulin concentrations are measured for the calculations. Insulin sensitivity is derived using the following formula:

$$\text{Insulin sensitivity} = 7.93 \times (\text{RISQI}) - 1.03$$

where RISQI is the reciprocal of the square root of basal insulin concentration. Acute insulin response to glucose can be calculated by using the following equation:

$$\text{AIRg} = 70.1 \times (\text{MIRG}) - 13.8$$

where $\text{MIRG} = [800 - 0.3 \times (\text{basal insulin concentration} - 50)^2] / (\text{basal glucose concentration} - 30)$ (Treiber *et al.* 2006b). These proxies compared favorably with

proxies used successfully in human studies when comparisons were made with insulin sensitivity and AIRg values obtained from the minimal model. The combined use of RISQI and MIRG facilitates distinction between compensated and uncompensated IR in horses (Treiber *et al.* 2005b). The aforementioned proxies were used in a study to establish metabolic predispositions and nutritional risk factors for development of pasture-associated laminitis in ponies (Treiber *et al.* 2006b). However, one disadvantage of using proxy measurements is that factors other than insulin sensitivity affect fasting insulin concentrations. Furthermore, if RISQI values of < 0.32 mU/L$^{0.5}$ are used to define IR, as suggested by Treiber *et al.* (2006b), any horse with a fasting insulin concentration $\geq$ 10 mU/L would be classified as insulin resistant, even though fasting insulin concentrations of 5 to 20 mU/L are usually considered normal in horses (Ralston 2002).

1.1.11 Quantitative methods for the evaluation of IR:

In contrast to nonspecific indications of insulin resistance, the euglycemic-hyperinsulinemic clamp technique allows the quantification of insulin sensitivity (Rijnen and van der Kolk 2003). During the EHC, euglycemia and hyperinsulinemia are induced by the concurrent infusion of glucose and insulin. Blood samples are collected during the procedure to confirm that blood glucose concentrations are maintained on a constant, euglycemic level, which is achieved by the adjustment of the glucose infusion rate. In a successful clamp, the rate of glucose infusion used to maintain euglycemia equals cellular glucose uptake mediated by the exogenously administered insulin. The glucose disposal rate is usually calculated from the mean glucose infusion rate used during the last 60 minutes of the 120- or 180-minute test. Arterial samples are preferable because insulin administration increases differences between arterial and venous blood glucose concentrations. Disadvantages of the method include its labour intensive nature and the use of supraphysiologic insulin concentrations (Kronfeld *et al.* 2005a). The EHC technique was successfully applied to determine reference range values indicative of insulin sensitivity in horses and ponies (Rijnen and van der Kolk 2003). Vick *et al.* (2008) also used the EHC technique in horses to demonstrate that lipopolysaccharide

administration induces a transient increase in insulin sensitivity followed by a reduction in insulin sensitivity at 24h. The effects of short term exercise training and the influence of dexamethasone administration on insulin sensitivity in horses have also been investigated using the EHC technique (Powell *et al.* 2002; Tiley *et al.* 2008).

The insulin suppression test is another quantitative method that has been used in people to measure the ability of a fixed rate insulin infusion to dispose of a glucose load (Kronfeld *et al.* 2005a). Endogenous insulin secretion is suppressed by administration of somatostatin or octreotide, and infusion rates are controlled to yield a relatively steady level of insulin and glucose concentration during the last 60 minutes of the test. The steady state plasma glucose concentration is determined as the mean glucose concentration of 6 blood samples obtained every 10 minutes during the last hour of the test. It reflects insulin-mediated glucose disposal which is directly related to IR. The limiting factors of this method are the adverse effects of insulin suppressant drugs, inconsistency of the steady-state, and an elevated steady-state glucose concentration which exceeds the renal threshold (Kronfeld *et al.* 2005a). To date, there are no reports detailing the use of this method in horses.

The minimal model of glucose-insulin dynamics has been used to interpret data from the FSIGTT. The minimal model partitions glucose disposal into glucose-dependent and insulin-dependent parts by fitting a nonlinear model to the data obtained from the FSIGTT. In the model, the glucose-time curve is described by two differential equations, one represents glucose-mediated glucose uptake, whereas the other explains insulin-mediated glucose disposal. The insulin modified FSIGTT involves the intravenous administration of a glucose bolus (300mg/kg of bwt) at 0 min followed by a bolus infusion of insulin (30 mU/kg of bwt) at 20 min (Kronfeld *et al.* 2005a). Over the course of the test, 31 blood samples are obtained at baseline, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 19, 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150 and 180 min relative to glucose infusion, to determine glucose and insulin concentrations. The FSIGTT has sometimes been extended to 240 min. Insulin sensitivity (**SI**) is calculated using the following equations:

$$X'(t) = -p_2 \times X(t) + (p_3 \times [I(t) - Ib])$$

$$SI = p_3 / p_2$$

Where X'(t) (min^{-2}) represents the change in insulin action over time; parameter p_2 represents the rate (min^{-1}) of decline of insulin action; X(t) is insulin action (min^{-1}) (i.e. the acceleration of glucose disposal at time t associated with insulin concentration above basal); parameter p_3 represents the rate (min^{-1}) of introduction of insulin into the interstitial space; I(t) represents the insulin concentration (mU/L) at time t; and Ib represents basal insulin concentration (mU/L). Assumptions were X(0) = 0 and {I(t)-Ib} = 0 if I(t) < Ib. Besides the SI value, the minimal model analysis of the FSIGTT also provides a measure of glucose effectiveness (**Sg**), a value representing the net fractional glucose clearance independent of insulin. The equation used to calculate Sg is:

$$G'(t) = -(X+Sg) \times G(t) + (Sg \times Gb)$$

Where G'(t) represents the rate of glucose clearance from plasma; X represents insulin action; G(t) represents plasma glucose concentration at time t with G(0) being the theoretical glucose concentration at time 0, as calculated using the minimal model; and Gb represents basal glucose concentration (Hoffman *et al.* 2003). Further variables provided by minimal model analysis of the insulin modified FSIGTT data include AIRg and the disposition index (**DI**; Kronfeld *et al.* 2005a). The AIRg is the increase in plasma insulin concentrations above baseline levels integrated from 0 to 10 min after glucose administration (Treiber *et al.* 2005a).

$$AIRg = \int [I(t) - Ib] \times dt$$

Where I(t) and Ib represents insulin concentrations at time t and baseline, respectively. This variable therefore characterizes the responsiveness of pancreatic β-cells to a glucose load. The DI represents the ability of the pancreatic β-cells to compensate for decreased insulin sensitivity and it is derived from the minimal model by multiplying AIRg with SI (Bergman 2005). The minimal model analysis of the insulin modified FSIGTT has been successfully used to investigate the effects of obesity and diet on insulin sensitivity in horses (Hoffman *et al.* 2003). Tiley *et al*. (2008) also applied the minimal model to establish the effects of dexamethasone on insulin sensitivity and glucose dynamics in Standardbreds. A study examining the repeatability of EHC versus minimal model

analysis of FSIGTT data demonstrated that EHC results were more repeatable under the experimental conditions used (Pratt *et al.* 2005). Further disadvantages of this technique include the potential loss of glucose into the urine during the FSIGTT, which is not accounted for in minimal model analysis. The AIRg values, which are calculated from insulin concentrations measured in the peripheral blood, are also confounded by hepatic insulin extraction and by the whole body insulin clearance rate (Toffolo *et al.* 1999). In human subjects, these confounding factors are controlled by using C-peptide measurements for the assessment of pancreatic β-cell function (Toffolo *et al.* 1999).

1.2 Endotoxin – endotoxemia:

1.2.1 Structure of the endotoxin:

Endotoxin is a heat stable macroamphiphile lipopolysaccharide (**LPS**) molecule found in the cell membrane of Gram-negative bacteria, which may cause severe physiological derangements when it enters the circulation in mammals (Rietschel *et al.* 1994). It constitutes approximately three quarters of the outer leaflet of the bacterial cell membrane and functions as a protective layer inhibiting the entry of toxic compounds. It is also important in the transport of nutrients, so it is essential for bacterial viability (Rietschel *et al.* 1994).

Lipopolysaccharide is composed of a hydrophilic heteropolysaccharide and a covalently bound lipid component, termed lipid-A. In certain bacteria, such as members of the *Enterobacteriaceae* genus, the heteropolysaccharide can be further divided into the O-specific chain and the core oligosaccharide (Rietschel *et al.* 1994). The O-specific chain is a polymer of oligosaccharides, containing one to eight glycosyl residues. The structure of the repeating units creates considerable variability between strains. It determines the serological specificity of the LPS and thus, functions as an important surface antigen (Rietschel *et al.* 1994).

The core region consists of a heterooligosaccharide which can be further divided into outer (O-specific chain proximal) and inner (lipid-A proximal) portions (Rietschel *et*

al. 1994). The outer core in *Salmonella* spp. and *Escherichia coli* serotypes contains hexoses such as D-glucose, D-galactose and N-acetyl-D-glucosamine, while the inner core is composed of heptoses and 3-deoxy-D-manno-octulosonic acid. Most of the structural diversity of the core region is related to the outer core while the innermost part appears to be highly conserved amongst Gram-negative bacteria (Rietschel *et al.* 1994).

Lipid-A constitutes the toxic and immunomodulating principle of LPS and it is the most conserved region of the endotoxin molecule. Chemically, it is a phosphoglycolipid containing D-gluco-configurated pyranosidic hexosamine residues present as a β-linked homo- or heterodimer. Changes in its hydrophilic backbone (ie, omission of phosphoryl groups) or addition of a fatty acid to its hydrophobic region both significantly reduce the bioactivity of lipid-A. Full endotoxic activity is expressed by a molecule containing two hexosamine residues, two phosphoryl groups and six fatty acids including 3-acyloxylacyl groups (Reed 2003; Rietschel *et al.* 1994).

1.2.2 Cellular recognition and signal transduction of endotoxin:

After endotoxin is released into the systemic circulation, it is recognized by the innate immune system, including macrophages and monocytes (Chow *et al.* 1999; Werners *et al.* 2005). The effects of LPS on macrophages are markedly enhanced by the presence of a protein called lipopolysaccharide-binding protein (**LBP**; Martin 2000; Werners *et al.* 2005). Lipopolysaccharide-binding protein mainly functions as a transport protein which disaggregates LPS and transfers it to targets on cellular membranes and to lipoproteins in solution. Lipopolysaccharide molecules bound to and transported by HDL are biologically inactive, therefore HDL binding provides a mechanism for detoxification of LPS in the plasma (Martin 2000). Lipopolysaccharide-binding protein is a 50-kDa polypeptide that is glycosylated and secreted as a 60-kDa glycoprotein primarily by the liver and lungs (Martin 2000; Wan *et al.* 1995). As a type I acute phase response protein, LBP mRNA expression is stimulated by LPS, IL-1, IL-6 and TNFα in primary rat hepatocyte cultures (Wan *et al.* 1995). Lipopolysaccharide-induced LBP mRNA expression was markedly enhanced in hepatocytes isolated from rats previously injected

with LPS, suggesting the existence of a priming mechanism which facilitates a more rapid and vigorous response to a subsequent challenge (Wan *et al.* 1995).

The LPS-LBP complexes are transported to the cluster-of-differentiation antigen 14 (**CD14**), a molecule existing in two forms (Werners *et al.* 2005). A tail composed of glycosylphosphatidylinositol may anchor CD14 molecules to the cell membrane or they may be present in a soluble form that lacks the glycosylphosphatidylinositol tail (Martin 2000; Werners *et al.* 2005). Binding of LPS-LBP complexes to CD14 present on monocytes, macrophages and neutrophil granulocytes leads to cellular activation and production of proinflammatory cytokines (Downey and Han 1998; Werners *et al.* 2005). Soluble CD14 receptors are also capable of binding LPS-LBP complexes and facilitate LPS-dependent activation of some CD14 negative cells such as endothelial, epithelial, and smooth muscle cells (Downey and Han 1998).

Once it was determined that CD14 lacks a cytoplasmic domain (Werners *et al.* 2005), it was then found that LPS-stimulated signal transduction occurs through Toll-like receptors (**TLR**; Chow *et al.* 1999). Lipopolysaccharide signaling through the TLR4 receptor, however, also requires the presence of myeloid differentiation factor 2, a protein physically associated with TLR4 on the cell surface (Shimazu *et al.* 1999; Werners *et al.* 2005; Yang *et al.* 2000). To date, at least 11 different TLRs have been cloned in mammalian species (Werners *et al.* 2005). Both TLR2 and TLR4 appear to play a role in LPS-induced signal transduction (Chow *et al.* 1999; Kirschning *et al.* 1998). Human embryonic kidney 293 cells transiently transfected with human TLR4 or TLR2 cDNA demonstrated a significantly increased responsiveness to LPS in the presence of CD14 as measured by the activation of nuclear factor κB (**NF-κB**; Chow *et al.* 1999).

After ligand binding, TLR4 dimerizes and undergoes a change in conformation that is necessary to initiate intracellular signaling events (Werners *et al.* 2005). The best characterized LPS-induced pathways are the NF-κB pathway and three MAPK-associated pathways (Yang *et al.* 2000):

- MAPK (extracellular signal regulated kinase)
- C-Jun NH_2-terminal kinase 1 and 2
- p38 MAPKs (Downey and Han 1998).

Endotoxin-induced activation of NF-κB via TLR4 is initiated by the association of the TLR4 receptor with myeloid differentiation protein. This protein stimulates the IL-1 receptor-associated kinase which in turn facilitates recruitment of TNF receptor associated factor-6 (Yang *et al.* 2000). The activation of these proximal signaling molecules leads to stimulation of members of the MAPK kinase kinase family that activate inhibitory kappa B kinases. Inhibitory kappa B kinases phosphorylate the inhibitory kappa B proteins, which are subsequently degraded resulting in the liberation of NF-κB and its translocation to the nucleus (Yang *et al.* 2000). Almost all genes important in the pathogenesis of LPS-induced systemic inflammation contain promoter elements for NF-κB such as TNFα, IL-1β, IL-6, IL-8, inducible nitric oxide synthase and cyclo-oxygenase (**COX**) -2 (Downey and Han 1998). In addition to the aforementioned cellular activation mechanisms, PKC and SRC family kinases were also found to be activated following LPS stimulation (Downey and Han 1998).

1.2.3 Pathogenesis of endotoxemia:

Endotoxin is liberated from the outer membrane of the cell wall of Gram-negative bacteria upon rapid proliferation or lysis of the organisms (Werners *et al.* 2005), thus the equine intestinal tract contains large amounts of LPS (Morris 1991). Normally there are three mechanisms preventing the access of LPS to the peripheral circulation: the mucosal cell barrier of the intestines, the ability of bile acids to bind LPS, and the clearance of LPS from the blood by the Kupffer cells of the liver (Hardie and Kruse-Elliott 1990a; Morris 1991). In order to cause disease, large amounts of endotoxin must be absorbed from the gastro-intestinal tract and then overwhelm the liver's detoxifying ability, or the number of Kupffer cells in the liver must be reduced due to pathologic processes (Hardie and Kruse-Elliott 1990a; Morris 1991).

In cases of endotoxemia the source of endotoxin can be endogenous or exogenous. The gastro-intestinal tract is an endogenous source and endotoxin release occurs during acute abdominal disease. In contrast, the source of endotoxin is exogenous in cases of metritis, pleuropneumonia, or neonatal septicemia (Werners *et al.* 2005). The

interaction of LPS with mononuclear phagocytes, and subsequent activation of these cells are pivotal early events in the pathogenesis of endotoxemia (MacKay 2001). Activated macrophages possess an increased ability of adherence and pseudopod formation (Morris 1991). Most importantly, activated macrophages secrete a wide variety of inflammatory mediators, such as cytokines, lipid mediators, and free oxygen radicals, which play a central role in the pathogenesis of endotoxemia (Hardie and Kruse-Elliott 1990a; Morris 1991; Werners *et al.* 2005). The most extensively studied cytokines, IL-1, IL-6, and TNFα are all pyrogens, and are capable of stimulating acute phase protein production by the liver (Werners *et al.* 2005).

Tumor necrosis factor-α is a polypeptide produced by activated macrophages, vascular smooth muscle cells, blood leukocytes, and mesothelial cells (Barton and Collatos 1999; Detmer *et al.* 2001; Hardie and Kruse-Elliott 1990a). The plasma half life of TNFα is short (approximately 6 min), and it rapidly binds to specific receptors in target tissues, such as lungs, kidneys, and liver (Hardie and Kruse-Elliott 1990a). As a proximal mediator of LPS effects, TNFα is important in neutrophil activation and adherence, alterations to the vascular endothelium, and decreased LPL synthesis (Hardie and Kruse-Elliott 1990a). This cytokine also enhances the synthesis of class I major histocompatibility complex antigen and triggers the secretion of the second wave of inflammatory cytokines, including IL-6 and granulocyte-monocyte colony stimulating factor (Hardie and Kruse-Elliott 1990a; MacKay 2001). In humans and mice, intravenous infusion of endotoxin induces a marked increase in TNFα concentrations at 2 hours, followed by a return to baseline levels by 4 hours post-infusion (Copeland *et al.* 2005). Stimulation with LPS significantly increased TNFα production in blood from 10 horses, however the magnitude of this response showed wide inter-animal variation (Werners *et al.* 2006).

Interleukin-1 plays a central role in the control of fever, neutrophil and T lymphocyte chemotaxis, expression of intracellular adhesion molecules in vascular endothelium, and also triggers an increase in the number of circulating immature neutrophils (Hardie and Kruse-Elliott 1990a). Macrophages and vascular smooth muscle cells secrete IL-1 in response to bacterial LPS or TNFα (Detmer *et al.* 2001; Hardie and

Kruse-Elliott 1990a). Increased plasma concentrations of IL-1β were detected in mice at multiple time points following intravenous LPS administration whereas in people, no circulating IL-1 was detected following endotoxin challenge (Copeland *et al.* 2005; van Deventer *et al.* 1990). Exposure to *Escherichia coli* O111:B4 LPS has also been shown to increase IL-1β mRNA expression in equine monocytes (Figueiredo *et al.* 2008).

Interleukin-6, a phosphoglycoprotein produced by fibroblasts, mononuclear, and endothelial cells, stimulates the final differentiation of B-lymphocytes into plasma cells and induces production of acute phase proteins by hepatocytes (Morris 1991). In mice and people, endotoxin administration increased IL-6 concentration with a peak registered 2 h after endotoxin infusion (Copeland *et al.* 2005). Van Deventer *et al.* (1990) demonstrated that release of IL-6 was delayed approximately 15 minutes relative to TNFα in human subjects receiving an LPS infusion, indicating that TNFα may stimulate the release of IL-6 from the monocyte/macrophage compartment, or alternatively, from endothelial cells. Interleukin-6 secretion was also registered in equine monocytes exposed to LPS (Figueiredo *et al.* 2008).

Lipopolysaccharide binding also destabilizes cell membranes and activates the nearby phospholipases A_2, C, and D (Stief 2007). Phospholipase A_2 cleaves the membrane phospholipids in the sn-2 position, thereby generating lysophospholipids and arachidonic acid (Stief 2007), with the latter further converted into leukotrienes via the 5-lipoxygenase pathway or into prostanoids via the COX pathway (Hardie and Kruse-Elliott 1990a). Thromboxane-A_2 is a COX end-product that causes vasoconstriction during endotoxemia and induces platelet aggregation (Morris 1991). Prostacyclin is another arachidonic acid derivate that is often released more gradually, and causes vasodilatation and inhibition of platelet aggregation (Morris 1991). There are four different leukotrienes released during endotoxemia. Of these, leukotriene-C_4, leukotriene-D_4, and leukotriene-E_4 are potent bronchoconstrictors and vasoconstrictors that also increase capillary permeability of postcapillary venules, whereas leukotriene-B_4 promotes neutrophil-endothelial adherence and is a potent chemoattractant for neutrophils (Hardie and Kruse-Elliott 1990a). Exposure to endotoxin has been shown to increase the expression of COX-2 protein and mRNA in equine digital vein endothelial

cells and equine digital artery smooth muscle cells, respectively (Menzies-Gow *et al.* 2008; Rodgerson *et al.* 2001).

Platelet activating factor is another important molecule mediating LPS effects (Carrick *et al.* 1993). It is synthesized by leukocytes, platelets, and endothelial cells through phospholipase A_2-mediated hydrolysis. Its biological effects include platelet and neutrophil activation and aggregation, bronchoconstriction, pulmonary hypertension, and increased vascular permeability (Carrick 1989; Carrick *et al.* 1993). Although treatment with a platelet activating factor receptor antagonist delayed the onset of fever, tachycardia, and leukopenia in horses that received endotoxin, the drug failed to change any of the clinical or laboratory data (Carrick *et al.* 1993).

1.2.4 Clinical conditions related to endotoxemia:

Endotoxin-induced systemic inflammatory response syndrome causes profound derangements in whole body homeostasis that ultimately affect multiple organ systems (Morris 1991). Endotoxemia most commonly occurs following gastro-intestinal compromise because of an increase in the permeability of the intestinal mucosa. Ischemic insults and severe bowel inflammation therefore facilitate endotoxin absorption from the lumen (Morris 1991). Absorbed endotoxin itself has a pronounced effect on the gastrointestinal tract. Lipopolysaccharide delays gastric emptying, decreases gastric acid output, and increases gastric pH (Doherty *et al.* 2003; Valk *et al.* 1998).

Platelet counts were lower and there was a trend towards increased coagulation times (thrombin time, prothrombin time and activated partial thromboplastin time) in ponies that received endotoxin intravenously (Fessler *et al.* 1982). Interestingly, the decline in platelet counts was more pronounced in ponies given flunixin meglumine, perhaps because this drug reduces the release of prostacyclin, which is known to inhibit platelet aggregation (Fessler *et al.* 1982). An endotoxin-induced elevation in fibrinogen concentration was also detected in ponies, which was prevented by the administration of dexamethasone, prednisolone succinate, or flunixin meglumine (Ewert *et al.* 1985).

One of the most characteristic consequences of endotoxemia is neutropenia. Tumor necrosis factor, IL-1, and LPS itself stimulate the expression of adhesion molecules such as p- and e-selectins on endothelial cells, and stimulates neutrophil adhesion (MacKay 2001; Morris 1991). Profound granulocytopenia was registered in human subjects 60 minutes after IV administration of LPS, which then shifted to granulocytosis two hours after infusion (Richardson *et al.* 1989). Granulocytosis persisted for 4 hours and returned to baseline levels by 24 hours. Rebound neutrophilia is attributed to an increase in colony stimulating factors released by macrophages following TNFα exposure (Morris *et al.* 1990). Endotoxin-induced monocytopenia and lymphopenia are also detected in humans (Richardson *et al.* 1989). Monocytopenia persists longer than neutropenia and lasts for up to 6 hours (Pernerstorfer *et al.* 1999). Significant lymphocytopenia is observed for 1 to 6 hours after endotoxin infusion (Richardson *et al.* 1989).

In a study of anesthetized ponies that received 0.2 mg/kg bwt endotoxin IV, multiple derangements in hemogram and blood-gas parameters were observed (Bottoms *et al.* 1981; Fessler *et al.* 1982). During the first 4 hours, there was a marked decrease in white blood cell count (**WBC**) due to a reduction in the number of neutrophils (Fessler *et al.* 1982). Significantly elevated packed cell volume (**PCV**) values were also detected in animals receiving LPS, which was likely to have been a consequence of catecholamine-mediated splenic contraction (Fessler *et al.* 1982). Higher circulating catecholamine concentrations have been previously detected in humans and laboratory animals with endotoxemia (Hinchcliff *et al.* 2005). Respiratory acidosis detected during the experiment was related to general anesthesia, however, significantly elevated blood lactate levels and lower venous pH values were suggestive of a shift from aerobic to anaerobic metabolism (Fessler *et al.* 1982). Flunixin meglumine administration ameliorated this shift to anaerobic metabolism, presumably by maintaining better peripheral blood flow, as demonstrated in a previous study (Bottoms *et al.* 1981; Fessler *et al.* 1982).

Circulating endotoxin has a direct toxic effect on tissues and initiates an exaggerated inflammatory response which causes vascular dysfunction (Hardie and Kruse-Elliott 1990a). This can result in distributive shock, multiple organ failure and

ultimately death. Toxic radicals released upon activation of leukocytes also contribute to cellular death (Hardie and Kruse-Elliott 1990a). Changes in blood biochemical values can reflect deteriorating organ function. Blood biochemical parameters were examined in anesthetized ponies receiving a lethal dose of endotoxin (Ewert *et al.* 1985). Elevated creatinine kinase activities were detected in all groups, but ponies that received dexamethasone, prednisolone succinate, or flunixin meglumine were less affected. Lactate dehydrogenase levels increased gradually during the shock period, with the highest levels detected in the group of ponies that did not receive anti-inflammatory drugs (Ewert *et al.* 1985).

Endotoxemia causes profound hemodynamic changes culminating in endotoxic shock, which is classified as a distributive shock (Muir 1998). Exposure of endothelial cells, neutrophils, and macrophages to LPS activates their inducible nitric oxide synthase enzyme leading to excessive generation of NO (Muir 1998). Nitric oxide causes vasodilatation, hypotension, and possibly arteriovenous shunting. Cardiac output is often elevated during the early phases of endotoxemia, which causes injected, warm mucous membranes. However, when endotoxin shock develops, arterial blood pressure and cardiac output decrease, resulting in reduced peripheral perfusion indicated by pale, cold mucosal membranes (Muir 1998). Compensatory responses include tachycardia and peripheral vasoconstriction mediated by thromboxane-A_2, ET-1, arginine-vasopressin, and angiotensin II. As the disease progresses, decompensation becomes apparent by progressive systemic hypotension, which is confounded by direct myocardial suppression of NO, and increased vascular permeability. Increased plasma concentrations of prostacyclin, prostaglandin-E_2, and bradykinin are often detected during the decompensation phase (Reed 2003). Increased blood levels of ET-1 have also been detected in various species during endotoxemia (Wanecek *et al.* 2000). Endothelin-1 is produced by vascular endothelial cells, mast cells, macrophages, and smooth muscle cells and is involved in the regulation of vascular tone. It causes concentration-dependent vasoconstriction which is mediated by endothelin-A and endothelin-B_2 receptors (Wanecek *et al.* 2000). During endotoxemia, ET-1 contributes to endotoxin-induced coronary vasoconstriction, late phase pulmonary hypertension, splanchnic

vasoconstriction, and acute renal failure (Wanecek *et al.* 2000). Endothelin-1 concentrations were elevated in laminar connective tissue collected from horses with laminitis, suggesting that ET-1 plays a role in the pathogenesis of this disease (Katwa *et al.* 1999).

1.2.5 Endotoxemia and IR:

In a study by Fessler *et al.* (1982), plasma insulin and glucose concentrations were evaluated following endotoxin administration. Ponies receiving endotoxin demonstrated an initial hyperglycemia without any alterations in insulin level, followed by hypoglycemia accompanied by transient hyperinsulinemia (Fessler *et al.* 1982). It was concluded that early hyperglycemia facilitated insulin release, which triggered subsequent rebound hypoglycemia (Fessler *et al.* 1982).

Endotoxemia caused whole body insulin resistance in rats with a 37% decrease in glucose disposal (Virkamaki and Yki-Jarvinen 1994). The decrease was partially attributed to a defect in muscle glycogen synthesis (Virkamaki and Yki-Jarvinen 1994). The same study examined the effects of α- and β-adrenergic blockade on glucose metabolism during endotoxemia. Although α- and β-blockade abolished the transient hyperglycemia observed after endotoxin administration, it failed to normalize glucose disposal or skeletal muscle glycogen synthesis, so LPS-induced insulin resistance was not corrected during the late (6-8 hrs) phase (Virkamaki and Yki-Jarvinen 1994). Continuous LPS infusion significantly decreased the number of insulin receptors, insulin-stimulated tyrosine phosphorylation of insulin receptors, abundance of IRS-1 molecules, and tyrosine phosphorylation of IRS-1 molecules in rat liver tissue, whereas only tyrosine phosphorylation of IRS 1 molecules was significantly affected in skeletal muscle (McCowen *et al.* 2001). The effect of endotoxin on IRS-1 is mediated by TNFα, which activates C-Jun NH_2-terminal kinase and I kappa B kinase resulting in the inhibition of IRS-1 signaling through serine^{307} phosphorylation (Aguirre *et al.* 2000; de Luca and Olefsky 2008).

In a prospective study of apparently healthy, middle aged women, elevated levels of the systemic inflammatory markers IL-6 and C-reactive protein were found to be determinants of risk for type 2 DM (Pradhan *et al.* 2001). In horses, Vick *et al.* (2008) investigated the effects of systemic inflammation on insulin sensitivity using the EHC method. Systemic inflammation induced by intravenous administration of 0.045 μg/kg bwt lipopolysaccharide induced a transient increase in insulin sensitivity followed by profound IR at 24 h (Vick *et al.* 2008). The IR that develops during the systemic inflammatory response likely aims to provide high glucose flow to cells involved in immune and inflammatory reactions and other non insulin-dependent cells (Fitzgerald 2004). In a study of humans where the EHC method was used to asses glucose dynamics during endotoxemia, LPS administration resulted in a biphasic response in glucose utilization, similar to that registered in horses (Agwunobi *et al.* 2000; Vick *et al.* 2008). The glucose infusion rate had to be increased 120 minutes after LPS administration, after which time it had to be progressively lowered. By the 420 minute time point, the glucose infusion rate was significantly lower in the group receiving LPS compared to control, indicating that insulin resistance had developed (Agwunobi *et al.* 2000). The difference in glucose infusion rate was attributed to alterations in nonoxidative glucose disposal. After an initial rise in glucose storage rate, it started to decline progressively and then reached statistical significance 420 minutes after LPS administration (Agwunobi *et al.* 2000). Endotoxin-mediated activation of TLR-4 receptors in 3T3-L1 adipocytes has also been shown to provoke insulin resistance (Song *et al.* 2006).

1.2.6 Endotoxemia and laminitis:

In horses, approximately 75% of acute laminitis is preceded by alterations involving the gastrointestinal system (Weiss *et al.* 1998). Changes in the caecal flora, including a concomitant increase in the number of lactic acid producing bacteria and reduction in the Gram-negative bacterial population, were demonstrated during the onset of acute laminitis induced by CHO overload in horses (Garner *et al.* 1978). These findings were further substantiated by registering that fecal and caecal bacterial

populations also change during oligofructose-induced laminitis from a predominantly Gram-negative population to one dominated by Gram-positive bacteria (Milinovich *et al.* 2007; Milinovich *et al.* 2006). Specifically, *Streptococcus lutetiensis*, a member of the *Streptococcus bovis/equinus* complex, demonstrated a marked population increase between 8 and 16h after OF administration (Milinovich *et al.* 2006). A sharp decline in the number of OF-utilizing organisms occurred between 16 and 24 hours and this was accompanied by a sharp decline in fecal pH, followed by the development of clinical laminitis between 24 and 32h (Milinovich *et al.* 2006). The lower pH likely contributes to the transient increase in intestinal mucosal permeability registered during the prodromal stages of alimentary laminitis (Weiss *et al.* 1998). Altered intestinal permeability allows endotoxin absorption into the circulation, as demonstrated by the elevated endotoxin levels registered in 11/13 horses developing Obel grade 3 laminitis following CHO overload (Sprouse *et al.* 1987). The role of endotoxemia in the development of laminitis has been further supported by results of a study investigating factors associated with the development of laminitis in hospitalized horses (Parsons *et al.* 2007). This study revealed that horses suffering from clinically evident endotoxemia have an odds ratio of 5 to develop laminitis when compared with horses that have no clinical signs of endotoxemia (Parsons *et al.* 2007). Additionally, an *in vitro* study implicated the streptococcal pyrogenic exotoxin B in the development of laminitis by demonstrating its ability to trigger laminar separation in equine hoof explants (Mungall *et al.* 2001).

The expression of extracellular MMPs, notably MMP-2 and MMP-9, is significantly higher in hoof laminar tissues obtained from laminitic horses versus hoof laminar tissues obtained from non-laminitic animals (Johnson *et al.* 1998). Endotoxin or inflammatory cytokine mediated activation of these gelatinases may further explain the strong association between endotoxemia and laminitis (Bailey *et al.* 2004; Johnson *et al.* 1998).

The vascular theory suggests that laminitis is a consequence of digital ischemia and subsequent reperfusion (Adair *et al.* 2000; Menzies-Gow *et al.* 2005). Equine digital smooth muscle cells exposed to endotoxin demonstrated an increased expression of

COX-2 mRNA (Rodgerson *et al.* 2001). Vasoactive prostanoids, produced via the COX-2 pathway, may be important in the vasoconstrictive events preceding laminitis (Rodgerson *et al.* 2001). Concentrations of ET-1, a potent vasoconstrictor, were also increased in laminar connective tissue of horses suffering from laminitis (Katwa *et al.* 1999). Decreased digital blood flow and high plasma ET-1 concentrations were found in horses with severe endotoxemia as well, providing yet another example of how endotoxemia and laminitis may be interconnected (Menzies-Gow *et al.* 2005). Microvascular thrombosis, demonstrated in ponies with carbohydrate-induced laminitis, likely contributes to alterations in digital blood flow (Weiss *et al.* 1994). Lipopolysaccharide-mediated activation of phospholipase A_2 increases cell membrane perturbation causing cell fragmentation (Stief 2007). These cell fragments subsequently activate the intrinsic (contact) pathway of the coagulation cascade by facilitating the conversion of prekallikrein to kallikrein (Stief 2007). Endotoxin has been shown to enhance tissue factor expression on equine peritoneal macrophages, which activates the extrinsic pathway of the coagulation cascade (Barton *et al.* 1996). In conclusion, endotoxin-mediated activation of both arms of the coagulation cascade is likely to contribute to the development of laminitis by altering digital blood flow.

1.2.7 Endotoxin tolerance and endotoxin hyporesponsiveness:

Repeated exposure to endotoxin can lead to the development of tolerance, which is reflected by a characteristic decrease in TNFα concentration (West and Heagy 2002). Endotoxin tolerance can be divided into early and late tolerance (Hardie and Kruse-Elliott 1990b). In one study, early endotoxin tolerance developed after lipopolysaccharide was administered twice 24 h apart (Allen *et al.* 1996). After the second injection, horses showed a decreased activity, lower concentration of TNFα, shorter duration of elevated rectal temperature, and smaller rise in blood pressure (Allen *et al.* 1996). Macrophages obtained from horses with acute gastrointestinal disease also produce significantly less TNFα *in vitro* when compared with similarly treated macrophages obtained from healthy animals (Barton *et al.* 1996). Early endotoxin tolerance seems to be a plausible

explanation for this latter phenomenon. During early phase endotoxin tolerance, impaired NF-κB activation and translocation, and altered IL-1 receptor-associated kinase and MAPK functions were demonstrated, leading to reduction in TNFα, IL-6, and IL-8 levels, while prostaglandin E_2 and IL-10 concentrations increased (West and Heagy 2002). LPS exposure induces translocation of p65 and p50 subunits of NFκB from the cytosol to the nucleus in both LPS responsive and LPS tolerant THP-1 cells (Chan *et al.* 2005). However, NFκB p65 binding to the IL-1β promoter is rapidly and transiently increased in LPS responsive monocytes whereas it is significantly limited in LPS tolerant cells. Concomitant alterations in chromatin remodeling are also present in LPS tolerant cells (Chan *et al.* 2005). Endotoxin-mediated overexpression of the dual specific phosphatase MKP-1 has been shown to contribute to the development of endotoxin tolerance by negatively regulating the p38 MAPK, which is an enzyme that is important for the post-transcriptional stabilization of TNFα mRNA (Nimah *et al.* 2005). While it has been shown in humans that early tolerance lasts only for a few days, late tolerance extends for several weeks (Hardie and Kruse-Elliott 1990b). Late tolerance is O-antigen dependent and may be mediated by specific antibodies (Hardie and Kruse-Elliott 1990b).

The ability of an individual to respond to endotoxin is highly variable (Arbour *et al.* 2000). Common, co-segregating missense mutations affecting the TLR4 receptor have been shown to be responsible for differences in the response to inhaled LPS in men (Arbour *et al.* 2000). Large individual differences in the response to LPS have also been documented in peripheral blood mononuclear cells and whole blood and alveolar macrophages obtained from horses (Werners *et al.* 2006). Genotyping TLR-4, myeloid differentiation factor 2, and CD-14 receptors in 10 horses, including 3 horses with significantly lower LPS-induced TNFα responses, revealed mutations involving the sequence of the TLR4 receptors (Werners *et al.* 2006). However, these changes could not be linked to the hyporesponsiveness to LPS (Werners *et al.* 2006). No mutations were found to affect the genes coding for either the myeloid differentiation factor 2 or CD-14 receptors, suggesting that these are more highly conserved structures (Werners *et al.* 2006).

CHAPTER 2

Effects of an intravenous endotoxin challenge on glucose and insulin dynamics in horses

This chapter is a revised version of a paper by the same name published in the *American Journal of Veterinary Research* in 2008 by Ferenc Tóth, Nicholas Frank, Sarah B. Elliott, Raymond J. Geor and Raymond C. Boston.

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2.1 Introduction

Endotoxin is a heat-stable lipopolysaccharide (**LPS**) located within the outer membrane of Gram-negative bacteria that can be released as a result of rapid bacterial growth or cell death (Morris 1991; Werners *et al.* 2005). In healthy animals, endotoxin is located within the intestines and is contained there by the mucosal barrier and mucus layer (Werners *et al.* 2005). If endotoxin enters the portal blood system via active transport or passive diffusion from the intestinal tract, LPS molecules are removed from circulation by the Kupffer cells of the liver (Morris 1991). Clinical signs of endotoxemia develop when body defenses are compromised and blood endotoxin concentration increases. In horses, endotoxemia is most commonly associated with gastrointestinal tract disturbances such as colic and colitis that involve bacterial overgrowth and increased intestinal wall permeability (French *et al.* 2002; Larsen 1997; Morris and Moore 1989). Endotoxemia develops when the mucosal barrier is compromised and there is movement of endotoxin into the blood and lymphatic system, or when endotoxin passes through the serosa into the peritoneal cavity (French *et al.* 2002; Larsen 1997; Morris and Moore 1989). Horses that develop retained fetal membranes, pleuropneumonia, wound

infections, or Gram-negative bacteremia are also at risk for endotoxemia (Morris 1991; Werners *et al.* 2005).

Insulin resistance is a state in which normal concentrations of insulin fail to elicit a normal physiologic response (Kahn 1978), and this condition develops as a consequence of endotoxemia in humans (Agwunobi *et al.* 2000) and rats (Virkamaki and Yki-Jarvinen 1994). This disturbance in glucose metabolism in horses is a concern because of its putative link with laminitis. Administration of a high dose of LPS (125 μg/kg) to ponies induces hyperglycemia within the first hour, followed by significant hypoglycemia compared to values registered before endotoxin administration, but plasma insulin concentrations remained unchanged (Ewert *et al.* 1985; Fessler *et al.* 1982). Effects of endotoxemia on insulin sensitivity are relevant because insulin resistance (**IR**) is a risk factor for pasture-associated laminitis in ponies (Treiber *et al.* 2006b), and there is *in vitro* evidence that hoof laminar tissues require an adequate supply of glucose to maintain structural integrity (Pass *et al.* 1998). If IR is an important risk factor for laminitis, then endotoxemia may exacerbate this disturbance in glucose metabolism and further increase the risk of disease.

Administration of exogenous LPS is used to experimentally induce endotoxemia but does not represent development of the disease clinically, which may explain why laminitis has not developed in LPS-treated horses (Barton *et al.* 2004; Doherty *et al.* 2003; Kiku *et al.* 2003; MacKay *et al.* 1999; Moore *et al.* 1981). However, endotoxemia may still play a role in the development of laminitis, and this method allows physiologic responses to LPS administration to be isolated and studied. Endotoxemia has been associated with acute laminitis in two studies (Parsons *et al.* 2007; Sprouse *et al.* 1987), although a causal relationship has not been established. Parsons *et al*. (2007) retrospectively evaluated horses that developed acute laminitis during hospitalization. This group determined that the odds of a horse with clinical endotoxemia developing acute laminitis during hospitalization were 5 times those of a horse with no clinical signs of endotoxemia. However, endotoxemia was only inferred from clinical signs and not confirmed by endotoxin assay. Sprouse *et al*. (1987) also reported that plasma endotoxin

concentrations significantly increased in 11 of 13 horses that developed Obel grade 3 laminitis after alimentary carbohydrate overload.

Clinical signs consistent with endotoxemia have been observed in horses during the development of laminitis after oligofructose administration (van Eps and Pollitt 2006). Horses that received 10 g of oligofructose/kg via intragastric administration developed diarrhea within 12 hours and had signs of depression, inappetence, fever, and tachycardia within the 48-h period following treatment (van Eps and Pollitt 2006). Mean white blood cell (**WBC**) and neutrophil counts initially decreased, but then increased within 48 hours of oligofructose administration. These findings suggest that endotoxemia contributes to the development of laminitis associated with carbohydrate overload in horses (Bailey *et al.* 2004). Carbohydrate overload causes an overgrowth of intestinal bacteria that lowers the intraluminal pH and increases intestinal wall permeability (Al Jassim *et al.* 2005; Elliott and Bailey 2006; Harris *et al.* 2006; van Eps and Pollitt 2004). Endotoxemia detected during the development of carbohydrate-induced laminitis may reflect this increase in intestinal wall permeability (Sprouse *et al.* 1987).

The purpose of the study reported here was to evaluate the effects of endotoxin administered intravenously on glucose and insulin dynamics in horses. We hypothesized that endotoxemia would induce alterations in glucose and insulin dynamics in horses. The frequently sampled intravenous glucose tolerance test (**FSIGTT**) was elected to test our hypothesis because data from this test can be assessed via minimal model analysis. The minimal model of glucose and insulin dynamics is a nonlinear model that uses data obtained from the FSIGTT to partition glucose disposal into glucose- and insulin-mediated fractions (Kronfeld *et al.* 2005a). It provides values for glucose effectiveness (**Sg**), insulin sensitivity (**SI**), and acute insulin response to glucose (**AIRg**; Hoffman *et al.* 2003; Kronfeld *et al.* 2005a; Treiber *et al.* 2006a). Glucose effectiveness describes the capacity of glucose to mediate its own disposal, whereas SI represents the ability of insulin to promote glucose disposal and decrease endogenous glucose production. The acute insulin response to glucose is a measure of initial phase endogenous insulin secretion in response to exogenous glucose. The ability of pancreatic beta cells to

increase insulin secretion in response to decreased insulin sensitivity is represented by the DI (Bergman 2005).

2.2 Materials and Methods:

Horses – Sixteen healthy mares from the University of Tennessee teaching and research herd were evaluated during the study period (February to June 2006). Horses were admitted to the University of Tennessee Large Animal Hospital in pairs, and each pair of horses remained hospitalized for 14 days. Only mares were selected to eliminate differences attributable to sex. The horses were 4 to 12 years old (mean age, 9.1 years; median, 9.5 years); breeds included mixed (n = 8), Quarter horse (3), Tennessee Walking Horse (1), Appaloosa (1), and Paint (3). Horses were weighed at the time of admission; weights ranged from 436 kg to 563 kg (mean weight, 493.6 kg; median, 498.6 kg). Body condition score (on a scale of 1 to 9) ranged from 4 to 6 (Henneke *et al.* 1983). The study protocol was approved by the University of Tennessee Institutional Animal Care and Use Committee.

Experimental design – A randomized crossover study design with repeated measures was used; each horse received the LPS and control treatments. Eight horses were randomly selected to receive an injection of LPS during the first of the 2-week period and then an injection of sterile saline (0.9% NaCl) solution (control treatment) alone the second week; the remaining 8 horses received the LPS and control treatments in the reverse order. Horses were weighed and physical examinations were performed on the first day (Friday), and then each horse was housed separately in 3.7 X 3.7-m stalls within the veterinary teaching hospital. Grass hay and water were provided *ad libitum* and each horse was acclimated to its new environment for approximately 72 hours. Horses were evaluated during each of the 2 study weeks; each week, procedures were performed according to the same schedule. On the first day of the week (Monday), an IV catheter was placed and a sham FSIGTT was performed. An FSIGTT was performed 24 hours before treatment (Tuesday; baseline [–24 hours]), and the infusion of LPS or saline solution was administered between 1200 and 1230 on Wednesday (designated time = 0).

Frequently sampled IV glucose tolerance test procedures were performed 24 (Thursday) and 48 hours (Friday) after treatment. All FSIGTTs were performed between 12:00 and 3:00 PM. Intravenous catheters were removed at the end of each study week.

Lipopolysaccharide administration – *Escherichia coli* O55:B5 LPS was mixed with 60 mL of sterile saline (0.9% NaCl) solution under a fume hood by an investigator (SBE) wearing gloves and a respirator to minimize exposure. The LPS solution (20 ng/kg) or 60 mL of saline solution alone was infused via the IV catheter during a 30-minute period. Horses were observed for signs of colic and physical examination variables, including rectal temperature, heart rate, respiratory rate, mucous membrane color, and capillary refill time with the results recorded every 15 min for the first 3 h, then every 30 min for the following 3 hours, and then every 2 hours for 18 hours.

CBC analysis – Blood was collected from the indwelling jugular catheter into tubes containing EDTA before the LPS or saline solution infusion was initiated (commenced at time = 0) and 3 hours later (ie, 2.5h after completing the LPS infusion). Samples were immediately transported to the clinical pathology laboratory for complete blood count (**CBC**) analysis.

FSIGTT procedure – On the first day of each study week, each horse was weighed and a 14-gauge polypropylene catheter[1] was inserted into the left jugular vein. During tests, the horse was allowed access to grass hay and water ad libitum. Patency of the IV catheter was maintained between tests by injection of 5 mL of saline solution containing heparin into the catheter every 6 hours. An injection cap and infusion set[2] (length, 30 cm; internal diameter, 0.014 cm) were attached to the catheter. The FSIGTT procedure first described for use in horses by Hoffman *et al.* (2003) was used. Briefly, a bolus (300 mg of glucose/kg) of a 50% (wt/vol) dextrose solution[3] was administered to each horse via the infusion line and catheter, followed by injection of saline solution containing heparin (4 U/mL). Blood samples were collected via the catheter immediately before and 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, and 19 min after infusion of dextrose. At

[1] Abbocath-T 14G X 140 mm, Abbott Laboratories, North Chicago, Ill.
[2] Butterfly, Abbott Laboratories, North Chicago, Ill.
[3] Dextrose 50% injection, Abbott Laboratories, North Chicago, Ill.

20 min, regular insulin[4] (30 mU/kg) was administered followed by another infusion of saline solution containing heparin. Blood samples were subsequently collected via the catheter at 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150, and 180 min after the dextrose infusion. At each time point, 3 mL of blood was withdrawn from the infusion line and discarded. A 6-mL blood sample was then collected, followed by infusion of 5 mL of saline solution containing heparin. Half the volume of the blood sample was transferred to a tube containing sodium heparin, which was immediately cooled on ice and then refrigerated. The remaining blood was transferred to a tube containing no anticoagulant. Those samples were allowed to clot at 22°C for 1h and then serum was harvested via low-speed (1,000 × *g*) centrifugation. Plasma and serum samples were stored at –20°C until further analyzed.

Plasma glucose and serum insulin concentrations – Plasma glucose concentrations were measured by use of a colorimetric assay[5] on an automated discrete analyzer[6]. Serum insulin concentrations were determined by use of a radioimmunoassay[7] that has been validated for use in horses (Freestone *et al.* 1991). Each sample was assayed in duplicate and intra-assay coefficients of variation < 5% or < 10% were required for acceptance of glucose and insulin assay results, respectively.

Interpretation of FSIGTT data by use of the minimal model – Values of SI, Sg, AIRg, and disposition index (**DI**) were calculated for each FSIGTT in accordance with the minimal model (Bergman *et al.* 1981) by use of commercially available software[8,9] and previously described methods (Hoffman *et al.* 2003). Disposition index was calculated via multiplication of AIRg by SI.

Statistical analysis – Horses were classified as responders or nonresponders according to their response to endotoxin, and groups were compared by use of the nonparametric Mann-Whitney *U* test. Mixed-model ANOVA for repeated measures was performed by use of statistical software[10] to determine the effects of treatment (LPS vs

[4] Humulin R, Eli Lilly & Co, Indianapolis, Ind.
[5] Glucose, Roche Diagnostic Systems Inc, Somerville, NJ.
[6] Cobas Mira, Roche Diagnostic Systems Inc, Somerville, NJ.
[7] Coat-A-Count insulin, Diagnostic Products Corp, Los Angeles, Calif.
[8] MinMod Millenium, version 6.10, Raymond Boston, University of Pennsylvania, Kennet Square, Pa.
[9] Stata 9.2, Stata Corp, College Station, Tex.
[10] PROC MIXED, SAS, version 9.1, SAS Institute Inc, Cary, NC.

saline solution) and time (−24, 24, or 48 hours) on Sg, SI, AIRg, and DI. When a significant treatment × time effect was detected, the Bonferroni test for multiple comparisons was used to identify significant differences between least squares means. Significance was defined at a value of $P < 0.05$.

2.3 Results

The response to endotoxin was determined via observation of signs of colic and detection of leukopenia, which was defined as WBC count < 5.6 X 10^3 WBCs/μL (Duncan *et al.* 1994) at 3 hours after LPS administration. Compared with horses that were classified as nonresponders (n = 3), rectal temperature, heart rate, WBC count, percentage decrease (from baseline) in WBC count, and neutrophil count differed significantly in horses that were identified as responders (n = 13; **Table 2.1**).

In responders, signs of colic detected within 3 hours of LPS administration included pawing, rolling, and stretching. Nonresponders did not develop signs of colic. Frequently sampled intravenous glucose tolerance tests were successfully performed, and mean ± SE plasma glucose and serum insulin concentrations were calculated (**Figures 2.1 and 2.2**). Data from a single FSIGTT performed 48 hours after LPS administration in 1 horse were excluded from the statistical analysis because the DI value was clearly an outlier and had a studentized residual (adjusted by dividing it by an estimate of its SD) of 7.44.

Compared with the control treatment, injection of LPS significantly decreased SI (time × treatment; $P = 0.040$) and increased AIRg (time × treatment; $P = 0.006$) over time **(Table 2.2)**. Mean ± SD SI significantly decreased from 2.9 ± 1.9 × 10^{-4} L•min^{-1}•mU^{-1} at baseline (ie, prior to LPS administration) to 0.9 ± 0.9 × 10^{-4} L•min^{-1}•mU^{-1} after 24 hours (69% reduction); however, the value at 48 hours did not differ significantly from the pretreatment value. Mean AIRg significantly increased from 520 ± 196 mU•min•L^{-1}at –24 hours to 938 ± 620 mU•min•L^{-1} and 712 ± 400 mU•min•L^{-1} at 24 and 48 hours after LPS treatment, respectively. After LPS treatment, mean DI significantly (time × treatment; $P = 0.024$) decreased from the baseline value of 14 ± 10 × 10^{-2} to

Table 2.1 – Mean ± SD physical examination variable and WBC count data in 16 horses that received *Escherichia coli* O55:B5 LPS in 60 mL of sterile physiologic saline (0.9% NaCl) solution (20 ng/kg) administered IV and developed signs of colic and leukopenia (responders) or did not respond to treatment (nonresponders).

Variable	Nonresponder group (n = 3)	Responder group (n = 13)
Maximal heart rate (beats/min)	49 ± 7	67 ± 17*
Maximal rectal temperature (ºC)	38.2 ± 0.6	39.1 ± 0.5*
Decrease in WBC count at 3 hours after treatment, compared with pretreatment value (%)	9.4 ± 5.1	45.1 ± 11.1*
WBC count at 3 hours after treatment ($\times 10^3$ cells/μL)	7.4 ± 1.4	4.0 ± 0.9*
Neutrophil count ($\times 10^3$ cells/μL)	5.5 ± 0.7	2.3 ± 0.8*

Leukopenia was defined as WBC count < 5.6×10^3 WBCs/μL. Reference range for heart rate: 28-40 beats/min. Reference range for rectal temperature: 37.8-38.8 ºC. Reference range for neutrophil count: 2.9-8.5 × 10^3 cells/μL.

*For this variable, value was significantly (Mann-Whitney *U* test; $P < 0.05$) different from that of the nonresponder group.

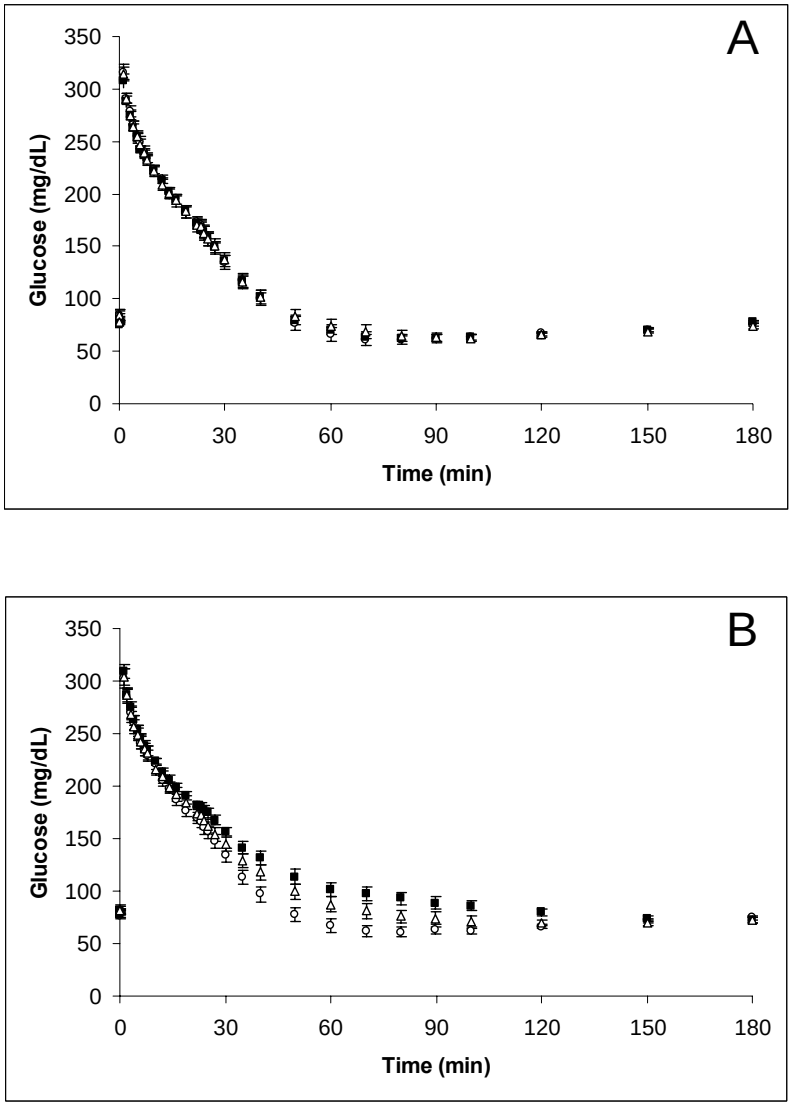


Figure 2.1 – Mean ± SE plasma glucose concentrations during FSIGTTs performed in 16 horses that received an IV infusion of saline (0.9% NaCl) solution (60 mL) or Escherichia coli O55:B5 LPS (endotoxin; 20 ng/kg) in 60 mL of saline solution during the first study week according to a randomized crossover design. Tests were conducted 24 hours before (white circles) and 24 (black squares) and 48 hours (white triangles) after administration of saline solution (panel A) or LPS (panel B). Values derived at 48 hours after LPS infusion are calculated from 15 horses.

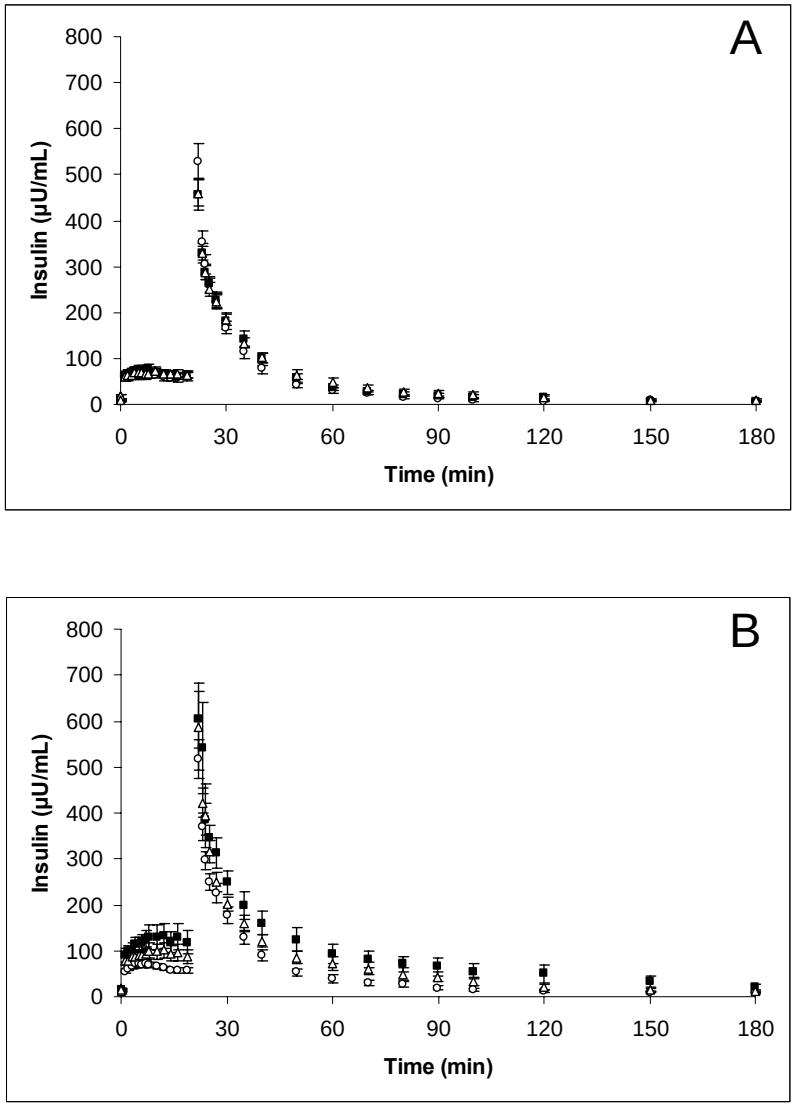


Figure 2.2 – Mean ± SE serum insulin concentrations during FSIGTTs performed in 16 horses that received an IV infusion of saline (0.9% NaCl) solution (60 mL) or Escherichia coli O55:B5 LPS (endotoxin; 20 ng/kg) in 60 mL of saline solution during the first study week according to a randomized crossover design. Tests were conducted 24 hours before (white circles) and 24 (black squares) and 48 hours (white triangles) after administration of saline solution (panel A) or LPS (panel B). Values derived at 48 hours after LPS infusion are calculated from 15 horses.

Table 2.2 – Mean ± SD minimal model analysis values obtained from FSIGTT data derived from 16 horses that received an IV infusion of physiologic saline solution (60 mL; control treatment) or E coli O55:B5 LPS in 60 mL of physiologic saline solution (20 ng/kg) during each week of a 2-week study period according to a randomized crossover design. The FSIGTTs were conducted 24 hours before and 24 and 48 hours after administration of the injection.

Variable	Treatment	Time point		
		Before treatment	**24 hours**	**48 hours ***
SI ($L \cdot min^{-1} \cdot mU^{-1}$) $\times 10^{-4}$	Control	3.0 ± 1.9^{a}	$2.7 \pm 1.5^{a,b}$	$2.7 \pm 1.3^{a,b}$
	LPS	$2.9 \pm 1.9^{a,b}$	0.9 ± 0.9^{c}	$1.5 \pm 0.9^{b,c}$
Sg (min^{-1}) $\times 10^{-2}$	Control	2.3 ± 0.6^{a}	2.5 ± 0.8^{a}	2.4 ± 0.8^{a}
	LPS	2.3 ± 0.6^{a}	1.9 ± 0.6^{a}	2.1 ± 0.7^{a}
AIRg ($mU \cdot min \cdot L^{-1}$)	Control	503 ± 260^{a}	$594 \pm 319^{a,b}$	$537 \pm 275^{a,b}$
	LPS	$520 \pm 196^{a,b}$	938 ± 620^{c}	$712 \pm 400^{c,b}$
DI $\times 10^{-2}$	Control	13 ± 8^{a}	14 ± 6^{a}	13 ± 6^{a}
	LPS	14 ± 10^{a}	6 ± 5^{b}	$10 \pm 9^{a,b}$

* Mean ± SD values for the endotoxin group are calculated from data derived from 15 horses.

[a-c] For variables with significant ($P < 0.05$) treatment × time effects, mean values with different superscripts differ significantly, as determined by use of an ANOVA for repeated measures and via comparison of least squares mean values by use of a Bonferroni adjustment. Treatment-time effects were detected for SI ($P = 0.041$), AIRg ($P = 0.006$), and DI ($P = 0.024$).

$6 \pm 5 \times 10^{-2}$ at 24 hours after endotoxin administration. No significant treatment × time effects were detected for Sg.

In the 3 horses that did not develop a clinical response to endotoxin administration, mean SI increased by 12% and then decreased by 27% at 24 and 48 hours following LPS infusion, respectively, compared with the value before treatment. Compared with the pretreatment value, mean AIRg was decreased by 2% and increased by 7% at 24 and 48 hours after injection, respectively; mean Sg was decreased by 36% and increased by 14% at 24 and 48 hours after injection. The DI values did not increase or decrease from baseline by > 1% after LPS administration. After the completion of the study horses were returned to the University of Tennessee teaching and research herd.

2.4 Discussion

In the present study, 13 of 16 healthy horses that received *Escherichia coli* O55:B5 LPS via IV infusion at a dose of 20 ng/kg developed signs of mild colic and leukopenia (ie, WBC count < 5.6 X 10^3 WBCs/μL). At 24 hours after LPS administration, insulin sensitivity was significantly lower than the value prior to treatment and AIRg increased to compensate for this alteration in glucose dynamics.

Signs of colic, fever, tachycardia, and leukopenia were detected in the horses that responded to LPS in the present study, and these findings are consistent with those of previous reports (Barton *et al.* 2004; Doherty *et al.* 2003; Kiku *et al.* 2003; MacKay *et al.* 1999; Moore *et al.* 1981). In 1 study (MacKay *et al.* 1999), IV administration of *Escherichia coli* O55:B5 LPS at a dose of 30 ng/kg decreased the WBC count, compared with baseline values, with the nadir occuring at 2 hours after infusion. Horses developed mild restlessness, sweating, increased respiratory effort, and tachypnea in response to LPS; mean rectal temperature increased over time, and a peak value > 39°C (102.2°F) was detected 4 hours after initiation of the endotoxin infusion. Plasma interleukin-6 and TNFα activities were also increased in treated horses. In another study (Barton *et al.* 2004), *E coli* O26:B5 LPS was administered to horses at the dose used in the present study (20 ng/kg). In those horses, mean rectal temperature was approximately 39°C at 3

hours after initiation of the LPS infusion; mean heart rate also significantly increased from baseline values, and peaked at approximately 50 beats/min after 1.5 hours. In that same study, neutrophil counts were assessed every 30 minutes and the lowest mean value (< 3,000 cells/μL) was detected 1 hour after LPS infusion. However, similar to the finding in the responder group of the present study, the mean neutrophil count remained less than the pretreatment value for 3 hours after LPS was infused.

In the study reported here, 3 horses failed to develop signs of colic or leukopenia. This lack of response may be attributable to circulating anti-endotoxin antibodies or genetic polymorphism within intracellular LPS signal transduction pathways (Arbour *et al.* 2000; Hardie and Kruse-Elliott 1990b). Toll-like receptor-4, myeloid differentiation factor-2, and cluster differentiation factor-14 can all be affected (Arbour *et al.* 2000). In humans, missense mutations affecting the extracellular domain of the Toll-like receptor 4 are associated with a diminished response to inhaled LPS (Arbour *et al.* 2000). However, a common mutation was not identified when this hypothesis was tested in horses that had blunted responses to LPS (Werners *et al.* 2006).

Glucose and insulin dynamics were assessed in horses by use of FSIGTT and minimal model analysis procedures in the present study. These methods have been previously used in horses and are preferred over the EHC technique, which is alternative method of quantifying SI (Hoffman *et al.* 2003; Kronfeld *et al.* 2005a; Pratt *et al.* 2005). One advantage of the minimal model is that it provides measures of the pancreatic response (AIRg) and ability of glucose to mediate its own disposal (Sg), as well as SI (Bergman 2005). The DI, which represents the ability of pancreatic beta cells to compensate for decreased insulin sensitivity, can also be calculated (Hoffman *et al.* 2003). Measures of AIRg, Sg, and DI are not provided by use of the EHC technique. The EHC technique is also more difficult to perform from a technical standpoint because an infusion line must remain connected to each horse throughout the 2- to 3-hour study period (Pratt *et al.* 2004), which provides more opportunity for equipment failure and stress.

Insulin sensitivity values prior to LPS infusion and those determined in horses during the week that they received the control treatment compared favorably with results

of previous studies (Hoffman *et al.* 2003; Treiber *et al.* 2005b). A mean SI value of 1.9×10^{-4} $L \bullet min^{-1} \bullet mU^{-1}$ was reported for nonobese Thoroughbred geldings (Hoffman *et al.* 2003) and the mean value for a group of 46 horses was 2.1×10^{-4} $L \bullet min^{-1} \bullet mU^{-1}$ (Treiber *et al.* 2005b). In contrast, lower mean SI values of 0.39×10^{-4} $L \bullet min^{-1} \bullet mU^{-1}$ and 0.08×10^{-4} $L \bullet min^{-1} \bullet mU^{-1}$ have been detected in healthy ponies and ponies that had previously had laminitis (Treiber 2005), which suggests that insulin sensitivity is generally lower in ponies than horses (Jeffcott *et al.* 1986; Rijnen and van der Kolk 2003). Mean SI significantly decreased following LPS administration in the horses of the present study. At 24 hours after treatment, mean SI was significantly lower than the mean value before LPS infusion; at 48 hours after treatment, mean SI was still lower than the mean value before LPS infusion, but this difference was not significant. A biphasic glucose response has been detected after LPS administration in humans (Agwunobi *et al.* 2000). Glucose infusion rates during EHC procedures were significantly higher 120 minutes after LPS was administered, compared with saline controls, but then progressively decreased and were significantly lower than what was registered in saline controls at 420 minutes after LPS administration. Endotoxemia also induces IR in rats, as evidenced by a 37% decrease in glucose disposal following LPS administration (Virkamaki and Yki-Jarvinen 1994). At the tissue level, continuous LPS infusion significantly decreased insulin receptor abundance, inhibited insulin-stimulated tyrosine phosphorylation of insulin receptors, and decreased the number of IRS-1 molecules within liver tissues collected from treated rats (McCowen *et al.* 2001). In contrast, only tyrosine phosphorylation of IRS-1 molecules was significantly affected in rat skeletal muscle tissue. Results of those studies suggest that IR can be rapidly induced through interference with tissue insulin signaling pathways; however, in those experiments, rats received markedly larger doses of LPS than those administered to horses in the study reported here.

Increases in serum TNFα activity have been detected in the plasma of horses following the experimental induction of endotoxemia, and this cytokine may mediate the development of IR in equids (Barton *et al.* 2004; MacKay *et al.* 1999; Rui *et al.* 2001). Results of a study (Rui *et al.* 2001) involving rodents indicated that TNFα induces serine phosphorylation of IRS-1, which disrupts the interaction of IRS-1 with the catalytic

domain of the insulin receptor and inhibits insulin-stimulated activation of the phosphatidylinositol 3-kinase cascade. Alternatively, IR may develop in horses after LPS administration because of increases in circulating cortisol concentrations. Administration of LPS significantly increases cortisol concentrations in ponies, and pretreatment of rat adipocytes with glucocorticoids inhibits insulin-mediated glucose uptake *in vitro* (Ewert *et al.* 1985; Fessler *et al.* 1982; Kawai *et al.* 2002). Increased catecholamine concentrations have also been detected in humans after injection of LPS and this response may lower SI (Krabbe *et al.* 2001). In an *in vitro* study (Hunt and Ivy 2002) of isolated rat muscle, physiologic concentrations of epinephrine inhibited insulin-mediated glucose uptake into tissues by modulating activation of IRS-1–associated phosphatidylinositol 3-kinase. Intravenous administration of epinephrine has also been shown to delay the ability of insulin to inhibit endogenous glucose production in humans (Vicini *et al.* 2002).

Glucose effectiveness in horses was not altered by LPS administration in the present study and values were consistent with those previously reported. In nonobese and moderately obese Thoroughbred geldings, mean ± SE Sg values of $1.43 \pm 0.16 \times 10^{-2}$ and $1.59 \pm 0.19 \times 10^{-2}$ have been detected, respectively, and for 46 healthy horses, a 95% confidence interval for Sg of 0.12 to 2.95×10^{-2} has been reported (Hoffman *et al.* 2003; Treiber *et al.* 2005b). In the study reported here, mean AIRg was 83% and 45% higher than the pretreatment value at 24 and 48 hours after LPS administration, respectively. Before LPS infusion and during the week that horses received the control treatment, AIRg values were higher than the mean value of 270 $mU \bullet min \bullet L^{-1}$ detected in 46 healthy horses, but were within the 95% reference interval of 67 to 805 $mU \bullet min \bullet L^{-1}$ previously reported for this variable (Treiber *et al.* 2005b). Minimal model analysis values may vary between study populations because of differences in evaluation techniques and breed, sex, age, or diet of the study horses.

The results of our study have indicated that pancreatic beta cells of horses respond to endotoxin-induced IR by secreting more insulin. Compensated IR has been previously described in ponies and is recognized by the presence of hyperinsulinemia in chronically insulin-resistant animals (Treiber *et al.* 2006b). However, in the horses of the present study, mean DI decreased by 57% over 24 hours in response to LPS, which suggests that

this compensatory response was inadequate. More time may be required for the pancreatic beta cells to fully respond to the decrease in SI or alternatively, endotoxin may directly inhibit pancreatic function.

The data obtained in the present study may be relevant to the pathogenesis of laminitis because laminar keratinocytes appear to have a high requirement for glucose (Pass *et al.* 1998). The presence of insulin-sensitive glucose transporter 4 protein within equine hoof tissues suggests that insulin plays an important role in glucose uptake (Mobasheri *et al.* 2004). *In vitro* experiments performed with freshly isolated hoof explants have also revealed that laminae separate at the dermoepidermal junction when glucose concentrations are decreased within the tissue culture medium (Pass *et al.* 1998). Insulin resistance might also predispose horses to laminitis by inducing endothelial cell dysfunction. This may increase endothelin-1 synthesis and cause a concurrent decrease in nitric oxide production in the endothelium, resulting in vasoconstriction (Kim *et al.* 2006). It has previously been shown that endothelin-1 concentrations are higher in laminar connective tissues from horses with laminitis (Eades *et al.* 2007; Katwa *et al.* 1999). In a recent study in horses, Eades *et al*. (2007) determined that digital venous blood endothelin-1 concentrations were significantly higher than baseline values 11 hours after carbohydrate was administered to induce laminitis.

In the present study, administration of 20 ng of *E coli* O55:B5 LPS/kg decreased insulin sensitivity while enhancing the acute insulin response to glucose and did not alter glucose effectiveness in horses. Results suggest that endotoxemia will further compromise glucose and insulin dynamics in chronically insulin-resistant horses, which may place them at higher risk for development of laminitis.

2.5 Evaluation of the euglycemic hyperinsulinemic clamp procedure

The initial protocol for this study included a comparison of FSIGTT and euglycemic hyperinsulinemic clamp (**EHC**) techniques. It was originally our intent to evaluate the effects of LPS on glucose and insulin dynamics using both techniques, with 8 horses evaluated with each procedure. However, results of preliminary studies revealed

unacceptably high coefficients of variation for consecutive EHC tests. A study was therefore designed to test the hypothesis that the EHC has a low repeatability when performed multiple times consecutively with only a short interval between procedures.

2.5.1 Methods

Four adult mares were evaluated and each mare underwent four EHC procedures 24 h apart. Horses were hospitalized in pairs and acclimated to their new environment for 72 h before testing commenced. A 14-gauge intravenous catheter was inserted into each jugular vein and a sham test was performed at the end of the acclimation period. The sham test consisted of repeated blood sample collections according to the EHC protocol. The first of four consecutive EHC procedures was then initiated 24 hours after completion of the sham test. During the tests, horses remained in their stalls and were provided with grass hay *ad libitum*.

During the EHC procedure, three blood samples were collected at – 10, – 5, and 0 min before initiating of the insulin and glucose infusions, to determine baseline glucose concentrations. At t = 0, a priming insulin dose of 0.018 IU/kg was administered intravenously, immediately followed by the continuous rate infusion (**CRI)** of regular insulin at a rate of 0.003 IU/min/kg for 3h. A CRI of 50% dextrose solution was concurrently administered using a syringe pump through the contralateral jugular catheter. Blood glucose concentrations were monitored every 5 min during the 3-hour clamp using a handheld glucometer.[11] These results were used to adjust the dextrose infusion rate to maintain euglycemia. At 15 min intervals, blood samples were collected for measurement of serum insulin and plasma glucose concentrations. The first 150 min period of each clamp was considered to be an equilibration period, so data from the final 30 min was used to calculate whole-body glucose uptake (**M**), steady state insulin concentration (**I**), and their ratio (**M/I**), which is a measure of insulin sensitivity (Pratt *et al.* 2005). Steady state insulin concentrations were calculated by averaging insulin

[11] Precision Q•I•D System, MediSense, Birmingham, UK

concentrations measured during the last 30 min of the clamp. The M value was derived as follows:

$$M = GIR - SC$$

where GIR is the glucose infusion rate (mmol/kg/min) and SC equals the space correction, which is calculated using the formula:

$$SC = (G_2 - G_1) \times (0.19 \times bwt) / (T \times bwt)$$

where G_1 and G_2 are the blood glucose concentrations at the beginning and end of each 5 min time interval (T) and (0.19 × bwt) is the glucose space in liters. Coefficient of variation (**CV**) values for the M/I ratio were calculated for the 4 procedures performed in each horse.

2.5.2 Results

No adverse effects were registered during EHC procedures; however 3 of 16 EHC procedures were unsuccessful because steady state glucose concentrations could not be maintained. Coefficient of variation values calculated for successful clamps in four horses were 20.2% (n = 4), 49.6% (n = 3), 13.3% (n = 3), and 20.2% (n = 3; **Table 2.3**). Mean ± SD interday CV for M/I ratios obtained from 4 horses undergoing 13 successful EHC procedures was 25.8 ± 16.2 %.

2.5.3 Discussion

Results of this study demonstrated higher CV for the EHC procedure than the value of 14.1 ± 5.7 % previously reported by Pratt *et al.* (2005). Furthermore, a steady state glucose concentration could not be attained in 3 of 16 clamps, which represents a 19% failure rate for this procedure. Calculation of an M/I ratio was not possible in these situations. One explanation for our results may be that Pratt *et al*. (2005) restrained horses in stocks during EHC procedures, thereby limiting their activity. Conversely, mares undergoing EHC procedures in this study were kept in stalls and their activity level varied from standing calmly to pacing nervously in their stalls, over the course of a single

test. Exercise enhances glucose delivery and facilitates glucose uptake in the muscle by increasing capillary recruitment and stimulating GLUT4 translocation to the sarcolemma, respectively (Wasserman and Ayala 2005). Therefore, altered muscle glucose delivery and uptake associated with different levels of activity, provide a plausible explanation for the higher CV registered in our study. Furthermore, changes in activity frequently observed within a single EHC procedure may explain the difficulty of attaining a steady state glucose concentration during some of the clamps.

Based on these results, it is apparent that horses should be restrained in standing stocks during the EHC procedure to control for alterations in glucose and insulin dynamics associated with differences in physical activity. Additionally, horses that are not accustomed to standing in stocks and being separated from their herd mates should be trained to tolerate long-term confinement to standing stocks before EHC procedures are performed in order to prevent stress-induced changes in insulin sensitivity (Harewood 2005; Irvine and Alexander 1994; Rosmond 2005). Housing the horse with a companion during the EHC procedure may also reduce the stress associated with separation.

Results demonstrated that the EHC had a 19% failure rate and low repeatability in our research setting, so the FSIGTT was selected to evaluate the effects of endotoxemia on glucose and insulin dynamics in horses.

Table 2.3 – Ratios of whole body glucose uptake and steady state insulin concentrations (M/I) registered in four horses during four euglycemic hyperinsulinemic clamps performed 24 h apart. Coefficient of variation values are calculated for the M/I ratios for successful clamps. Euglycemic hyperinsulinemic clamps that failed, due to unsuccessful clamping of blood glucose concentrations during the last 30 min of the procedures, are indicated with the word "failed".

Horse	M/I ratio ($\times 10^{-4}$)				CV (%)
	24h clamp	48h clamp	72h clamp	96h clamp	
1	1.47	1.37	1.34	0.89	20.22
2	Failed	0.43	0.4	0.14	49.64
3	0.55	0.71	Failed	0.65	13.29
4	Failed	0.3	0.24	0.21	20.21

CHAPTER 3

Evaluation of the insulin-modified frequently sampled intravenous glucose tolerance test for use in horses.

3.1 Introduction

Insulin resistance (**IR**), is an important condition in horses, and has become a frequent subject of publications in equine medicine in recent years (Hurley and Moore 2007). Insulin resistance is a state in which normal concentrations of insulin fail to elicit a normal physiologic response (Kahn 1978). Previous studies have suggested an association between IR and various clinical conditions, including laminitis and reduced reproductive performance (Hurley and Moore 2007; Johnson 2002; Pass *et al.* 1998; Treiber *et al.* 2006a; Treiber *et al.* 2006b).

There are several methods available to evaluate glucose and insulin dynamics in the horse. Nonspecific indicators of IR include basal hyperglycemia, basal hyperinsulinemia, decreased glucose tolerance and altered results of proxy measurements (Kronfeld *et al.* 2005a; Treiber *et al.* 2005b). Ponies suffering from prelaminitic metabolic syndrome have been successfully identified using the modified insulin-to-glucose ratio and reciprocal of the square root of insulin proxy measurements (Treiber *et al.* 2006b). Data obtained from the combined glucose-insulin test (**CGIT**) has facilitated the estimation of insulin sensitivity (**SI**), glucose effectiveness (**Sg**) and net insulin response in mares (Frank *et al.* 2005a; Frank *et al.* 2005b). Nevertheless, quantitative, specific assessment of insulin sensitivity in horses can only be achieved by using the euglycemic hyperinsulinemic clamp (**EHC**), the minimal model analysis of the frequently sampled intravenous glucose tolerance test (**FSIGTT**) or the insulin suppression test (Kronfeld *et al.* 2005a). Although it was demonstrated in one study that minimal modeling of the FSIGTT has lower repeatability than the EHC (Pratt *et al.* 2005), the minimal model provides additional information about glucose and insulin

dynamics such as Sg and the acute insulin response to glucose (**AIRg**), which are not supplied by the EHC (Kronfeld *et al.* 2005a).

Minimal modeling of FSIGTT data was first applied to horses by Hoffmann *et al*. (2003), using the same technique utilized in man. Dosages of dextrose and insulin administered during this established FSIGTT are identical to those used in humans (Avogaro *et al.* 1996; Bergman *et al.* 1987; Hoffman *et al.* 2003; Trout *et al.* 2007). However, plasma glucose concentrations above 350 mg/dL are often detected during this established FSIGTT, which exceeds the estimated renal threshold for glucose of 160 to 180 mg/dL for horses (Carlson 2002).

If urinary glucose spilling is present during the established FSIGTT it is likely to influence data supplied by minimal model analysis, because urinary glucose loss is not taken into account in this model (Avogaro *et al.* 1996). The extent to which this deficiency influences minimal model indices has not yet been investigated (Avogaro *et al.* 1996). Glucose concentrations exceeding the estimated renal threshold occur early in the FSIGTT, so Sg values, calculated mainly between 8 and 20 min after glucose bolus, are most likely to be affected (Avogaro *et al.* 1996). Furthermore, plasma glucose concentrations can remain above renal threshold beyond 20 min in horses (Toth *et al.* 2008), which could influence SI values.

The pancreatic insulin response to different glucose loads has never been studied in horses. Intravenous administration of 0.33 g/kg bwt dextrose to horses as a bolus resulted in maximum glucose and insulin concentrations 1 and 2 min relative to glucose infusion, respectively, but measurements were only performed for 30 min (Giraudet *et al.* 1994). Altered pancreatic function has been associated with IR in several species, including the horse (Brunton *et al.* 2006; Hoffman *et al.* 2003; Toth *et al.* 2008), which suggests that this aspect of glucose metabolism warrants further investigation. Given that the horse has been recently proposed as a potential model for human metabolic syndrome, careful investigation of methods used for measuring insulin and glucose dynamics in horses is also warranted (Hodavance *et al.* 2007).

In the study reported here we aimed to evaluate the effects of different dosages of dextrose and insulin on the glycemic and insulin response in horses during the FSIGTT.

We also aimed to develop a new FSIGTT that minimized urine glucose spillover and compare it with the established FSIGTT and the CGIT, which is another commonly used test for IR in horses. It was hypothesized that lowering the dextrose dosage administered during the FSIGTT would eliminate urinary glucose spillover, while still providing sufficient data for minimal model analysis. We also hypothesized that marked urinary glucose spillover occurs during the established FSIGTT and CGIT.

3.2 Materials and Methods

Horses – Six mares from the University of Tennessee teaching and research herd were evaluated during the study period (February to June 2007). Horses were admitted to the teaching hospital in pairs. To eliminate differences attributable to sex, only mares were selected. Horses were 6 to 13 years of age (mean age, 9.3 years; median, 9 years) and breeds of horse included Quarter horse / Tennessee Walking horse crossbred (n = 3), Quarter horse (2) and Standardbred (1). Horses were weighed at the time of admission; weights ranged from 461 kg to 523 kg (mean weight, 497 kg; median, 501 kg) with body condition scores (on a scale of 1 to 9) ranging from 4 to 6 (Henneke *et al.* 1983). The study protocol was approved by the University of Tennessee Institutional Animal Care and Use Committee.

Experimental design – Effects of six different dextrose and insulin dosages on glucose and insulin dynamics were assessed in six mares across a 5–week period. Urine glucose spillover was also measured during the last week of the testing period. Horses were admitted to the hospital 3 days before the beginning of the study period to facilitate acclimation to their new environment. Animals were weighed and physical examinations were performed upon admission, and each horse was housed separately in 3.7-m × 3.7-m stall. Grass hay and water were provided ad libitum over the course of the study period. Mares were examined to ensure that they were not pregnant and 2.2 mg/mL altrenogest[1] (10mL; orally once a day) was administered throughout the study to control for the

[1] Regu-Mate®, Intervet Inc, Millsboro, Del

confounding effects of estrus cycle. Each week during the study, an IV catheter was placed on day 1 and left in place for 4 days.

In the first phase of the study, each horse received six different dosages of dextrose[2] (50, 100, 150, 200, 250 or 300 mg/kg bwt) on days 2, 3, 4 of weeks one and two, according to a Latin square design. Insulin was not administered during this phase of the study. Blood samples were collected in accordance with the FSIGTT protocol.

During the second phase of the study, on days 2, 3, 4 of weeks three and four, each horse was given 300 mg/kg bwt dextrose followed by a variable dosage of regular insulin[3] (5, 10, 15, 20, 25 and 30 mU/kg bwt) 20 min later, with the order of insulin administration determined by the Latin-square design. Blood samples were again collected in accordance with the FSIGTT protocol.

During the third phase of the study, on days 2, 3 and 4 of week five, each horse was given the following three different combinations of dextrose and insulin: 300 mg/kg bwt dextrose followed by 30 mU/kg insulin 20 min later (established FSIGTT), 100 mg/kg bwt dextrose followed by 20 mU/kg insulin 20 min later (new FSIGTT) and 150 mg/kg bwt dextrose and 100 mU/kg insulin administered sequentially at the beginning of the test (CGIT). The sequence of treatments was determined by a crossover study design. A 24-French Foley catheter with a 30 mL inflatable balloon was inserted into the urinary bladder and urine samples were collected immediately before dextrose administration and then every 10 min for 3 h thereafter. Blood samples were collected according to the FSIGTT and CGIT protocols. Urinary catheters were removed every day at the conclusion of the study.

After completing the first three phases of the study an additional fourth phase was designed to determine total urinary glucose loss during the established FSIGTT and new FSIGTT and CGIT in two of the mares. Urine collection was achieved using the same methodology as that described above, except that all urine produced was collected during the procedure, including urine that was voided around the catheter.

[2] Dextrose 50% injection, Abbott Laboratories, North Chicago, Ill
[3] Humulin R, Eli Lilly and Co, Indianapolis, Ind.

FSIGTT – On the first day of each study week, horses were weighed and a 14-gauge polypropylene catheter[4] was inserted into the jugular vein. Horses were given access to grass hay and water *ad libitum* during the test period. Patency of the IV catheter was maintained between tests by infusion of 5 mL of 0.9% saline solution containing heparin (4 U/mL) every 6h. Dextrose (50% wt/vol) solution was infused intravenously at six different dosages (50, 100, 150, 200, 250 and 300 mg of glucose/kg) via an infusion set[5] (length, 30 cm; internal diameter, 0.014 cm) attached to the catheter, followed by injection of saline solution containing heparin. No insulin was administered during this part of the study. Blood samples were collected via the catheter immediately before and 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16,19, 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150, and 180 min after dextrose infusion. At each time point, 3 mL blood was withdrawn from the infusion line and discarded. A 6 mL blood sample was then collected, followed by infusion of 5 mL of saline solution containing heparin. Half the volume of blood collected was transferred to a tube containing sodium heparin, which was immediately cooled on ice and refrigerated. The remaining blood sample was allowed to clot at 22°C for 3h and serum was harvested via low-speed (1,000 × *g*) centrifugation. Plasma and serum samples were stored at –20°C until further analysis.

During the third and fourth week of the study, a 300 mg/kg bolus infusion of dextrose (50% wt/vol) solution was administered to each horse via the infusion line and catheter, followed by 5 ml of saline solution containing heparin. Blood samples were collected again via the catheter immediately before and 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, and 19 min after infusion of dextrose. At 20 min, regular insulin was infused intravenously at one of six different dosages (5, 10, 15, 20, 25 and 30 mU/kg) according to the Latin square design, and then 20 mL saline solution containing heparin was infused to clear the catheter. Further blood samples were collected at 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150, and 180 minutes. Samples were handled identically to that described above.

CGIT procedure – The method first described by Eiler *et al.* (2005), was used with increased sampling frequency during the first 30 min of the procedure. At t = 0,

[4] Abbocath-T 14G X 140mm, Abbott Laboratories, North Chicago, Ill
[5] Butterfly, Abbott Laboratories, North Chicago, Ill.

dextrose solution (50% wt/vol) was infused intravenously at a dosage of 150 mg/kg bwt, immediately followed by regular insulin at a dosage of 100 mU/kg bwt via the infusion set attached to the catheter, followed by infusion of 20 mL saline solution containing heparin. Blood samples were collected via the catheter immediately before and 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16,19, 22, 23, 24, 25, 35, 45, 60, 75, 90, 105, 120, 135, 150, 165, and 180 min after dextrose infusion. Samples were handled identically to that described for the FSIGTT procedure.

Urine collection – To facilitate urine collection for the third and fourth phases of the study, a 24-French Foley catheter, with a 30 mL inflatable balloon, was inserted into the urinary bladder, and left *in-situ* during the course of procedures. In the third phase, the bladder was emptied and urine samples were collected every 10 min to measure urinary glucose concentrations.

During the fourth phase of the study, urine production was established in two mares by collecting all urine from the bladder every 10 min over the course of each test. If any urine was lost due to leakage around the Foley catheter it was collected from the floor, which was covered with a plastic sheet to facilitate collection. At the end of each procedure, urine samples from each horse were pooled, and total urine volume and urine glucose concentration were determined. Urinary glucose loss was calculated as the product of urine volume and urine glucose concentration, and expressed as a percentage of the total amount of glucose administered.

Plasma and urine glucose concentrations and serum insulin concentrations – Glucose concentrations were measured by use of a colorimetric assay[6] on an automated discrete analyzer (D'Agord Schaan *et al.* 2003). Insulin concentrations were determined by use of a radioimmunoassay[7] that has been validated for equine insulin (Freestone *et al.* 1991). Each sample was assayed in duplicate and intra-assay coefficients of variation of less than 5% or 10% were required for acceptance of glucose and insulin assay results, respectively.

Interpretation of FSIGTT data by the minimal model – Values for SI, Sg, AIRg, and disposition index (**DI**) were calculated for each FSIGTT in accordance with

[6] Glucose, Roche Diagnostic Systems Inc, Somerville, NJ.
[7] Coat-A-Count insulin, Diagnostic Products Corp, Los Angeles, Calif.

the minimal model (Bergman *et al.* 1981) by use of commercially available software[8,9] and previously described methods (Hoffman *et al.* 2003). Disposition index was calculated by multiplying AIRg by SI.

Statistical analysis – Area under the curve for glucose (**AUCg**) and area under the curve for insulin (**AUCi**) values were calculated for each treatment in each phase of the study from the glucose and insulin concentrations measured during both FSIGTT procedures and the CGIT, by use of the trapezoidal method, using commercially available computer software (SAS 9.1)[10]. During phases one and two, mixed model analysis of variance with orthogonal polynomial contrasts was used to compare AUCi and AUCg values as well as minimal model variables, including SI, Sg, AIRg and DI, among treatments. To evaluate urinary glucose spilling, urinary AUCg (**uAUCg**) values were calculated during the third phase of the study based on urine glucose concentrations, using the trapezoidal method. Mixed model analysis of variance was used to evaluate the effects of treatments on AUCi, AUCg, uAUCg, and minimal model variables. Non-normally distributed data were log transformed for statistical analysis. If normal distribution could not be achieved by transformation, then the non-parametric Mann-Whitney U test was applied. When a significant treatment × time effect was detected, a protected Least Significant Difference mean separation method for multiple comparisons was used to identify significant differences between least squares means. Results are reported as true means ± SD or medians and 25 and 75 percentiles. Correlation was calculated for AUCi and AIRg values obtained throughout the study. Linear regression was performed using urinary glucose measurements during the third phase of study to predict the time when urinary glucose spillover ceased. Significance was defined at $P < 0.05$.

[8] MinMod Millenium, version 6.10, Raymond Boston, University of Pennsylvania, Kennet Square, PA.
[9] Stata 9.2, Stata Corporation, College Station, TX.
[10] PROC MIXED, SAS, version 9.1, SAS Institute Inc, Cary, NC.

3.3 Results

No abnormalities were detected during daily physical examinations. Experimental procedures remained well tolerated by each horse, with the exception of one mare which showed mild signs of abdominal pain following insertion of the urinary catheter, necessitating its removal 80 min after insertion. Collection of a urine sample was unsuccessful at 28 of 285 time points during the study, including 10 samples lost from the mare that did not tolerate the urinary catheter. Evaluation of baseline insulin concentrations and SI values revealed that one horse included in the study was insulin resistant. The mean resting insulin concentration for this horse was 58.3 μU/mL and mean SI was determined to be 0.23×10^{-4} L•min^{-1}•mU^{-1}. Therefore, data from this horse were excluded from statistical analyses and results reported here are for 5 horses.

In the first phase of the study, a significant linear relationship ($P < 0.001$) was detected between the dextrose dosage administered and AUCg, AUCi, and AIRg values, with all three variables increasing as the dextrose dosage increased (**Table 3.1**, **Figures 3.1 and 3.2**). A plateau for AUCg was detected as dextrose dosages of 200 mg/kg or above were administered (**Figure 3.3**).

During the second phase of the study, a strong inverse linear relationship ($P = 0.023$) was apparent between insulin dosage and AUCg during the FSIGTT (**Figure 3.4)**, with the AUCg decreasing as the insulin dosage increased. However, altering the insulin dosage did not significantly affect AUCi ($P = 0.482$), SI ($P = 0.878$), Sg ($P = 0.460$), AIRg ($P = 0.731$) and DI ($P = 0.948$**; Table 3.2**).

In the third phase of the study, AUCg ($P = 0.003$) and AUCi ($P < 0.001$) differed significantly among tests (**Figure 3.5**). No significant difference was registered for SI ($P = 0.424$), Sg ($P = 0.134$), AIRg ($P = 0.502$) and DI ($P = 0.812$) when the new and established FSIGTT were compared (**Table 3.3**). A strong correlation ($R = 0.709$, $P < 0.001$) was found between AUCi and AIRg values measured throughout the study.

Urine AUCg differed significantly ($P < 0.001$) among tests, with values of 161.4 g/dL•min, 34.2 g/dL•min and 58.9 g/dL•min detected for the established FSIGTT, new FSIGTT, and CGIT, respectively (**Figure 3.5**).

Table 3.1 – Mean ± SD AUCg and AUCi values median and 25th and 75th percentile AIRg values, and obtained from 5 horses following intravenous administration of 50, 100, 150, 200, 250 and 300 mg/kg bwt 50% dextrose solution. Procedures were conducted over a period of two weeks.

Variable		Glucose dosage (mg/kg bwt)						Trt Effect *P*
		50	**100**	**150**	**200**	**250**	**300**	
AUCg (g/dL•min)	mean ± SD	17 ± 0.6 [a]	17.7 ± 0.6 [a]	18.3 ± 0.8 [a]	21.2 ± 2.5 [b]	21.3 ± 3.3 [b]	21.3 ± 2.8 [b]	< 0.001
AUCi (mU/mL•min)	mean ± SD	3.8 ± 2.5 [a]	4.4 ± 1.6 [a,b]	5.5 ± 2.9 [b]	6.3 ±3.5 [b,c]	8.3 ± 4.8 [c]	9.6 ± 6.3 [c]	< 0.001
AIRg (mU•min•L^{-1})	median	417 [a]	561 [a]	644 [b,c]	519 [b,a]	756 [c]	785 [c]	
	25th	409	504	614	482	543	711	< 0.001
	75th	523	573	686	619	787	1018	

[a-c] For variables with significant ($P < 0.05$) treatment effects, values with different superscripts differ significantly, as determined by use of an ANOVA and via comparison of least squares mean values by use of a Least Significant Difference adjustment. Nonparametric Mann-Whitney U test was used to compare AIRg values among treatments.

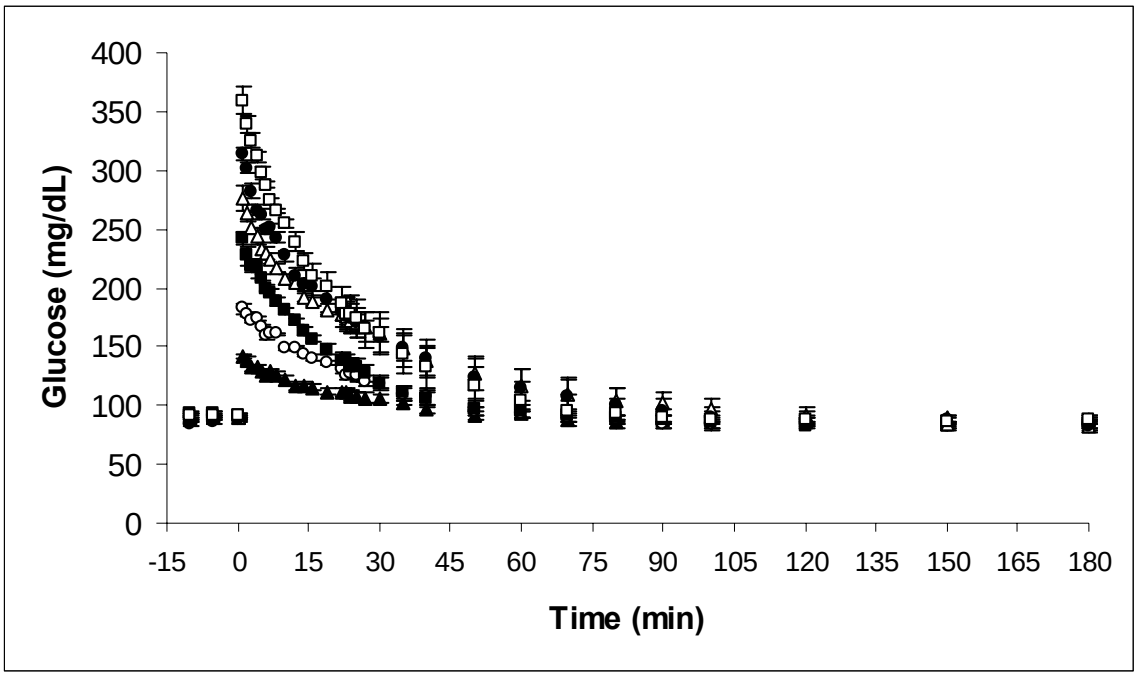


Figure 3.1 – Mean ± SE plasma glucose concentrations registered during the first phase of the study following intravenous administration of six different dosages of 50% dextrose solution to 5 horses. Dosages: 50 mg/kg (solid triangles), 100 mg/kg (open circles), 150 mg/kg (solid squares), 200 mg/kg (open triangles), 250 mg/kg (solid circles) and 300 mg/kg (open squares).

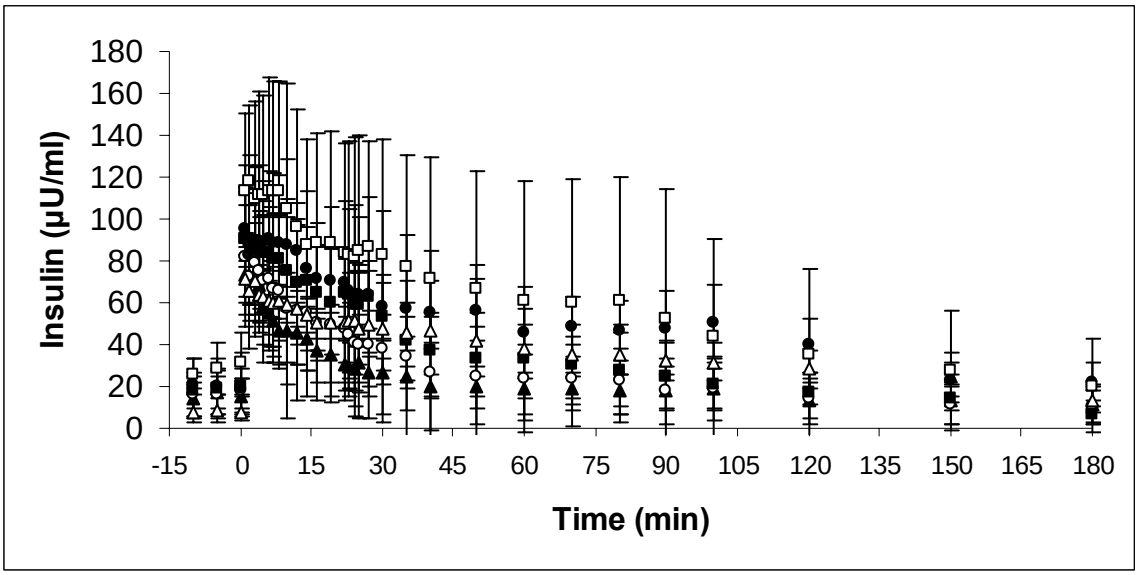


Figure 3.2 – Mean ± SE blood insulin concentrations registered during the first phase of the study following intravenous administration of six different dosages of 50% dextrose solution to 5 horses. Doses included: 50 mg/kg (solid triangles), 100 mg/kg (open circles), 150 mg/kg (solid squares), 200 mg/kg (open triangles), 250 mg/kg (solid circles) and 300 mg/kg (open squares).

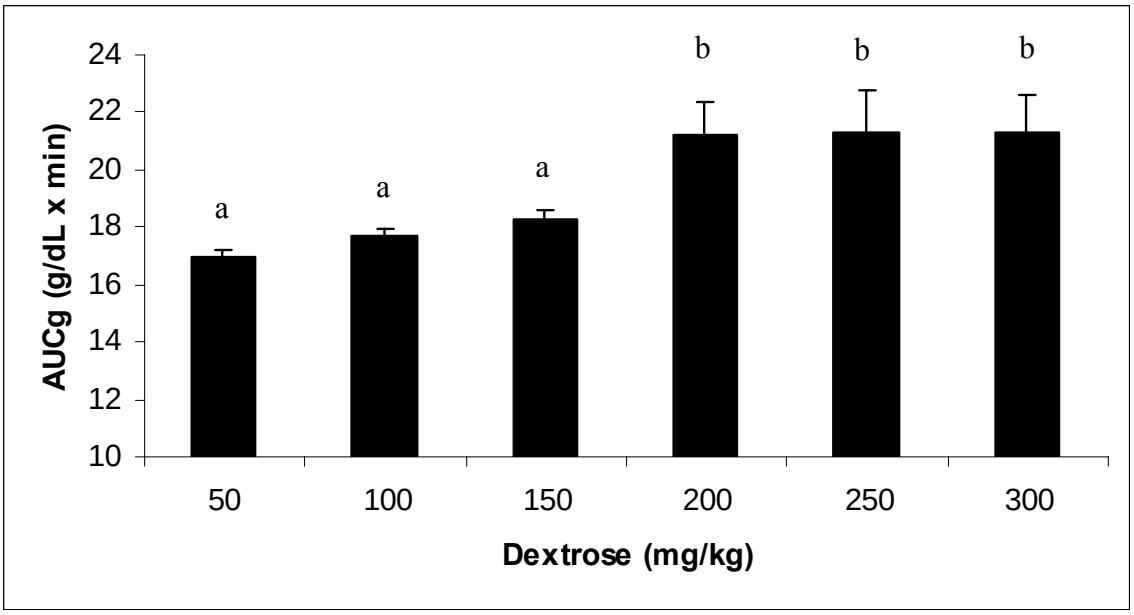


Figure 3.3 – Mean ± SE area under the curve for glucose (AUCg) values calculated during the first phase of the study following intravenous administration of six different dosages of 50% dextrose solution to 5 horses.

[a-b] Values with different superscripts differ significantly ($P < 0.05$), as determined by ANOVA and comparison of least squares mean values by use of a Least Significant Difference adjustment.

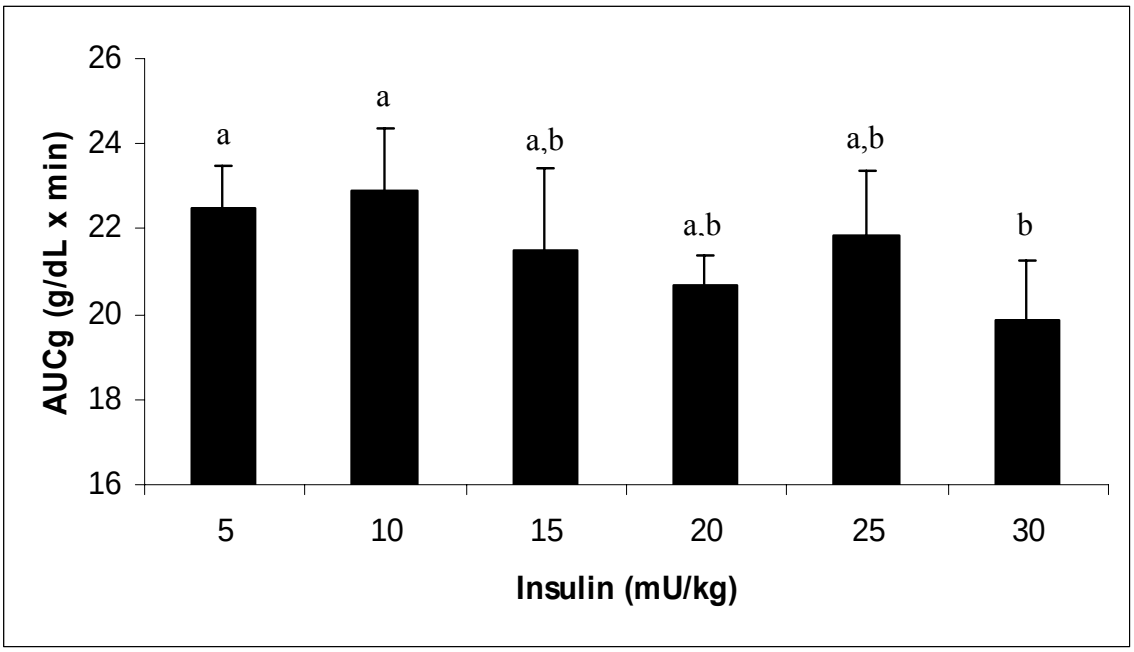


Figure 3.4 – Mean ± SE area under the curve for glucose (AUCg) values calculated in 5 horses during the second phase of the study after intravenous administration of 300 mg/kg 50% dextrose solution at t = 0 min followed by 6 different dosages of insulin (5, 10, 15, 20, 25 and 30 mU/kg) at t = 20 min.

[a-b] Values with different superscripts differ significantly ($P < 0.05$), as determined by ANOVA and comparison of least squares mean values by use of a Least Significant Difference adjustment.

Table 3.2 – Mean ± SD AUCg and AUCi values, and median and 25th and 75th percentile minimal model analysis values from 5 horses following intravenous administration of 300 mg/kg bwt 50% dextrose solution at t = 0, followed by the administration of 5, 10, 15, 20, 25 and 30 mU/kg bwt insulin at t = 20 min.

		Insulin dosage (mU/kg bwt)						Trt effect
		5	**10**	**15**	**20**	**25**	**30**	***P***
AUCg (g/dL•min)	mean ± SD	22.5 ± 2.3 [a]	22.9 ± 3.3 [a]	21.5 ± 4.3[a,b]	20.7 ± 1.5[a,b]	21.9 ± 3.4[a,b]	19.9 ± 3.1 [b]	0.026
AUCi (mU/mL•min)	mean ± SD	12.3 ± 7.2	12.1 ± 4.7	10.4 ± 4.5	12 ± 4.6	12.6 ± 6	13.6 ± 7.2	0.482
SI ($L \cdot min^{-1} \cdot mU^{-1}$) $\times 10^{-4}$	median	1.75	1.17	1.33	1.04	1.71	1.97	
	25th	1.04	0.94	0.72	1.01	0.31	0.62	0.878
	75th	3.22	1.7	1.68	1.17	2.74	2.32	
Sg (min^{-1}) $\times 10^{-2}$	median	0.52	2.45	2.92	2.07	1.77	1.95	
	25th	0.35	0.9	1.81	1.69	1.12	1.87	0.460
	75th	1.49	2.64	2.93	2.15	2.21	2.41	
AIRg ($mU \cdot min \cdot L^{-1}$)	median	588	651	549	646	654	732	
	25th	551	588	547	597	594	562	0.731
	75th	751	759	712	765	719	816	
DI $\times 10^{-2}$	median	9.63	10.44	8.36	8.59	10.18	11.09	
	25th	7.96	3.93	7.27	6.72	2.00	4.54	0.948
	75th	24.16	12.92	9.13	8.97	19.70	11.68	

[a-c] For variables with significant ($P < 0.05$) treatment effects, mean values with different superscripts differ significantly, as determined by use of an ANOVA and via comparison of least squares mean values by use of a Least Significant Difference adjustment. Nonparametric Mann-Whitney U test was used to compare SI, Sg, AIRg and DI values among treatment groups.

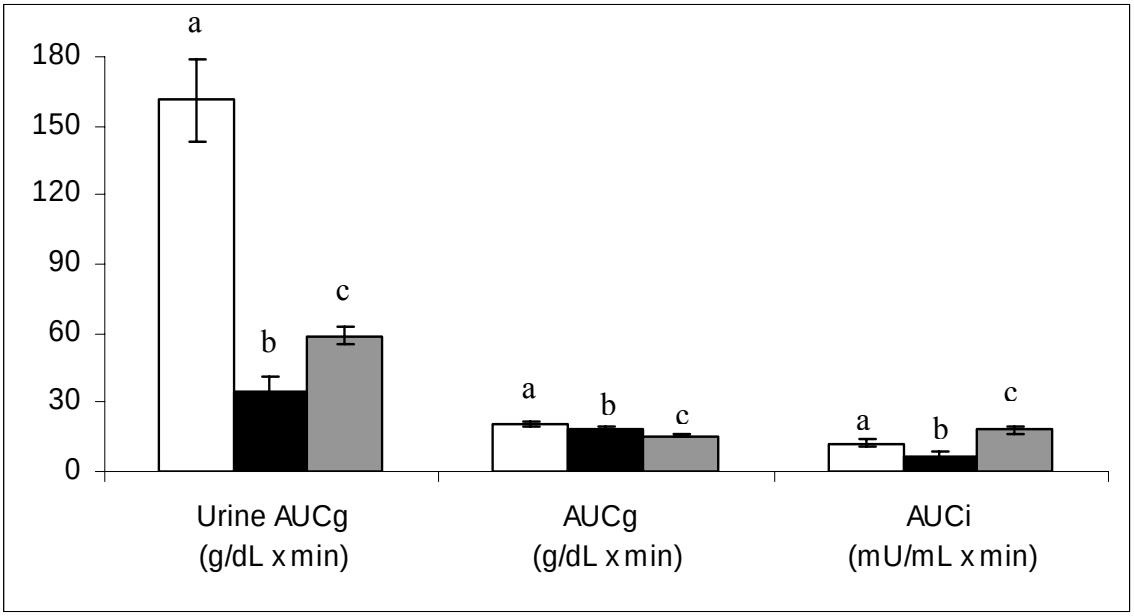


Figure 3.5 – Mean ± SE area under the curve for urinary glucose (uAUCg), area under the curve for glucose (AUCg) and area under curve for insulin (AUCi) values calculated for 5 horses during the third phase of the study. Each horse underwent three tests according to a crossover study design: established FSIGTT (300 mg/kg dextrose and 30 mU/kg insulin; white column), new FSIGTT (100 mg/kg dextrose and 20 mU/kg insulin; solid column) and the CGIT (150 mg/kg dextrose and 100 mU/kg insulin; shaded column).

[a-c] Within each variable, mean values with different superscripts differ significantly ($P < 0.05$), as determined by ANOVA and comparison of least squares mean values by use of a Least Significant Difference adjustment.

Table 3.3 – Mean ± SD urine AUCg, AUCi, AUCg and minimal model analysis values obtained from 6 horses using the established, new FSIGTT, and CGIT method. Tests were conducted 24h apart.

	Treatment			Trt effect
	Established FSIGTT	**New FSIGTT**	**CGIT**	***P***
Urine AUCg (g/dL•min)	161.4 ± 40.2[a]	34.2 ± 16.4 [b]	58.9 ± 8.6 [c]	< 0.001
AUCg (g/dL•min)	20.8 ± 2.2 [a]	18.4 ± 1.5 [b]	15.7 ± 1.2 [c]	0.003
AUCi (mU/mL•min)	12.4 ± 3.7 [a]	7 ± 2.7 [b]	18.3 ± 3.8 [c]	< 0.001
SI ($L \cdot min^{-1} \cdot mU^{-1}$) $\times 10^{-4}$	1.64 ± 0.92	2.02 ± 1.54	N/A	0.424
Sg (min^{-1}) $\times 10^{-2}$	2.3 ± 0.7	1.6 ± 0.8	N/A	0.133
AIRg ($mU \cdot min \cdot L^{-1}$)	707 ± 121	632 ± 255	N/A	0.502
DI ($\times 10^{-2}$)	11.25 ± 5.72	11.44 ± 8.54	N/A	0.811

[a-c] For variables with significant ($P < 0.05$) treatment effects, mean values with different superscripts differ significantly, as determined by use of an ANOVA and via comparison of least squares mean values by use of a Least Significant Difference adjustment.

N/A = Not applicable; minimal model analysis was not performed.

Linear regression calculated from the urinary glucose concentrations during the linear phase revealed that the projected time for urinary glucose spillover to cease was 53.7 min, 27.6 min and 44.8 min, respectively for the tests evaluated. Mean ± SD % urinary glucose loss relative to the mass of glucose administered intravenously for two horses undergoing the additional urine collection procedure was 10.11 ± 1.51 %, 2.66 ± 0.58 % and 4.97 ± 2.14 %, respectively for the established FSIGTT, new FSIGTT and CGIT (**Table 3.4**).

3.4 Discussion

The present study demonstrated that increasing the dosage of dextrose administered during the FSIGTT caused parallel increases in AUCg, AUCi and AIRg values. A plateau was registered for the AUCg values when the glucose dosage reached 200 mg/dL. Increasing the insulin dosage, however, only influenced the AUCg and left minimal model and AUCi values unchanged. Marked urinary glucose spilling was apparent when the established FSIGTT was performed in horses. The new FSIGTT, which is performed by infusing 100 mg/kg dextrose and 20 mU/kg insulin, provided sufficient data for minimal model analysis while minimizing urinary glucose loss.

In the first phase of the study, AUCg increased in parallel with the increase in dextrose dosage, however, a plateau was reached with dextrose dosages of 200 mg/kg or above. Although no urinary glucose measurements were performed in this phase of the study, we assume that this plateau resulted from increased urinary glucose loss. The sudden decline in blood glucose concentrations immediately following the peak after 200, 250, and 300 mg/kg dextrose was administered may have been a result of immediate urinary glucose loss, as well as mixing within the body glucose space. The same factors were implicated for the steep decrease in blood glucose concentrations following the peak after intravenous glucose boluses were administered in two studies performed in dogs (Kaneko et al. 1978; Rottiers et al. 1981). Additionally, increased blood glucose concentrations are expected to facilitate glucose disposal by their mass effect. This ability of glucose per se to normalize its concentration through actions on glucose

Table 3.4 – Net glucose mass infused, total urine volume, urine glucose concentration, net urinary glucose loss and glucose lost (%) registered in two horses during the established FSIGTT, new FSIGTT and CGIT.

	Horse	Established FSIGTT	New FSIGTT	CGIT
Net glucose mass administered (g/horse)	A	163.6	54.5	81.8
	B	155.5	51.8	77.7
Total urine volume (mL)	A	750	630	570
	B	960	480	455
Urine glucose concentration (mg/dL)	A	1973.1	265.9	495.9
	B	1809.9	242.5	1107.5
Net urinary glucose loss (g)	A	14.8	1.7	2.8
	B	17.4	1.2	5
Glucose lost (%)	A	9	3.1	3.5
	B	11.2	2.3	6.5

production and utilization, independent of insulin, is represented by the parameter Sg in the minimal model (Pacini *et al.* 1998). Indeed, Sg values appeared to be higher in the third phase of the study for the established FSIGTT when 300 mg/kg dextrose was administered, compared with the new FSIGTT when 100 mg/kg dextrose was infused, although the difference was not significant. Our finding of a strong linear relationship between glucose dosage and AUCg is in agreement with the work of Rottiers *et al.* (1981). This group demonstrated that administration of five progressively increasing glucose loads caused significantly higher successive post-infusion plasma glucose concentrations in dogs (Rottiers *et al.* 1981). In the study reported here, AUCi and AIRg also increased as the dextrose dosage increased. Since AIRg provides a measure of insulin secretion over the first 10 minutes following glucose administration (Tiley *et al.* 2007) it was expected that changes in AIRg would mirror the changes in AUCi. Several studies have demonstrated that total insulin secretion, which is represented by AIRg in the minimal model, increases as the glucose dosage increases, which is an observation that is similar to the increasing AUCi values registered in our experiment (Lerner and Porte 1971; Rottiers *et al.* 1981).

It was interesting to note that insulin secretion occurred as a single phase response in horses included in this study. Attributes of biphasic insulin secretion, including a nadir and a second insulin peak were absent in the horses studied here. Our findings are consistent with those of Giraudet *et al.* (1994). This group found that insulin concentrations abruptly increased and then gradually decreased in horses that received 0.33 g/kg bwt glucose intravenously (Giraudet *et al.* 1994). In humans and rats, the sudden, sustained increase in plasma glucose concentration triggers biphasic insulin secretion, characterized by a rapid increase with a peak, an interpeak nadir and a subsequent slower increasing phase (Caumo and Luzi 2004; Henquin *et al.* 2002). The first phase of the insulin response comes from the readily releasable pool granules located adjacent to the cell membrane of the pancreatic β-cells (Bratanova-Tochkova *et al.* 2002). For the second phase to occur, replenishment of the readily releasable pool from the reserve pool is necessary (Bratanova-Tochkova *et al.* 2002). Second phase insulin secretion also requires the augmenting action of glucose, and the magnitude of the second

phase response increases with the concentration of glucose administered (Henquin *et al.* 2002). Indeed, prolonged elevation of glucose concentration during the hyperglycemic clamp facilitated clearer determination of the second phase insulin response than the intravenous glucose tolerance test (Caumo and Luzi 2004).

Species differences have been also described for biphasic insulin secretion (Henquin *et al.* 2002). Second phase insulin response to glucose in isolated Islets of Langerhans from rats usually increase above the first phase peak, whereas the second phase of glucose-induced insulin secretion is often flat and lower than the first phase peak in mice (Henquin *et al.* 2002). Administration of glucose to dogs at different dosages has also failed to elicit biphasic insulin secretion (Kaneko *et al.* 1978; Rottiers *et al.* 1981). Furthermore, secondary plasma insulin peaks registered in human subjects undergoing glucose tolerance tests correspond to exogenous insulin administration or injection of the insulin secretagogue tolbutamide (Bergman *et al.* 1987; Katz *et al.* 2000). In the first phase of the study reported here, dextrose was administered as a single bolus and blood glucose concentrations were not sustained, neither was any insulin or insulin secretagogue administered following the glucose infusion, which may explain the absence of a distinct second phase of insulin secretion.

During the second phase of the study, AUCg decreased as the insulin dosage increased, however, no significant differences were registered for AUCi, SI, Sg, AIRg and DI. Our inability to detect significant differences in AUCi values in spite of administering markedly different dosages of exogenous insulin was an unexpected finding. In retrospect, high variance of AUCi values provides a plausible explanation for the lack of significant difference between treatments. The higher coefficient of variation values encountered when plasma insulin concentrations were measured, relative to those obtained for plasma glucose, may be partially responsible for the high variance observed for AUCi. Additionally, individual differences in stress-induced cortisol responses (Ralston 2002) can influence the insulin response, and by doing so introduce further variability into AUCi values. The absence of a dosage effect on minimal model variables may indicate that even small insulin doses are sufficient to facilitate cellular glucose uptake. Indeed the original FSIGTT design did not include administration of exogenous

insulin; this was introduced later to facilitate minimal modeling in diabetic patients (Pacini *et al.* 1998). Previous studies performed in horses used insulin dosages for the FSIGTT that ranges from 1.5 mU/kg bwt (Treiber *et al.* 2005a) to 30 mU/kg bwt (Hoffman *et al.* 2003).

In the third phase of the study, our primary goal was to compare the newly developed FSIGTT procedure with the established FSIGTT and CGIT with respect to urinary glucose spillover. A dextrose dosage of 100 mg/kg bwt was selected for the new FSIGTT because measurements of blood glucose during the first phase of the study suggested that this dosage could eliminate urinary glucose spillover. Additionally, administration of a lower dextrose dosage than the 300 mg/kg bwt used during the established FSIGTT facilitates rapid administration via the IV catheter. Rapid infusion of dextrose is advantageous because the minimal model assumes that the glucose is supplied to the system with a pulse width of zero (personal communication; Ray Boston 2008). The insulin dosage used in the new FSIGTT was selected (20 mU/kg bwt) with the aim of facilitating glucose disposal in horses, without causing clinical hypoglycemia. This insulin dosage was also recommended by other investigators after completion of a study involving the established FSIGTT (Treiber *et al.* 2005a).

Analysis of uAUCg revealed that urinary glucose spillover was significantly lower during the new FSIGTT, when compared with the established FSIGTT or CGIT, but was not completely abolished. Increased urine production was also detected during the established FSIGTT, suggesting that osmotic diuresis was occuring as a result of glucose spilling into the urine. On average, more than 10 % of glucose administered during the established FSIGTT was lost to the urine, compared with less than 3 % for the new FSIGTT. These results are in agreement with previous findings in dogs demonstrating that urinary glucose excretion varies directly with the size of glucose load (Kaneko *et al.* 1978). Regression analysis of urinary glucose concentration demonstrated that cessation of urinary glucose spilling occurred the earliest with the new FSIGTT, and the latest with the established FSIGTT. Although glucose was still apparent in the urine beyond the time suggested by the regression analysis, this was most likely a result of inadequate clearance of glucose-containing urine from the bladder. Incomplete emptying

of the bladder would be expected to affect subsequent samples. Glucose is normally freely filtered at the glomerulus and most of the filtered glucose is reabsorbed in the proximal tubules with a smaller percentage in the more distal segments of the nephron (Brodehl *et al.* 1987). Urine typically contains only minimal amounts of glucose, however, it has been reported that glucosuria develops when blood glucose concentrations exceed 160 to 180 mg/dL in horses (Carlson 2002), which is when the resorptive capacity of the renal tubules is exceeded (Brodehl *et al.* 1987).

Minimal model variables obtained for the new and established FSIGTT did not differ significantly. Mean SI values for the new and established FSIGTT were 2.02×10^{-4} $L \cdot min^{-1} \cdot mU^{-1}$ and 1.64×10^{-4} $L \cdot min^{-1} \cdot mU^{-1}$, respectively and these values compare favorably with results of previous studies (Hoffman *et al.* 2003; Treiber *et al.* 2005b). In these studies mean SI values of 2.1×10^{-4} $L \cdot min^{-1} \cdot mU^{-1}$ and 1.9×10^{-4} $L \cdot min^{-1} \cdot mU^{-1}$ were registered for 46 horses (Treiber *et al.* 2005b) and in 4 non-obese thoroughbred geldings (Hoffman *et al.* 2003), respectively. However, higher (3.0 and 2.9×10^{-4} $L \bullet min^{-1} \bullet mU^{-1}$) mean SI values were detected in a previous study of sixteen mares performed by our group (Toth *et al.* 2008). Lower mean SI values of 0.39×10^{-4} $L \cdot min^{-1} \cdot mU^{-1}$ and 0.08×10^{-4} $L \cdot min^{-1} \cdot mU^{-1}$ were also measured in healthy ponies and in ponies with history of laminitis, respectively (Treiber 2005). However, there is evidence to suggest that ponies have lower insulin sensitivity than horses (Rijnen and van der Kolk 2003).

Glucose effectiveness values measured in the present study compared favorably with results obtained in previous studies (Hoffman *et al.* 2003; Toth *et al.* 2008; Treiber *et al.* 2005b). Mean Sg values registered for non-obese and moderately obese thoroughbred geldings were 1.43×10^{-2} min^{-1} and 1.59×10^{-2} min^{-1}, respectively (Hoffman *et al.* 2003).

When the minimal model is applied, AIRg is estimated from serum insulin concentrations measured during the first 10 minutes following exogenous glucose administration (Tiley *et al.* 2007), therefore the high correlation between AUCi and AIRg recorded throughout the study was expected. Conversely, it was surprising that AIRg values did not differ significantly between the new FSIGTT and established FSIGTT, despite the significant difference registered for AUCi when the two tests were compared.

This latter finding may be explained by the fact that different exogenous insulin dosages administered at 20 min contributed to the significant difference in AUCi between the two tests. In contrast, only insulin concentrations measured during the first 10 minutes of the FSIGTT, prior to exogenous insulin administration, are included in the minimal model when AIRg is estimated. Mean AIRg values previously detected in 16 mixed breed (Quarter Horse / Tennessee Walking Horse) mares (Toth *et al.* 2008) were 503 and 520 $mU \cdot min \cdot L^{-1}$ and these values are consistent with results obtained in the current study. However, AIRg values measured in non-obese, moderately obese, and obese Thoroughbred horses were 211, 221, and 408 $mU \cdot min \cdot L^{-1}$, respectively (Hoffman *et al.* 2003), which are lower than those reported here.

The CGIT was completed in each horse without complications during the third phase of the study. After its initial description by Eiler *et al.* (2005), the CGIT has been used in several studies to identify horses with IR, and to establish glucose and insulin dynamics (Frank *et al.* 2005a; Frank *et al.* 2006). In the study reported here, the mean ± SD AUCg value registered during the CGIT was $15.7 \pm 1.2 \times 10^3$ mg/dL•min, which compares favorably to 12.7×10^3 mg/dL•min registered in eight healthy mares (Frank *et al.* 2005a) and to the median value of 15.5×10^3 mg/dL•min reported for obese horses with IR (Frank *et al.* 2006). In contrast the mean AUCi value of 18.3×10^3 μU/mL•min measured in this study was somewhat higher than the range of $10.6 – 13.2 \times 10^3$ μU/mL•min reported for 5 nonobese horses (Frank *et al.* 2006) but lower than 25×10^3 μU/mL•min demonstrated in 8 healthy mares. Serum insulin and blood glucose concentrations were measured more frequently during the CGIT in this study than recommended in the original report (Eiler *et al.* 2005). Calculation of AUCg and AUCi from a larger number of samples should increase accuracy, and this may explain why our results differed from those of previous studies.

In conclusion, results of this study indicate that glucose dosages equal to or higher than 200 mg/kg bwt result in AUCg plateau, which suggests that the 300 mg/kg bwt dosage used in the established FSIGTT is too high. Furthermore, profuse urinary glucose spilling was detected during the established FSIGTT. Although we were unable to demonstrate significant difference in minimal model values between the new and

established FSIGTT procedures because of high variability, it is likely that urinary glucose loss confounds minimal model analysis results, since one assumption of the model is that glucose exiting the blood stream is used by peripheral tissues. A new FSIGTT was developed for use in horses to minimize urinary glucose loss. In addition, the smaller dextrose dosage used in the new FSIGTT facilitates more rapid administration, which may also improve the accuracy of minimal model analysis.

CHAPTER 4

Evaluation of C-peptide dynamics and clearance in horses using a double antibody human C-peptide radioimmunoassay

4.1 Introduction

Insulin is a peptide hormone containing A and B chain that is synthesized by the β-cells of the pancreatic islets of Langerhans (Wahren *et al.* 2000; Wilcox 2005). Upon translation of the insulin mRNA, pre-proinsulin is generated and this molecule is comprised of a signal peptide, the B chain, the connecting peptide (**C-peptide**) and the A chain (Wilcox 2005). Proinsulin is synthesized by removal of the signal peptide from pre-proinsulin in the ribosomes of the rough endoplasmic reticulum of β-cells. Proinsulin is then transported to the Golgi apparatus, where it forms soluble, zinc containing hexamers. Enzymes acting outside the Golgi convert proinsulin to insulin by cleaving the C-peptide from the molecule during the formation of immature storage vesicles. C-peptide has an important role in insulin synthesis by linking the A and B chains of insulin in a manner that facilitates folding and interchain disulfide bond formation (Wahren *et al.* 2000). Proteolytic removal of C-peptide from proinsulin allows the carboxy terminal of the B-chain of the insulin molecule to assume a conformation which facilitates interaction with the insulin receptor. Insulin and C-peptide are cosecreted in equimolar amounts when mature granules release their contents into the portal circulation (Wilcox 2005).

Until recently, C-peptide was generally accepted to possess little or no biological activity. Lately, however, specific binding of C-peptide to cell surfaces has been demonstrated, suggesting the presence of G-protein coupled membrane receptors for this protein (Wahren *et al.* 2000). C-peptide has been shown to elicit concentration-dependent stimulation of Na^+-K^+ ATPase in proximal segments of nephrons obtained from rats (Ohtomo *et al.* 1996). Indeed, patients suffering from type I diabetes mellitus (**DM**) frequently develop glomerular hyperfiltration early in the course of their disorder, which can be significantly decreased by infusion of exogenous C-peptide (Johansson *et*

al. 1992). Addition of C-peptide to the treatment regime of type I diabetic human subjects significantly decreased urinary albumin excretion, when compared with insulin treatment alone (Wahren *et al.* 2000). Through its stimulatory effect on Na^+-K^+ ATPase, C-peptide has also been suggested to ameliorate nerve dysfunction in type I diabetes (Wahren *et al.* 2000).

Compensatory hyperinsulinemia associated with insulin resistance typically predates the development of type II DM in human subjects (Brunton *et al.* 2006). Similarly, horses suffering from decreased insulin sensitivity triggered by endotoxemia or obesity exhibit an augmented pancreatic insulin response to exogenous glucose challenge, indicated by higher acute insulin response to glucose (**AIRg**) values (Hoffman *et al.* 2003; Toth *et al.* 2008). In humans, overproduction of insulin by pancreatic β-cells eventually induces β-cell exhaustion, impaired insulin secretion, and relative insulin deficiency (Brunton *et al.* 2006). As glucose levels rise, β-cell function further deteriorates, with diminishing sensitivity to glucose, ultimately resulting in the development of type II DM (Wilcox 2005). Insulin-resistant horses and ponies rarely develop pancreatic β-cell insufficiency and usually maintain a state of compensated IR (Johnson *et al.* 2005; Treiber *et al.* 2005b). Nevertheless, DM has been described in a Spanish Mustang in association with IR (Johnson *et al.* 2005) and hyperglycemia was also registered in 2 ponies suffering from insulin resistance, which indicates failed compensation (Treiber *et al.* 2006b). Thus, estimation of the ability of pancreatic β-cells to secrete insulin is important for the accurate assessment of glucose homeostasis.

During minimal model analysis of the frequently sampled intravenous glucose tolerance test (**FSIGTT**), serum insulin concentrations measured during the first 10 minutes after exogenous glucose administration provide an estimate for AIRg (Tiley *et al.* 2007). The AIRg reflects the ability of pancreatic β-cells to secrete insulin after glucose challenge. However, it has been demonstrated in humans that approximately 60 percent of insulin secreted into the portal vein is removed by the liver (Wilcox 2005), so serum insulin concentrations measured in peripheral blood provide only a crude estimate of pancreatic function (Hovorka *et al.* 1998; Kjems *et al.* 2000). It is therefore preferable to measure serum C-peptide concentrations when assessing pancreatic function, because

this peptide is not extracted by the liver (Hovorka *et al.* 1998; Kjems *et al.* 2000). Higher AIRg values have been associated with IR in horses (Hoffman *et al.* 2003; Toth *et al.* 2008), but it has not been determined whether this occurs as a result of increased pancreatic insulin secretion or reduced hepatic insulin extraction. This could be investigated by simultaneously measuring insulin concentrations in blood samples obtained from both the portal vein and hepatic vein, but the measurement of C-peptide concentrations from peripheral blood samples is a more practical alternative.

A literature search revealed only one report describing C-peptide measurements in horses; but the authors did not state whether the test was validated for the horse (Johnson *et al.* 2005). Characteristics of C-peptide dynamics in horses, including clearance rate and plasma half life have not been reported to date. C-peptide metabolism has been established in dogs by blocking endogenous C-peptide secretion using a somatostatin infusion (Polonsky *et al.* 1983). Exogenous C-peptide was then administered to investigate hepatic metabolism and metabolic clearance rate of C-peptide.

The aims of the study reported here were 1) to measure C-peptide concentrations in equine serum using a human double antibody C-peptide radioimmunoassay (**RIA**), 2) to establish clearance rate of C-peptide, and 3) to describe C-peptide dynamics during the FSIGTT in horses. We hypothesized that the human double antibody C-peptide RIA would be able to detect equine C-peptide and that concentrations would increase as the pancreas responds to exogenous glucose during the FSIGTT.

4.2 Materials and Methods

Horses – Six mares from the University of Tennessee teaching and research herd were evaluated during the study period (Oct 2006 to March 2007). Horses were admitted to the teaching hospital in pairs and housed separately in 3.7-m × 3.7-m stalls. To eliminate differences attributable to sex, only mares were selected. Horses were 6 to 13 years of age (mean age, 9.3 years; median, 9 years); breeds included Quarter horse / Tennessee Walking horse crossbreds (n = 3), Quarter horse (2) and Standardbred (1). Horses were weighed at the time of admission and weights ranged from 461 kg to 523 kg

(mean weight, 497 kg; median, 501 kg). Body condition score (on a scale of 1 to 9) ranged from 4 to 6 (Henneke *et al.* 1983). Daily physical examinations were performed throughout the study period. Grass hay and water were provided ad libitum, and each horse was acclimated to its new environment for approximately 72 h before experiments started. The study protocol was approved by the University of Tennessee Institutional Animal Care and Use Committee.

Experimental design – The study reported here comprises two phases performed three months apart. The aim of the first phase was to evaluate C-peptide clearance in horses after suppression of pancreatic insulin secretion by somatostatin, while the second phase involved measurement of C-peptide concentrations in equine sera obtained during the FSIGTT in the same six horses.

C-peptide dynamics – On the first day of the study, a 14-gauge polypropylene catheter[1] was inserted into each jugular vein. Patency of IV catheters was maintained by infusion of 5 mL of saline solution containing heparin (4 U/mL) every 6 hours. Tests were performed 24 h after catheter insertion. At –90 min relative to C-peptide infusion, each horse was administered a 500 μg bolus of (D-Trp^8)-somatostatin-14[2] followed by a continuous rate infusion (**CRI**) of 500 μg/h somatostatin for 330 min through one of the intravenous catheters. Blood samples were collected at –100, –95, –90, – 75, – 60, – 45, – 30, – 15, – 10, – 5, 0 min using an injection cap and infusion set[3] (length, 30 cm; internal diameter, 0.014 cm) attached to the contralateral intravenous catheter. At 0 min, a 50 nmol bolus of biosynthetic human C-peptide[4] was administered intravenously. Further blood samples were collected at 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 19, 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150, 180, 210, and 240 min (n = 43 samples). At each time point, 3 mL of blood was withdrawn from the catheter and discarded. A 4-mL blood sample was subsequently collected then the catheter was flushed with 5 mL of saline solution containing heparin. Blood was transferred to a tube without anticoagulant. Samples were allowed to clot at 22 ^{o}C for 1h and then serum was

[1] Abbocath-T 14G X 140mm, Abbott Laboratories, North Chicago, Ill
[2] Bachem Americas, Inc. 3132 Kashiwa Street, Torrence, CA 90505
[3] Butterfly, Abbott Laboratories, North Chicago, Ill.
[4] GenScript Co., 120 Centennial Ave., Piscataway, NJ 08854

harvested via low-speed (1,000 × *g*) centrifugation. Serum samples were stored at –20°C until analyzed.

FSIGTT procedure – This procedure was performed during the second phase of the study. Each horse was weighed and a 14-gauge polypropylene catheter was inserted into the left jugular vein one day before the FSIGTT. Patency of the IV catheter was maintained by infusion of 5 mL of saline solution containing heparin into the catheter every 6h. During tests, horses were allowed access to grass hay and water *ad libitum*. An injection cap and infusion set (length, 30 cm; internal diameter, 0.014 cm) were attached to the catheter. The FSIGTT procedure first described for use in horses by Hoffman *et al*. (2003) was followed. Briefly, a 300 mg/kg bolus of 50% (wt/vol) dextrose[5] solution was administered to each horse via the infusion line and catheter, followed by infusion of 20 mL saline solution containing heparin. Blood samples were collected via the catheter immediately before and 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, and 19 min after infusion of dextrose. At 20 min, regular insulin[6] (30 mU/kg) was administered followed by another infusion of 20 mL saline solution containing heparin. Blood samples were subsequently collected via the catheter at 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150, and 180 min after the dextrose bolus infusion. At each time point, 3 mL of blood was withdrawn from the infusion line and discarded. A 6-mL blood sample was then collected, followed by infusion of 5 mL of saline solution containing heparin. Half the volume of the blood sample was transferred to a tube containing sodium heparin, which was immediately cooled on ice and then refrigerated. The remaining blood was transferred to a tube containing no anticoagulant. These samples were allowed to clot at 22 °C for 1 hour and then serum was harvested via low-speed (1,000 × *g*) centrifugation. Plasma and serum samples were stored at –20°C until further analyzed.

C-peptide RIA – Serum concentrations of C-peptide were measured using a human, double antibody RIA kit[7] previously used, but not validated in horses (Johnson *et al.* 2005). Samples were assayed in duplicate in accordance with instructions provided

[5] Dextrose 50% injection, Abbott Laboratories, North Chicago, Ill.
[6] Humulin R, Eli Lilly and Co, Indianapolis, Ind.
[7] Siemens Medical Solutions Diagnostics, 5210 Pacific Concourse Dr., Los Angeles, CA 90045-6900 (formerly: Diagnostics Products Co.)

by the manufacturer. The accuracy of the assay was assessed by recovery and parallelism. Dilutional parallelism was evaluated by diluting three aliquots of six separate equine serum samples to 1:2, 1:4 and 1:8 dilutions of their initial concentration using sterile nanopure water. Once the concentration for the undiluted sample was obtained, expected (**E**) concentrations were calculated according to the dilutions used. Observed (**O**) concentrations were compared with expected values and a ratio was calculated, which was expressed as a percentage (**%O/E**). Spiking and recovery was assessed by adding 50 μL solutions containing 195.3, 71.2, and 21.8 ng/mL human C-peptide to 950 μL of three aliquots of six different equine serum samples. Expected values were calculated by calculating the contribution of the added human C-peptide plus the initial value for horse serum alone. For each spiked sample, observed concentrations were compared with expected values and %O/E was calculated. Since human antibody RIA was used in this study, results are expressed as human equivalents (**HE**) of immunoreactive (**ir**) C-peptide. C-peptide values were converted from ng/mL to pmol/mL using a conversion factor of 0.333.

Serum insulin concentrations – Insulin concentrations were determined by use of a radioimmunoassay[8] previously validated for equine insulin (Freestone *et al.* 1991). Each sample was assayed in duplicate and intra-assay coefficients of variation of less than 10% were required for acceptance of assay results. Insulin values were converted from μU/mL to pmol/mL using a conversion factor of 6.945×10^{-3}.

Statistical analysis – Serum C-peptide concentrations were measured and clearance was calculated using commercially available software.[9] Statistical analysis was performed according to a randomized blocked design, blocked on horse. Serum C-peptide concentrations measured before, and during the first 90 min of the somatostatin CRI were averaged for each horse and compared using mixed model analysis of variance (one-tailed test). Mixed model analysis of variance with repeated measures was used to compare C-peptide concentrations measured before and after dextrose administration

[8] Coat-A-Count insulin, Siemens Medical Solutions Diagnostics, 5210 Pacific Concourse Dr., Los Angeles, CA 90045-6900 (formerly: Diagnostics Products Co.)
[9] WinNonlin 5.1, Pharsight, Mountain View, CA

using statistical software.[10] During the FSIGTT, C-peptide-to-insulin ratios were averaged in each horse for the 3 baseline samples and for samples obtained between 1 and 19 min (n = 13 samples) and subsequently compared using mixed model analysis of variance. No comparisons were made after the 19 min time point because of the confounding effect of exogenous insulin. Square root or log transformation was used to correct for non-normally distributed data. Significance was defined at a value of $P < 0.05$.

4.3 Results:

Experimental procedures remained well tolerated throughout the study with no abnormalities registered during daily physical examinations. Evaluation of baseline insulin concentrations and insulin sensitivity (**SI**) values revealed that one horse included in the study suffered from IR. The mean resting insulin concentration for this horse was 58.3 μU/mL and mean SI was determined to be 0.23×10^{-4} L•min^{-1}•mU^{-1}. Data from this horse were therefore handled separately.

Mean ± SD percent O:E ratio for the 6 serially diluted samples was 119 ± 25% (**Table 4.1**) and parallelism was observed. Observed-to-expected spike and recovery ratios for 6 samples with 3 spiking concentrations ranged from 112 to 136% with a mean ± SD of 123.09 ± 8.02% (**Table 4.2**).

Due to malfunction of the intravenous fluid pump, delivery of somatostatin failed in one horse, so mean C-peptide clearance rate was calculated using data obtained from four horses. Mean ± SD ir-C-peptide HE concentration measured before (0.23 ± 0.12 pmol/mL) and during the first 90 min (0.11 ± 0.06 pmol/mL) of the somatostatin CRI differed significantly ($P = 0.030$; one-tailed test). Mean ir-C-peptide HE concentration (2.96 ± 0.67 pmol/mL) peaked 1 min after intravenous administration of 50 nmol biosynthetic human C-peptide and remained significantly ($P = 0.003$ at 50 min) elevated for 50 min (**Figure 4.1**) relative to baseline. Mean ± SD clearance rate of ir-C-peptide HE was calculated to be 1.74 ± 0.74 mL/min/kg bwt.

[10] PROC MIXED, SAS, version 9.1, SAS Institute Inc, Cary, NC.

Table 4.1 – Dilutional parallelism of human equivalents of immunoreactive C-peptide as measured in six different equine serum samples using a human double antibody C-peptide radioimmunoassay kit.

Sample	Dilution	Observed (ng/mL)	Expected (ng/mL)	O/E %
A	1 in 1	0.9	NA	NA
	1 in 2	0.41	0.45	91 %
	1 in 4	0.26	0.23	116 %
	1 in 8	0.11	0.11	97 %
B	1 in 1	1.47	NA	NA
	1 in 2	0.71	0.73	98 %
	1 in 4	0.47	0.37	128 %
	1 in 8	0.11	0.18	60 %
C	1 in 1	0.78	NA	NA
	1 in 2	0.42	0.39	108 %
	1 in 4	0.30	0.19	153 %
	1 in 8	0.14	0.10	146 %
D	1 in 1	1.37	NA	NA
	1 in 2	0.75	0.69	109 %
	1 in 4	0.45	0.34	132 %
	1 in 8	0.24	0.17	141 %
E	1 in 1	1.23	NA	NA
	1 in 2	0.69	0.61	112 %
	1 in 4	0.34	0.31	111 %
	1 in 8	0.17	0.15	113 %
F	1 in 1	1.29	NA	NA
	1 in 2	0.77	0.65	119 %
	1 in 4	0.45	0.32	139 %
	1 in 8	0.25	0.16	161 %

O/E = ratio of observed (O) concentration to expected (E) concentration

NA = not applicable

Table 4.2 – Recovery of human equivalents of immunoreactive C-peptide as measured from six different equine serum samples spiked with biosynthetic human C-peptide using a human double antibody C-peptide radioimmunoassay kit.

Sample	Spiking concentration (ng/mL)	Observed (ng/mL)	Expected (ng/mL)	O/E %
A	0	0.9	NA	NA
	195.2	14.2	10.6	134 %
	71.2	6.0	4.4	136 %
	21.8	2.5	2.0	128 %
B	0	1.5	NA	NA
	195.2	12.6	12.0	113 %
	71.2	5.8	5.0	117 %
	21.8	2.8	2.5	112 %
C	0	0.8	NA	NA
	195.2	13.2	10.5	125 %
	71.2	5.6	4.3	131 %
	21.8	2.3	1.8	124 %
D	0	1.4	NA	NA
	195.2	14.0	11.1	127 %
	71.2	6.2	4.9	128 %
	21.8	2.7	2.4	112 %
E	0	1.2	NA	NA
	195.2	12.5	10.9	114 %
	71.2	6.00	4.7	126 %
	21.8	2.6	2.3	114 %
F	0	1.3	NA	NA
	195.2	13.0	11.0	118 %
	71.2	6.4	4.8	133 %
	21.8	2.9	2.3	125 %

O/E = ratio of observed (O) concentration to expected (E) concentration

NA = not applicable

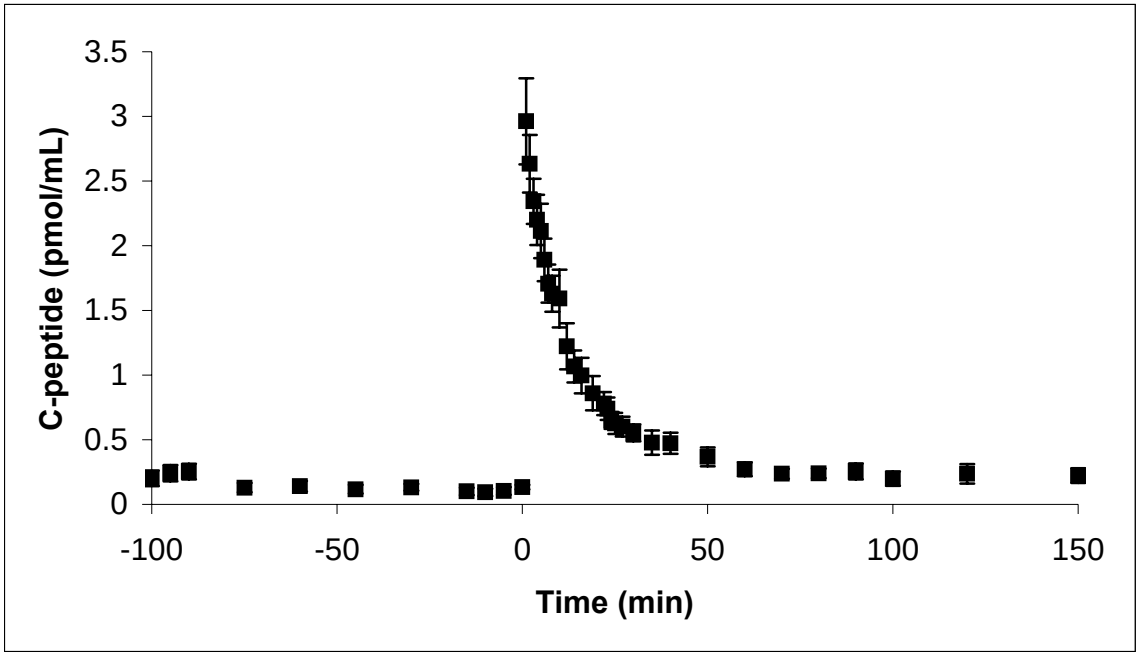


Figure 4.1 – Mean ± SE concentration of human equivalents of immunoreactive C-peptide in serum collected from horses (n = 4) after administration of 50 nmol biosynthetic human C-peptide at 0 min. Endogenous C-peptide secretion was suppressed by infusion of 500 μg biosynthetic human somatostatin at –90 min followed by 500 μg/h for 330 min.

Measurements of ir-C-peptide HE were successfully performed from blood samples collected during the FSIGTT from all five horses. For 100 min after intravenous glucose administration, ir-C-peptide HE concentrations remained significantly elevated ($P = 0.042$ at 100 min) relative to baseline (**Figure 4.2**). Mean ± SD ir-C-peptide HE-to-insulin ratio was 3.6 ± 1.95 preceding glucose administration, and this ratio significantly decreased ($P = 0.004$) to 1.03 ± 0.18 during the first 20 min following dextrose administration (**Figure 4.3**).

In the horse with preexisting IR, the resting concentration (0.24 pmol/mL) and clearance rate (1.62 mL/kg/min) of ir-C-peptide HE were both similar to those registered in healthy horses. However, mean ir-C-peptide HE-to-insulin ratio values for the three baseline samples and those from the first 20 min of the FSIGTT appeared to be lower relative to healthy mares (0.66 ± 0.16 and 0.48 ± 0.09, respectively; **Figure 4.4**).

4.4 Discussion

Serum C-peptide concentrations were successfully measured in equine serum, and the release of C-peptide and insulin from the pancreas were suppressed in horses by intravenously administering somatostatin. This enabled determination of the C-peptide clearance rate in horses. Administration of dextrose during the FSIGTT was associated with higher concentrations of ir-C-peptide HE relative to baseline and a lower C-peptide-to-insulin ratio.

Somatostatin is a peptide hormone that has two biologically active isoforms somatostatin-14 and somatostatin-28 containing 14 and 28 amino acid, respectively (Ludvigsen 2007). This peptide hormone is present in the brain, and is also secreted by the enteroendocrine D cells and δ-cells of the pancreas. Pancreatic somatostatin secretion is influenced by changes in blood glucose concentrations, and hormonal effects are exerted in an autocrine or paracrine fashion through binding to one of five somatostatin receptor subtypes (Low 2004). In mice, somatostatin receptor subtype 5 is responsible for the inhibition of insulin secretion from the β-cells and somatostatin receptor subtype 2, present on the α-cells, is essential for inhibition of glucagon secretion (Low 2004). In

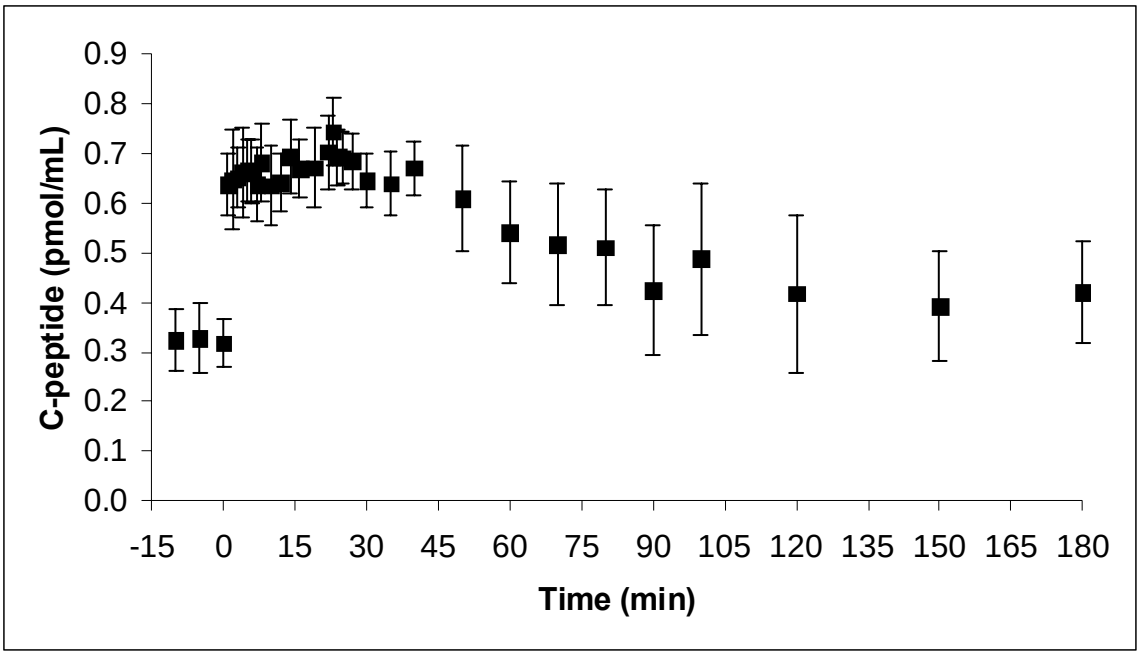


Figure 4.2 – Mean ± SE concentrations of human equivalents of immunoreactive C-peptide measured during the frequently sampled intravenous glucose tolerance test in 5 horses. Dextrose (300 mg/kg) was infused intravenously at time = 0 followed by 30 mU/kg insulin after 20 min

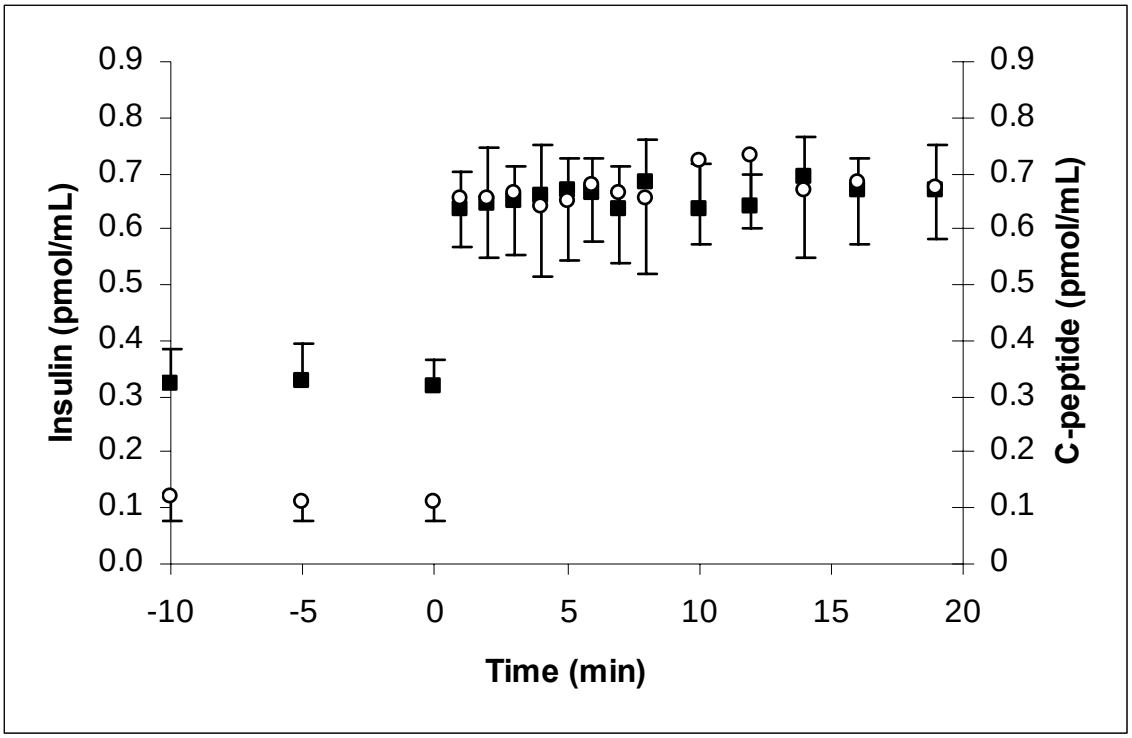


Figure 4.3 – Mean ± SE concentrations of human equivalents of immunoreactive C-peptide (solid squares) and insulin (open circles) as measured from three baseline samples and samples collected during the first 19 min of the frequently sampled intravenous glucose tolerance test in 5 horses.

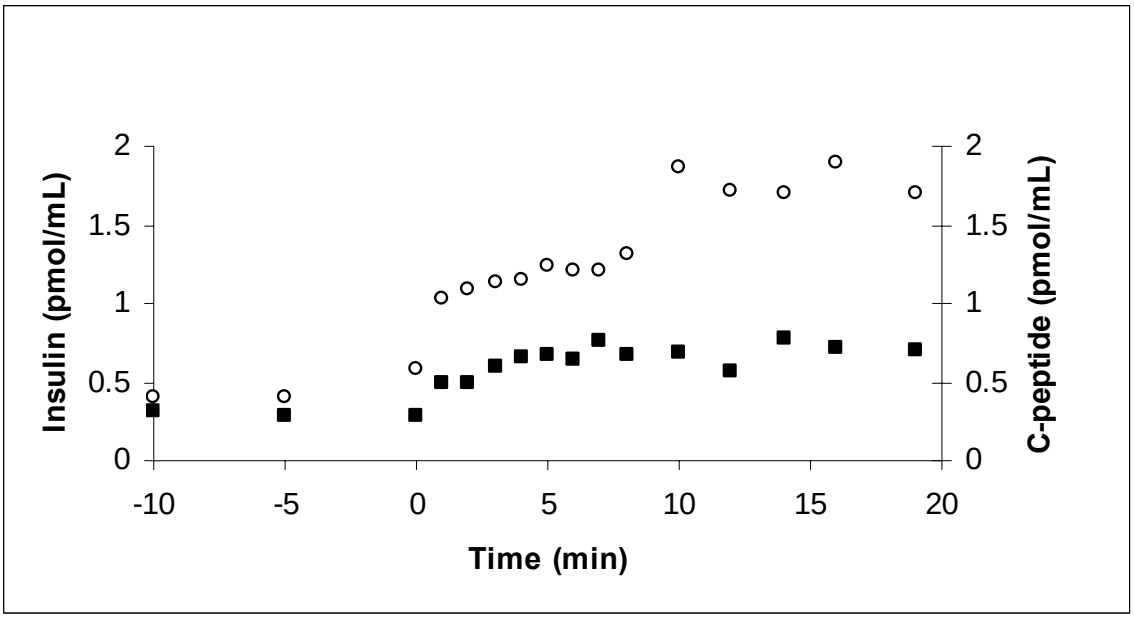


Figure 4.4 – Concentrations of human equivalents of immunoreactive C-peptide (solid squares) and insulin (open circles) as measured from three baseline samples and samples collected during the first 19 min of a frequently sampled intravenous glucose tolerance test in a horse suffering from insulin resistance

humans, however, somatostatin inhibits insulin secretion by binding to somatostatin receptor subtype 2 (Low 2004).

Successful suppression of C-peptide secretion in horses was evident by the significantly lower ir-C-peptide HE concentrations detected after initiation of the somatostatin CRI. However, detection of trace amounts of ir-C-peptide HE during the same period suggests that suppression was incomplete. These findings are consistent with previous observations in humans, as intravenous administration of somatostatin, or its analogue octreotide, resulted in only partial suppression of C-peptide release to 48% and 27% of baseline concentrations, respectively (Hwu *et al.* 2001). In dogs, however, an intravenous bolus of 50 μg somatostatin followed by a CRI of 800 ng/kg/min, suppressed C-peptide concentrations below the detection limit of the assay (Polonsky *et al.* 1983). Complications associated with somatostatin infusion such as gastrointestinal discomfort, diarrhea or fainting observed in human subjects (Hwu *et al.* 2001) were not detected in this study.

The resting mean ir-C-peptide HE concentration measured in this study (0.23 ± 0.12 pmol/mL) corresponds well with those reported previously for samples obtained from the femoral artery (0.24 ± 0.04 pmol/mL) and hepatic vein (0.33 ± 0.06 pmol/mL) of fasted dogs (Polonsky *et al.* 1983) or from fasted human subjects (range: 0.14 to 0.35 pmol/mL; Faber *et al.* 1978). Our results compare favorably with plasma C-peptide concentrations of 0.18 pmol/mL and 0.64 ± 0.17 pmol/mL registered in one diabetic Spanish Mustang and five healthy horses, respectively (Johnson *et al.* 2005).

Mean clearance rate for ir-C-peptide HE measured in this study (1.74 ± 0.74 mL/min/kg) was lower than the values of 4.4 mL/min/kg and 4.7 mL/min/kg detected in healthy and diabetic human subjects, respectively (Faber *et al.* 1978), and an even higher endogenous C-peptide metabolic clearance rate of 11.5 ± 0.8 mL/kg/min has been detected in dogs (Polonsky *et al.* 1983). In humans, C-peptide is metabolized by the kidney and partially excreted into the urine (Zavaroni *et al.* 1987). In the basal state, renal C-peptide metabolism is characterized by a high fractional extraction of approximately 26 % and very low urinary clearance of only 14 % (Zavaroni *et al.* 1987). These values indicate that most of the C-peptide taken up by the kidneys is metabolized

by renal tissues. Differences in kidney function may therefore explain the wide variation in C-peptide clearance rate registered in different species.

Concentrations of ir-C-peptide HE increased and remained significantly higher than baseline for 100 min during the FSIGTT. This observation was expected because insulin and C-peptide are co-secreted from the pancreas in equimolar amounts, and increased insulin concentrations are routinely demonstrated during the FSIGTT in horses (Hoffman *et al.* 2003; Hovorka *et al.* 1998). Similar observations were made in dogs during a 60 min intravenous glucose infusion, with elevated C-peptide and insulin concentrations detected (Polonsky *et al.* 1983). When intravenous glucose tolerance tests (**IVGTT**) are performed in humans, higher C-peptide concentrations are detected after glucose infusion (Kjems *et al.* 2001).

Characteristic biphasic C-peptide secretion occurs in humans following glucose infusion (Kjems *et al.* 2001), but this was not observed in this study. The most likely explanation for this finding is that glucose concentrations are not sustained during the FSIGTT in horses, so concentrations may not remain high enough to stimulate second phase insulin secretion. It has been suggested that the second phase insulin secretion requires the augmenting action of glucose (Henquin *et al.* 2002). Indeed, prolonged elevation of glucose concentration during the hyperglycemic clamp facilitates clearer determination of the second phase insulin response than the intravenous glucose tolerance test (Caumo and Luzi 2004). For this reason, the hyperglycemic clamp is considered the gold standard method for the assessment of biphasic insulin response *in vivo* (Caumo and Luzi 2004). Furthermore, tolbutamide is often administered to humans during the IVGTT to trigger the insulin/ C-peptide response at t = 20 min (Kjems *et al.* 2001), whereas only insulin is administered during the FSIGTT performed in horses (Hoffman *et al.* 2003; Toth *et al.* 2008). Tolbutamide is a first generation sulfonylurea drug that stimulates pancreatic insulin secretion (Krentz and Bailey 2005). Finally, the second phase insulin/ C-peptide response may blend with the first phase in horses, resulting in a secretory pattern similar to that demonstrated in mice (Henquin *et al.* 2002). In isolated mouse pancreatic islets, second phase insulin response to glucose is flatter and lower than the first phase (Henquin *et al.* 2002).

One of the most intriguing findings of this study was the significant decrease in C-peptide-to-insulin ratio from a baseline of 3.6 ± 1.9 to 1.0 ± 0.2 after dextrose administration during the FSIGTT. Similar results have been obtained in human subjects as the C-peptide-to-insulin molar ratio decreased from a fasting level of 5.0 to values between 2.0 and 3.0 following β-cell stimulation (Faber *et al.* 1978). The authors of that study hypothesized that the lower C-peptide-to-insulin ratio was due to the longer half-life of C-peptide. In dogs, the C-peptide-to-insulin molar ratio remained unchanged after initiation of a glucose infusion lasting 60 min (Polonsky *et al.* 1983). One possible explanation for this change in proportions is that hepatic insulin extraction decreases following glucose challenge in horses, causing the peripheral blood C-peptide-to-insulin ratio to approach 1:1 after stimulation of pancreatic β-cells. It is unlikely that increased hepatic extraction of C-peptide contributes to the shift in ratio, since very little or no hepatic C-peptide extraction has been demonstrated in dogs (Polonsky *et al.* 1983) or humans (Polonsky *et al.* 1986). Reduced hepatic insulin extraction may be a normal physiological response to dextrose infusion if it results in more insulin being left in circulation to act on insulin-sensitive tissues that store glucose. Alternatively, renal clearance of C-peptide might increase in response to dextrose, which would lower serum concentrations and alter the C-peptide-to-insulin ratio. In human subjects, renal uptake, renal clearance and fractional extraction of C-peptide markedly increased when elevated plasma C-peptide concentrations were induced by the consumption of amino acids (Zavaroni *et al.* 1987).

One interesting observation from this study was that a horse suffering from preexisting insulin resistance exhibited a lower C-peptide-to-insulin ratio than that registered in healthy horses, both during the resting period and following dextrose administration. This difference was attributed to higher insulin concentrations because C-peptide concentrations did not appear to be affected. These findings suggest that higher concentrations of insulin detected in horses and ponies with IR are a consequence of reduced insulin clearance rather than enhanced secretion (Hoffman *et al.* 2003; Toth *et al.* 2008; Treiber *et al.* 2006b). However, caution must be exercised when interpreting this result because data were only collected from a single insulin-resistant horse.

One of the weaknesses of the study reported here is our inability to measure equine specific C-peptide, due to the lack of a species-specific C-peptide RIA or biosynthetic equine C-peptide. However, amino acid sequences of equine and human C-peptide are homologous, differing only in seven residues (Wahren *et al.* 2000), which suggests a high potential for cross reactivity. Another limiting factor of this study was the small number of horses evaluated, which was compounded by the exclusion of one horse because of pre-existing IR and malfunction of the infusion pump. Nevertheless, detection of statistical significance was still possible.

Measurement of ir-C-peptide HE may provide an additional tool for the assessment of glucose and insulin dynamics in horses, facilitating precise reconstruction of the prehepatic insulin secretion profile. Evaluation of C-peptide concentration during IVGTT has been used to accurately assess prehepatic insulin secretion in healthy subjects and people suffering from type II diabetes (Kjems *et al.* 2000; Kjems *et al.* 2001). The use of the C-peptide minimal modeling of the insulin modified IVGTT was also demonstrated to correctly assess β-cell function in 15 healthy human subjects (Toffolo *et al.* 1999).

Estimation of pancreatic insulin secretion is important in horses because decreased insulin sensitivity and hyperinsulinemia have been associated with laminitis (Bailey *et al.* 2007; Carter *et al.* 2009; Treiber *et al.* 2006b). Insulin concentrations higher than 32 mU/L predict incipient pasture-associated laminitis in ponies before exposure to pastures rich in non-structural carbohydrates (Carter *et al.* 2009). Development of laminitis in ponies undergoing experimental induction of hyperinsulinemia (1,036 ± 55 μU/mL) as euglycemia was being maintained provides evidence that insulin plays a role in the pathogenesis of laminitis (Asplin *et al.* 2007b). Insulin resistance, established from increased insulin concentrations via the proxy measure, reciprocal of the square root of insulin, was one of the criteria used to identify ponies suffering from prelaminitic metabolic syndrome; a condition associated with a 10-fold increase in the incidence of pasture-associated laminitis (Treiber *et al.* 2006b). In the same study, euglycemia was registered in all but 2 horses suffering from IR,

indicating that increased insulin secretion managed to sustain insulin-mediated glucose disposal in spite of the decreased insulin sensitivity (Treiber *et al.* 2006b).

Decompensation develops when increased pancreatic insulin secretion is unable to offset reduced insulin sensitivity and hyperglycemia develops (Treiber *et al.* 2006b). Increased expression of matrix metalloproteinases 2 and 9 has been described in horses suffering from laminitis (Souza *et al.* 2006) and in endothelial cells and macrophages exposed to high glucose concentrations (Death *et al.* 2003). These findings suggest that a connection exists between the hyperglycemia associated with decompensation and laminitis. Hence, accurate description of prehepatic insulin secretion based on peripheral C-peptide concentration will likely enhance our understanding and treatment of IR and comorbid conditions in horses.

In conclusion, we demonstrated that C-peptide can be detected and quantified in equine serum using a double antibody human C-peptide RIA. Changes in the proportions of ir-C-peptide HE and insulin were demonstrated in association with dextrose infusion suggesting that hepatic insulin extraction may be influenced by changes in blood glucose levels. Precise quantification of C-peptide concentration from equine serum along with further studies investigating the relationship between serum C-peptide levels and pancreatic insulin release are required to facilitate accurate evaluation of β-cell function in horses. More insulin-resistant horses must be evaluated in the future to confirm or refute our observation that higher insulin concentrations detected in one horse with IR were a result of reduced insulin clearance rather than enhanced pancreatic secretion.

CHAPTER 5

Effects of pretreatment with dexamethasone or levothyroxine sodium on endotoxin-induced insulin resistance in horses

5.1 Introduction

Insulin resistance (**IR**) is a state in which normal concentrations of insulin fail to elicit the expected physiologic response (Kahn 1978). Development of IR in horses has previously been associated with endotoxemia (Toth *et al.* 2008; Vick *et al.* 2008), obesity (Hoffman *et al.* 2003), pituitary pars intermedia dysfunction (**PPID**; Keen *et al.* 2004) and dexamethasone administration (Tiley *et al.* 2007). The importance of decreased insulin sensitivity (**SI**) is underscored by its purported role in the development of laminitis (Asplin *et al.* 2007b; Bailey *et al.* 2008; Treiber *et al.* 2006b), osteochondrosis (Pagan 2001) and decreased fertility (Sessions *et al.* 2004).

Previous studies have demonstrated that ponies predisposed to laminitis suffer from decreased insulin sensitivity (Bailey *et al.* 2008; Treiber *et al.* 2006b). Treiber *et al.* (2006b) showed that 25% of ponies with preexisting IR, but no clinical evidence of laminitis in March developed clinical laminitis by May. The appearance of clinical laminitis in these ponies coincided with a significantly higher starch content measured within pasture grass in May relative to March (Treiber *et al.* 2006b). Excessive consumption of non-structural carbohydrates including starches can lead to rapid fermentation within the equine hindgut causing intraluminal acidosis, Gram-negative bacterial death, and increased intestinal permeability (Longland and Byrd 2006; Weiss *et al.* 1998). Studies specifically examining hindgut bacterial populations during the development of carbohydrate-induced laminitis revealed that proliferation of bacteria belonging to the *Streptococcus bovis/equinus* complex consistently preceded the development of laminitis, implicating these specific microorganisms in the pathogenesis of alimentary laminitis (Milinovich *et al.* 2007; Milinovich *et al.* 2006). Horses grazing on pastures rich in soluble carbohydrate may therefore develop laminitis as a result of

intestinal events caused by carbohydrate overload, exacerbation of preexisting IR, or both problems occurring concurrently.

Endotoxin is a heat stable lipopolysaccharide (**LPS**) found in the outer membrane of Gram-negative bacteria, which is released upon bacterial death and during bacterial multiplication (Rietschel *et al.* 1994). Under physiological circumstances, only a small amount of endotoxin enters the bloodstream, and it is subsequently removed by the Kupffer cells of the liver (Hardie and Kruse-Elliott 1990a). Clinically apparent endotoxemia develops when the liver is unable to clear all of the circulating endotoxin, or when excessive amounts of endotoxin enter the blood (Hardie and Kruse-Elliott 1990a; Morris 1991). In horses, endotoxemia most commonly results from compromise of the gastrointestinal mucosal barrier, which allows endotoxin to move from the intestinal lumen to the bloodstream (Morris 1991). Other conditions associated with endotoxemia include Gram-negative bacteremia, pleuropneumonia, and metritis (Morris 1991; Werners *et al.* 2005).

Endotoxemia may contribute to the development of laminitis in horses. Elevated endotoxin concentrations were detected in 85% of horses developing Obel Grade 3 laminitis after experimental carbohydrate overload (Sprouse *et al.* 1987). In a previous study, we demonstrated that transient IR develops after LPS administration (Toth *et al.* 2008), which leads to the hypothesis that endotoxin-induced alterations in glucose and insulin dynamics contribute to the development of laminitis in horses. It has also been recognized that hospitalized horses suffering from endotoxemia have 5 times the odds of developing laminitis than horses with no evidence of endotoxemia (Parsons *et al.* 2007). This finding supports the role of endotoxemia in the development of laminitis and also raises the question of whether stress induced by stall confinement (Harewood 2005; Mal 1991) increases the risk of disease.

Chronic IR in pastured ponies and stress associated with hospitalization are pre-existing factors that may exacerbate the reduction in insulin sensitivity induced by endotoxemia. If this is the case, ponies and horses affected by these conditions may undergo exaggerated responses to endotoxin that lower insulin sensitivity even further and raise the likelihood of laminitis. This may enhance the negative impact of minor

intestinal events occurring in grazing ponies if endotoxemia develops in these animals. We therefore hypothesized that differences in resting insulin sensitivity affect the magnitude of changes in glucose and insulin dynamics induced by endotoxin administration.

Dexamethasone (**DEX**) and levothyroxine sodium (**L-T_4**) treatments were administered to alter resting insulin sensitivity prior to endotoxin administration in this study. It has previously been demonstrated that SI decreases when DEX is administered to horses (Tiley *et al.* 2007). Insulin sensitivity significantly decreased in healthy adult horses when DEX was administered orally at a dosage of 0.08 mg/kg, IV, q 48h for 21 days (Tiley *et al.* 2007). In contrast, we have previously shown that SI increases in healthy adult mares treated with L-T_4 for 8 or 48 weeks (Frank *et al.* 2008; Frank *et al.* 2005b). The mechanisms responsible for this improvement in SI have not been determined in horses, but it may be the result of increased glucose transport through the cell membrane in both intra and extrahepatic sites (Haber *et al.* 1995; Romero *et al.* 2000; Weinstein *et al.* 1994). Thyroid hormone has been shown to increase glucose transporter 4 (**GLUT4**) gene expression as well as translocation of glucose transporters to cell membranes (Romero *et al.* 2000; Weinstein *et al.* 1994).

5.2 Materials and methods

Horses – Twenty-four adult mares from the University of Tennessee teaching and research herd were included in the study. The study was performed over the course of five months between September 2007 and January 2008. Horses were admitted to the University of Tennessee Veterinary Teaching Hospital in groups of four and remained hospitalized for 22 days. At the conclusion of the study, horses were returned to the research herd. To eliminate differences attributable to gender only mares were selected. Mean ± SD weight of horses upon admission was 519 ± 36 kg. The age of horses ranged from 5 to 16 years (mean ± SD: 10.4 ± 3.6 years) and breeds included Quarter horse (11), Quarter horse / Tennessee Walking horse crossbred (10), Tennessee Walking Horse (1), Standardbred (1) and Thoroughbred (1). Body condition scores (on a scale from 1 to 9)

ranged from 4 to 6 (Henneke *et al.* 1983). The study protocol was approved by the University of Tennessee Institutional Animal Care and Use Committee.

Experimental design – Using a completely randomized design, horses were assigned to one of the three treatment groups, each containing eight horses. The treatment groups included:

Control group (n = 8)	horses receiving 200g of oats and no other medications
L-T_4 group (n = 8)	horses receiving 48 mg of L-T_4 powder[1] mixed with 200g of oats once daily
DEX group (n = 8)	horses were administered 20 mg dexamethasone[2] (2 mg/mL solution) orally using a syringe and 200g of oats once daily

Horses were weighed and physical examinations were performed on the first day (Friday) of the study, then each horse was housed separately in 3.7-m X 3.7-m stalls within the veterinary teaching hospital. Grass hay was fed in amounts equivalent to two percent of body weight (**bwt**) and *ad libitum* water was provided, and each horse was acclimated to its new environment for approximately 72h. On the fourth day of the study (Monday) a 14G intravenous catheter[3] was inserted into the left jugular vein and left in place for approximately 24h. A baseline frequently sampled IV glucose tolerance test (**FSIGTT**) was performed on the fifth day (Tuesday) starting at 9:00 AM. Treatments were administered each morning for 15 days, starting on the sixth day (Wednesday). On the twentieth day (Wednesday) horses were weighed, and intravenous catheters were inserted and left in place for approximately 48h. The post-treatment FSIGTT procedure was performed on day 21 (Thursday) starting at 0900. On this day, treatments were administered at 1200, after completion of the FSIGTT. Additionally, at 1300 all horses received an intravenous LPS infusion, and 20h later horses underwent the post-LPS FSIGTT starting again at 0900 AM. Horses in the DEX and L-T_4 groups received

[1] Thyro L, Lloyd Inc, Shenandoah, IA
[2] Dexamethasone, VetOne, Bimeda-MTC Animal Health Inc., Cambridge, ON, Canada
[3] Abbocath-T 14G X 140mm, Abbott Laboratories, North Chicago, IL.

tapering doses of their respective treatments for another 6 days after the conclusion of the study.

Lipopolysaccharide administration – *Escherichia coli* O55:B5 LPS[4] was mixed with 30 mL of sterile saline (0.9% NaCl) solution under a fume hood with the investigator wearing gloves and a respirator to minimize exposure. The LPS solution (20 ng/kg) was infused via the IV catheter during a 15-minute period. Horses were observed for signs of colic and physical examination variables, including rectal temperature, heart rate, respiratory rate, mucous membrane color, and capillary refill time were recorded every 30 min for the first 3h, then every hour for the following 3h, and then every 2h for 6h.

Complete blood count (CBC) analysis – To evaluate individual responses to endotoxin, CBC analyses were performed in each horse. Blood was collected from the indwelling jugular catheter into tubes containing EDTA before the LPS infusion was initiated and 2h (ie, 1h 45 min after completing the LPS infusion). Samples were immediately transported to the clinical pathology laboratory for CBC analysis.

FSIGTT procedure – One day preceding the FSIGTT, a 14-gauge polypropylene catheter was inserted into the left jugular vein. During tests, horses were allowed access to grass hay and water. Patency of the IV catheter was maintained before the test by infusion of 5 mL of saline solution containing heparin (4 U/mL) into the catheter every 6 hours. During the FSIGTT, an injection cap and infusion set[5] (length, 30 cm; internal diameter, 0.014 cm) were attached to the catheter. The FSIGTT procedure first described by Hoffman (Hoffman *et al.* 2003) and modified by our research group (Chapter 3) was used. Briefly, a bolus (100 mg of glucose/kg) of a 50% (wt/vol) dextrose solution[6] was administered to each horse via the infusion line and catheter, followed by injection of saline solution containing heparin. Blood samples were collected via the catheter 10, 5 and 1 min before and 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, and 19 min after infusion of

[4] Sigma Chemical Co., St Louis, MO
[5] Butterfly, Abbott Laboratories, North Chicago, IL.
[6] Dextrose 50% injection, Abbott Laboratories, North Chicago, IL.

dextrose. At 20 min, regular insulin[7] (20 mU/kg) was administered followed by another infusion of saline solution containing heparin. Blood samples were subsequently collected via the catheter at 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150, and 180 min relative to the dextrose infusion. At each time point, 3 mL of blood was withdrawn from the infusion line and discarded. A 6-mL blood sample was then collected, followed by infusion of 5 mL of saline solution containing heparin. Half of the volume of blood was transferred to a tube containing sodium heparin, which was immediately cooled on ice and then refrigerated. The remaining blood was transferred to a tube containing no anticoagulant. Those samples were allowed to clot at 22°C for 1h and then serum was harvested via low-speed (1,000 × *g*) centrifugation. Plasma and serum samples were stored at –20°C until further analyzed.

Plasma glucose and serum insulin concentrations – Plasma glucose concentrations were measured by use of a colorimetric assay[8] on an automated discrete analyzer.[9] Serum insulin concentrations were determined by use of a radioimmunoassay[10] that has been validated for use in horses (Freestone *et al.* 1991). Each sample was assayed in duplicate and intra-assay coefficients of variation < 5% or < 10% were required for acceptance of glucose and insulin assay results, respectively.

Interpretation of FSIGTT data by use of the minimal model – Values of SI, glucose effectiveness (**Sg**), acute insulin response to glucose (**AIRg**), and disposition index (**DI**) were calculated for each FSIGTT in accordance with the minimal model (Bergman *et al.* 1981) by use of commercially available software[11,12] and previously described methods (Hoffman *et al.* 2003). Disposition index was calculated via multiplication of AIRg by SI.

[7] Humulin R, Eli Lilly and Co, Indianapolis, IN.
[8] Glucose, Roche Diagnostic Systems Inc, Somerville, NJ.
[9] Cobas Mira, Roche Diagnostic Systems Inc, Somerville, NJ.
[10] Coat-A-Count insulin, Diagnostic Products Corp, Los Angeles, CA.
[11] MinMod Millenium, version 6.10, Raymond Boston, University of Pennsylvania, Kennet Square, PA.
[12] Stata 9.2, Stata Corporation, College Station, TX.

Statistical analysis – To evaluate the effects of LPS administration on physical parameters and white blood cell (**WBC**) count, measurements obtained before and after endotoxin administration were compared using mixed model analysis of variance. In each horse, area under the curve for glucose (**AUCg**) and for insulin (**AUCi**) were calculated for all three FSIGTT procedures (baseline, post-treatment and post-LPS) from the glucose and insulin concentrations measured during the FSIGTT, by use of the trapezoidal method and commercially available computer software.[13] To determine the effects of treatments and time (baseline, post-treatment and post-LPS) on Sg, SI, AIRg, DI, AUCi and AUCg mixed model analysis of variance was used. If data was non-normally distributed or unequal variance was encountered, square root or log transformation was applied. When a significant treatment × time effect was detected, protected Least Significant Difference mean separation method was used. Significance was defined at a value of $P < 0.05$.

5.3 Results

Mean ± SD body weight of horses receiving L-T_4 (n = 8) decreased significantly ($P < 0.001$) relative to baseline (528.9 ± 25.3 kg) by the end of the 15-day pretreatment period (511 ± 28 kg), whereas mean body weight remained unchanged in the DEX and control groups.

Mean ± SE baseline SI values did not differ significantly ($P = 0.942$) between groups (**Table 5.1**), but one horse within the L-T_4 group had a low baseline SI value (0.59×10^{-4} L•min^{-1}•mU^{-1}) at the beginning of the study that was judged to be an outlier (studentized residual of 2.14), so data from this animal were excluded from the analysis of treatment and time effects on glucose and insulin dynamics. Insulin sensitivity significantly decreased ($P < 0.001$) over time in all groups across the 15-day pretreatment period. Mean SI values decreased by 47%, 52%, and 78% relative to baseline for control, L-T_4 and DEX groups, respectively. A significant ($P = 0.021$) treatment × time effect was detected; mean SI for the DEX group was significantly ($P = 0.032$) lower than

[13] PROC MIXED, SAS, version 9.1, SAS Institute Inc, Cary, NC

control group by the end of the pretreatment period. Significant treatment × time effects were also detected for AUCg ($P = 0.001$) and AUCi ($P < 0.001$). Mean AUCg significantly increased ($P < 0.001$) over time in the DEX group, but did not change in the control ($P = 0.626$) and L-T_4 ($P = 0.878$) groups relative to baseline. Mean AUCi

Table 5.1 – Mean ± SD minimal model variables and AUCi and AUCg registered in horses undergoing 15 days of control, levothyroxine sodium (L-T4) or dexamethasone treatment followed by intravenous administration of 20 ng/kg LPS. Measurements were taken on day 5 (after acclimation; baseline), before LPS administration (pre LPS) and 20h after LPS infusion (post LPS). Respective treatments (control/L-T_4/dexamethasone) were also administered on day 21 preceding the LPS administration.

		Treatment			Trt × time
		Control (n = 8)	**L-T_4 (n = 7)**	**Dexamethasone (n = 8)**	***P***
SI ($L \cdot min^{-1} \cdot mU^{-1}$) $\times 10^{-4}$	baseline	3.24 ± 1.7^{a}	3.48 ± 1.35^{a}	3.34 ± 1.82^{a}	0.021
	Pre LPS	1.72 ± 1.25^{b}	$1.67 \pm 1.16^{b,c}$	$0.73 \pm 0.63^{c,d}$	
	Post LPS	$0.63 \pm 0.59^{d,e}$	$1.48 \pm 1.2^{b,c,d}$	0.21 ± 0.23^{e}	
Sg (min^{-1}) $\times 10^{-2}$	baseline	2.02 ± 0.99	2.29 ± 1.36	2.68 ± 1.31	0.288
	Pre LPS	2.48 ± 1.06	2.39 ± 1.12	1.65 ± 0.88	
	Post LPS	2.29 ± 1.7	1.41 ± 1.06	1.53 ± 0.98	
AIRg ($mU \cdot min \cdot L^{-1}$)	baseline	516 ± 263	434 ± 162	416 ± 221	0.099
	Pre LPS	664 ± 428	545 ± 191	884 ± 263	
	Post LPS	1190 ± 1150	712 ± 108	1635 ± 741	
DI $\times 10^{-2}$	baseline	14.9 ± 9	14.6 ± 7.4	11.7 ± 4.1	0.103
	Pre LPS	8.8 ± 4.1	7.9 ± 6	6.9 ± 6.9	
	Post LPS	5.5 ± 5.4	9.6 ± 6.7	2.4 ± 3.1	
AUCi (mU/mL•min)	baseline	$5.12 \pm 1.33^{a,b}$	$5.81 \pm 2.3^{a,b,c}$	4.85 ± 1.85^{a}	< 0.001
	Pre LPS	10.2 ± 4.91^{c}	$9.1 \pm 7.49^{a,b,c}$	23.23 ± 20.74^{d}	
	Post LPS	26.9 ± 33.32^{d}	$9.61 \pm 4.5^{b,c}$	57.25 ± 47.35^{e}	
AUCg (g/dL•min)	baseline	$17.27 \pm 0.94^{a,b,c}$	16.65 ± 1.52^{a}	15.83 ± 1.02^{a}	0.001
	Pre LPS	$16.92 \pm 1.27^{a,b}$	16.77 ± 2.72^{a}	$19.57 \pm 2.39^{c,d}$	
	Post LPS	19.87 ± 3.52^{d}	$18.67 \pm 2.63^{b,c,d}$	22.43 ± 3.38^{e}	

[a-c] For variables with significant ($P < 0.05$) treatment × time effects, mean values with different superscripts differ significantly, as determined by repeated measures ANOVA and comparison of least squares means using Least Significant Difference adjustment.

increased over this 15-day period in the control ($P = 0.001$) and DEX ($P < 0.001$) groups, but remained unchanged in the L-T_4 group ($P = 0.153$). No significant changes in Sg, AIRg, or DI were detected.

All horses included in this study responded to LPS by exhibiting colic signs, and leukopenia defined by a WBC count < 5.4×10^3 WBCs/μL (Smith 2002) that was detected 2h after LPS administration (**Table 5.2**). One horse had a baseline WBC count of 5.1×10^3 cells/μL which further decreased to 2.4×10^3 cells/μL following endotoxin administration. Heart rate, respiratory rate and rectal temperature increased ($P < 0.001$) and WBC count decreased ($P < 0.001$) in all treatment groups following LPS administration. Horses in the DEX group had significantly higher maximal heart rates after LPS administration than L-T_4 ($P < 0.001$) and control ($P = 0.006$) group animals, and maximal respiratory rates were higher in DEX group, compared with the control group during the same period ($P = 0.006$).

Insulin sensitivity significantly decreased after LPS administration in control ($P = 0.002$) and DEX ($P = 0.015$) groups, but remained unaffected ($P = 0.695$) in the L-T_4 group. Additionally, mean AUCi ($P < 0.001$) and AUCg ($P < 0.001$) values were significantly higher in the DEX and control groups after LPS administration, compared with values obtained beforehand **(Figure 5.1 and 5.2)**. Mean AUCg increased ($P = 0.016$) over time in the L-T_4 group, but AUCi remained unchanged ($P = 0.329$) relative to values obtained before LPS administration. Mean SI measured 20h after LPS administration was significantly ($P = 0.002$) higher in the L-T_4 group than the DEX group, and there was a trend ($P = 0.075$) towards higher SI values in the L-T_4 group relative to control group. No significant treatment × time effects were detected for Sg, AIRg, or DI.

5.4 Discussion

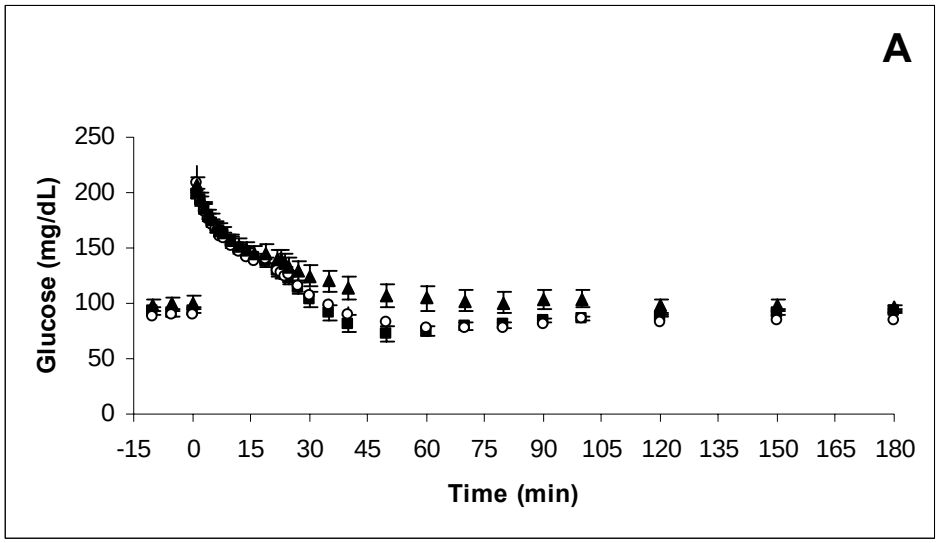
In the present study, SI decreased significantly across the 15-day pretreatment period in all treatment groups, which was an unexpected finding. Possible explanations for this finding include stress associated with hospitalization, changes in exercise, or

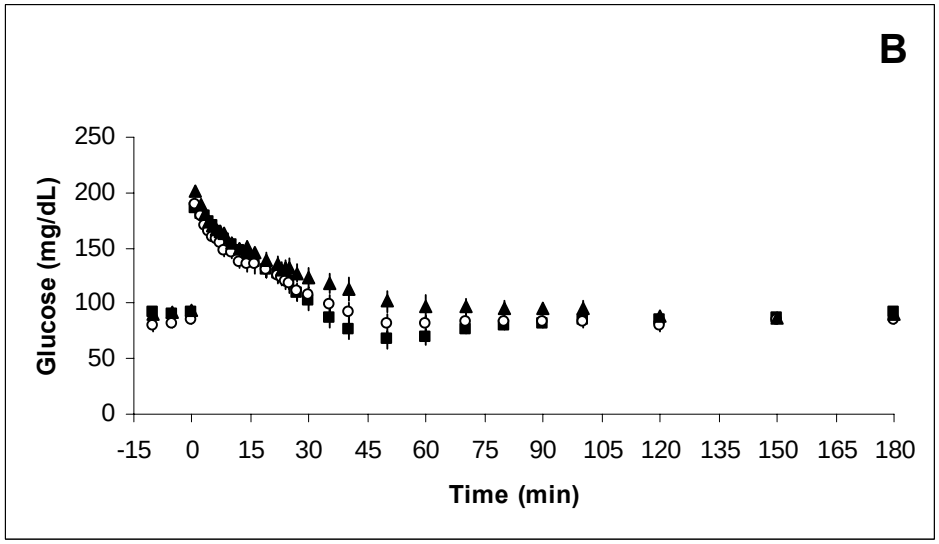
Table 5.2 – Mean ± SD maximal heart rate (HR), maximal respiratory rate (RR), maximal rectal temperature (T) and white blood cell (WBC) count measured in horses undergoing 15 days of control, levothyroxine sodium (L-T_4) or dexamethasone treatment, followed by intravenous administration of 20 ng/kg LPS. Measurements of WBC were performed on day 20 immediately before LPS administration (Pre LPS) and 2h after the initiation of the LPS infusion (Post LPS). Physical examinations were performed on day 20 before LPS administration (Pre LPS) and within 12h after the initiation of the LPS infusion (Post LPS). Respective treatments (control/L-T_4/dexamethasone) were also administered on day 20 preceding the LPS administration.

		Treatment		
		Control (n = 8)	**L-T_4 (n = 8)**	**Dexamethasone (n = 8)**
HR bpm	Pre LPS	32.6 ± 7 [a]	38.8 ± 5.1 [a]	39.3 ± 5.2 [a]
	Post LPS	64.3 ± 16.4 [b]	55 ± 6.8 [b]	79.8 ± 16.1 [c]
RR b/min	Pre LPS	17.6 ± 5.9 [a]	17.3 ± 3.5 [a]	17.5 ± 6.7 [a]
	Post LPS	38 ± 7.1 [b]	53.3 ± 20.4 [c,b]	59.8 ± 16.2 [c]
T ºC	Pre LPS	37.3 ± 0.3 [a]	37.4 ± 0.3 [a]	37.3 ± 0.3 [a]
	Post LPS	38.6 ± 0.3 [b]	38.9 ± 0.4 [b]	39.2 ± 0.6 [b]
WBC $\times 10^3/\mu L$	Pre LPS	7.26 ± 1.35 [a]	6.94 ± 0.71 [a]	7.38 ± 0.82 [a]
	Post LPS	3.04 ± 0.89 [b]	3.03 ± 0.86 [b]	2.94 ± 0.90 [b]

[a-c] For variables with significant ($P < 0.05$) treatment × time effects, mean values with different superscripts differ significantly, as determined by ANOVA for repeated measures and comparison of least squares mean values by use of a Least Significant Difference adjustment.

Figure 5.1 – Mean ± SE plasma glucose concentration during the FSIGTT in horses undergoing 15 days of control (panel A), L-T_4 (panel B) or dexamethasone (panel C) treatment followed by intravenous administration of 20 ng/kg LPS. Testing was performed on day 5 at baseline (solid squares), day 21 before LPS administration (empty circles), and on day 22 after LPS infusion (solid triangles).


A
Glucose (mg/dL)
250
200
150
100
50
0
-15 0 15 30 45 60 75 90 105 120 135 150 165 180
Time (min)


B
Glucose (mg/dL)
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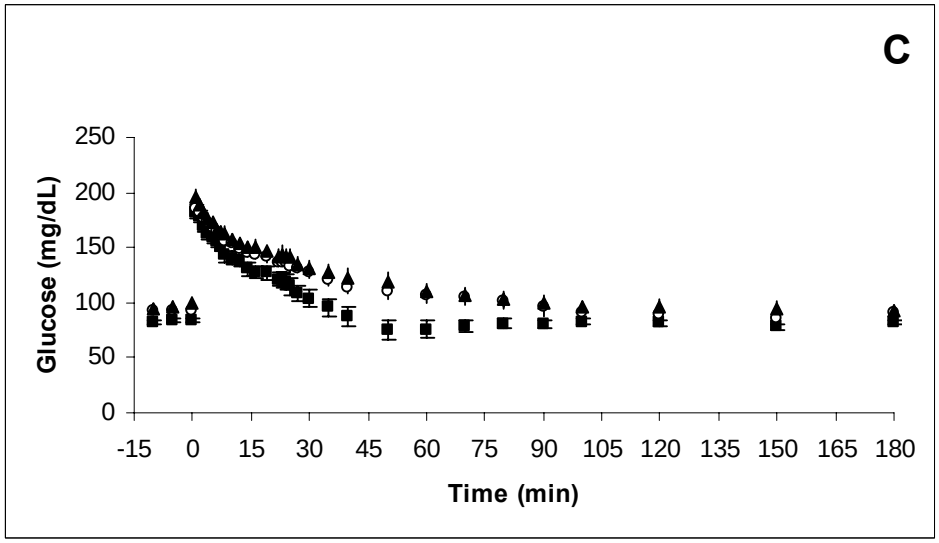
-15 0 15 30 45 60 75 90 105 120 135 150 165 180
Time (min)


C
Glucose (mg/dL)
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200
150
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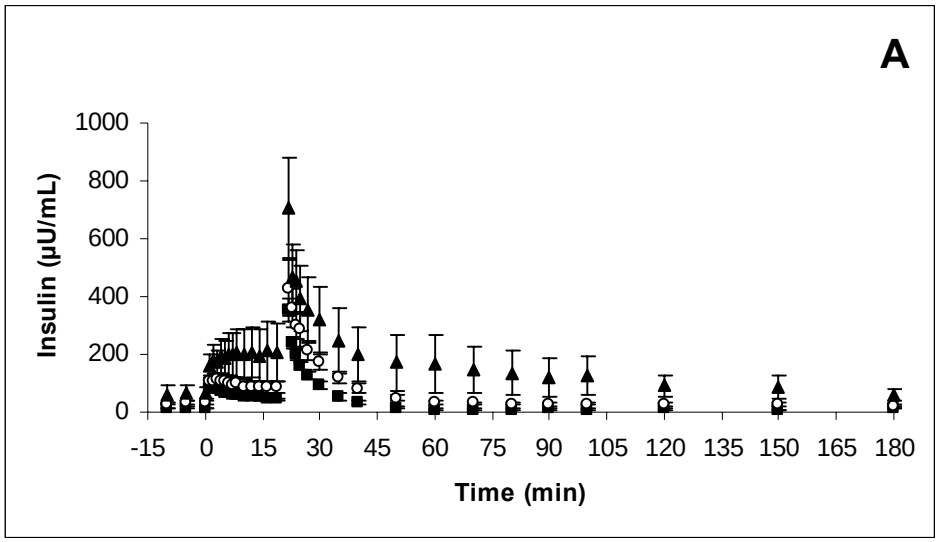
50
0
-15 0 15 30 45 60 75 90 105 120 135 150 165 180
Time (min)

Figure 5.2 – Mean ± SE serum insulin concentration, during the FSIGTT in horses undergoing 15 days of control (panel A), L-T_4 (panel B) or dexamethasone (panel C) treatment followed by intravenous administration of 20 ng/kg LPS. Testing was performed on day 5 at baseline (solid squares), day 21 before LPS administration (open circles) and day 22 after LPS infusion (solid triangles).


A
Insulin (µU/mL)
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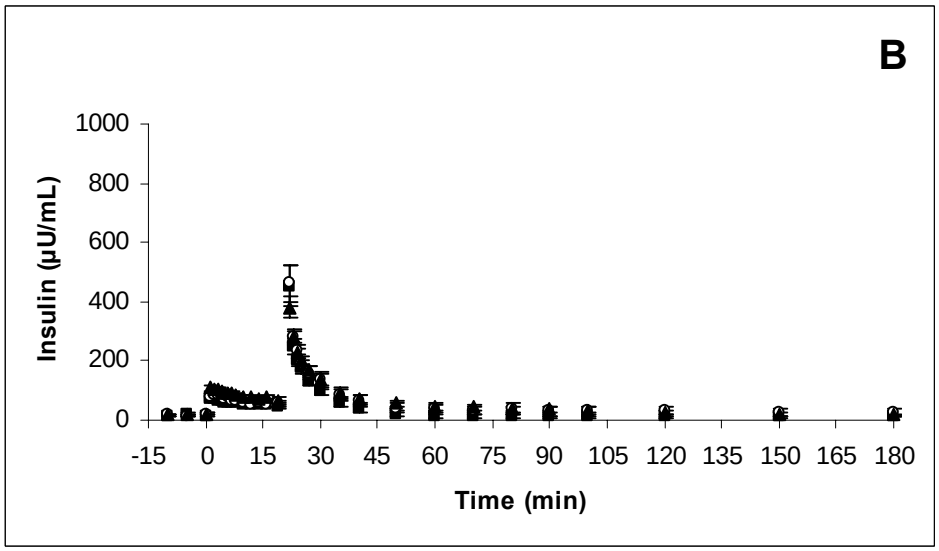
800
600
400
200
0
-15 0 15 30 45 60 75 90 105 120 135 150 165 180
Time (min)


B
Insulin (µU/mL)
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200
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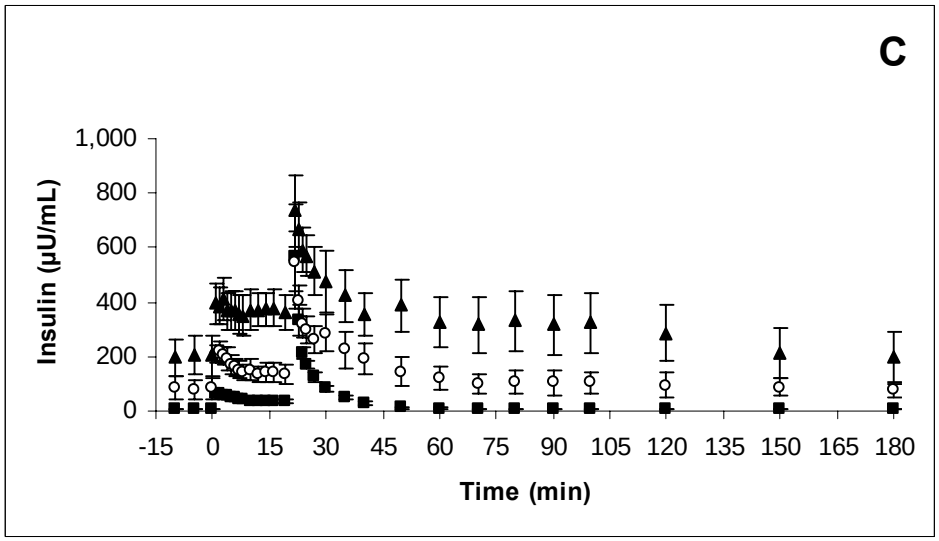
-15 0 15 30 45 60 75 90 105 120 135 150 165 180
Time (min)


C
Insulin (µU/mL)
1,000
800
600
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200
0
-15 0 15 30 45 60 75 90 105 120 135 150 165 180
Time (min)

alterations in diet. At the end of the pretreatment period, the lowest mean SI value was detected in the DEX group. Administration of LPS exacerbated IR causing an additional 71.2 % and 63.4 % decrease in SI in the DEX and control groups, respectively. However, the degree of change did not differ between these groups, so our hypothesis that resting insulin sensitivity influences the magnitude of endotoxin-induced IR was not supported. Nevertheless, mean SI after LPS administration was less than 0.5 × 10–4 L•min-1•mU-1 in the DEX group, indicating an additive effect. In contrast to our expectations, pretreatment with L-T4 did not increase SI, but did prevent endotoxin-induced exacerbation of IR. After endotoxin infusion, horses receiving L-T4 treatment had significantly higher SI values than horses undergoing dexamethasone treatment, and there was a trend towards higher insulin sensitivity in the L-T4 group, when compared with control results.

Two methods were used to evaluate glucose and insulin dynamics and estimate insulin sensitivity. One of these methods was minimal model analysis, which partitions glucose disposal into glucose- and insulin-mediated parts by fitting a nonlinear model to data obtained from the FSIGTT (Kronfeld *et al.* 2005a). Although the repeatability of this method was shown to be lower than the euglycemic-hyperinsulinemic clamp (**EHC**) in one study (Pratt *et al.* 2005), this technique is preferred because it is technically simpler and provides estimates of Sg, AIRg and DI as well as SI. The second method involved calculation of area under the curve values for glucose and insulin concentrations during the FSIGTT. It has previously been demonstrated that horses suffering from IR have significantly higher AUCi and AUCg than their healthy counterparts (Frank *et al.* 2006).

Dexamethasone and L-T_4 treatments alter insulin sensitivity in horses (Frank *et al.* 2008; Frank *et al.* 2005b; Tiley *et al.* 2007). In a previous study, insulin sensitivity significantly increased when horses were treated with L-T_4 for 8 weeks. These changes were paralleled by significant decreases in plasma triglyceride, total cholesterol and very low density lipoprotein concentrations (Frank *et al.* 2005b). When the duration of L-T_4 treatment was extended to 48 weeks, increased insulin sensitivity persisted throughout the study and was accompanied by significant weight loss (Frank *et al.* 2008). It is

speculated that SI improves in response to L-T_4 treatment because weight loss in induced, but thyroid hormones may also exert direct effects on glucose uptake (Frank *et al.* 2008). However, SI did not increase across the 15-day pretreatment period in the study reported here. On the contrary, insulin sensitivity was significantly lower by the end of the 15d pretreatment period. Since identical changes were noted in the control group, it was assumed that insulin sensitivity decreased because of factors common to all treatment groups.

Before enrollment in the study, horses were kept on pasture without additional feed provided. During the study however, horses were moved into stalls, exercise was restricted, and only grass hay was fed. It has previously been demonstrated that short-term exercise training increases SI in both obese and lean horses (Powell *et al.* 2002), and skeletal muscle GLUT4 content increases 2- to 3-fold after 6 weeks of exercise (McCutcheon *et al.* 2002). Sedentary behavior is often implicated in the development of IR in humans (Muoio and Newgard 2008), so this is one possible explanation for the reduction in SI across the pretreatment period. Alternatively, stress associated with stall confinement during hospitalization (Harewood 2005; Mal 1991) may have contributed to the decrease in SI. It has been previously demonstrated that minor perturbations in housing conditions, such as removal of a horse from its accustomed environment, are sufficient to alter the normal circadian rhythm of cortisol concentrations, which would raise total cortisol secretion across a 24-hour period (Irvine and Alexander 1994).

Mean insulin sensitivity significantly decreased to $0.73 \pm 0.63 \times 10^{-4}$ L•min^{-1}•mU^{-1} in horses that received dexamethasone for 15d relative to baseline, which compares favorably with the mean value of $0.53 \pm 0.13 \times 10^{-4}$ L•min^{-1}•mU^{-1} detected in horses that received 0.08 mg/kg bwt dexamethasone intravenously every other day for 21 days (Tiley *et al.* 2007). Dexamethasone induces IR by altering insulin signaling pathways in adipocytes (Kawai *et al.* 2002) and skeletal muscle (Ruzzin *et al.* 2005) and liver (Saad *et al.* 1993) tissues. Phosphorylation of phosphoinositol-3-kinase, a central molecule in insulin signal transduction, significantly decreased in rat liver following dexamethasone treatment (Saad *et al.* 1993). In skeletal muscle, glucocorticoid-induced IR is accompanied by reduced insulin-mediated phosphorylation of protein kinase B, which in

turn leaves the enzyme glycogen synthase kinase-3 in its unphosphorylated active form. Active glycogen synthase kinase-3 phosphorylates and thereby inactivates glycogen synthase, which inhibits glycogen synthesis and contributes to the development of IR (Ruzzin *et al.* 2005). Protein kinase Cβ appears to play an important role in glucocorticoid-induced IR in adipocytes (Kawai *et al.* 2002). In horses, treatment with dexamethasone for 21 days impairs insulin-mediated phosphorylation of glycogen synthase kinase-3 in skeletal muscle but does not alter the expression of GLUT4 or protein kinase B, or affect insulin-stimulated phosphorylation of protein kinase B (Tiley *et al.* 2008).

All horses included in the study exhibited characteristic signs of endotoxemia after LPS administration, including leukopenia, elevated rectal temperature, heart rate and respiratory rate. These findings are in agreement with previous observations of endotoxemic horses (Barton *et al.* 2004; MacKay *et al.* 1999). It was surprising however, that clinical signs of endotoxemia were not ameliorated by dexamethasone treatment. On the contrary, horses in the DEX group had significantly higher heart and respiratory rates than horses in the control group. It has previously been demonstrated that dexamethasone pretreatment suppresses LPS-induced interleukin-1β and interleukin-6 responses in rats (O'Connor *et al.* 2003). These mediators play a central role in the development of the LPS-induced inflammatory reaction (Morris 1991). Nevertheless, the same study also showed that exposure to an acute stressor preceding dexamethasone administration eliminates the inhibitory effect of this drug on proinflammatory cytokine production and release (O'Connor *et al.* 2003). In ponies, dexamethasone administration 5 min 3, 9 and 24h after endotoxin infusion did not prevent the endotoxin-induced reduction in WBC count (Ewert *et al.* 1985).

In the study reported here, SI was lower twenty hours after LPS administration in horses undergoing control and dexamethasone treatments, but remained unaffected in the L-T_4 group. Results of two independently conducted studies, including one performed by our research group, have established that SI decreases in response to LPS infusion in horses (Toth *et al.* 2008; Vick *et al.* 2008). In humans, assessment of glucose and insulin dynamics in response to LPS revealed a biphasic response with an initial increase in

glucose utilization followed by a progressive decrease in SI (Agwunobi *et al.* 2000). Endotoxin-mediated IR involves hepatic, skeletal muscle and adipose tissues (McCowen *et al.* 2001; Song *et al.* 2006). Lipopolysaccharide-induced activation of Toll-like receptor 4 in 3T3-L1 adipocytes stimulated the expression of inflammatory cytokines and provoked IR (Song *et al.* 2006). In rats, sustained endotoxemia adversely affected the early steps of the insulin signaling pathway by inhibiting tyrosine phosphorylation of the insulin receptor substrate-1 molecule in skeletal muscle and hepatocytes (McCowen *et al.* 2001).

Both SI and AUCi remained unchanged 20h following LPS infusion in horses receiving L-T_4. This finding was attributed to the effects of thyroid hormone on glucose transport at both hepatic and extrahepatic sites (Haber *et al.* 1995; Romero *et al.* 2000; Weinstein *et al.* 1994). In 3T3-L1 adipocytes, treatment with tri-iodothyronine (T_3) was shown to increase GLUT1 and GLUT4 content and favor partitioning to plasma membranes, which would facilitate transmembrane glucose transport into cells (Romero *et al.* 2000). These changes were observed in both the presence and absence of insulin (Romero *et al.* 2000). An *in vivo* study performed in rats treated with L-thyroxine yielded similar results, demonstrating that insulin-stimulated glucose transport increased in association with higher GLUT1 and GLUT4 content and functional activity (Matthaei *et al.* 1995). Thyroid supplementation has been shown to increase both basal and insulin-stimulated glucose uptake in skeletal muscle, with the change being proportional to the increase in GLUT4 content (Weinstein *et al.* 1994). In liver-derived ARL-15 cells, T_3 stimulates glucose transport by increasing GLUT1 content and enhancing its partition to the cell surface (Haber *et al.* 1995).

In conclusion, results of this study indicate that thyroid supplementation protects against endotoxin-induced IR when administered to horses for 15 days beforehand. It was also demonstrated that horses receiving dexamethasone treatment developed exacerbated IR following LPS administration, which may explain why hospitalized horses with endotoxemia are more likely to develop laminitis. These animals may have higher cortisol concentrations as a result of stress and confinement. Insulin sensitivity may also be lower in these animals because exercise is restricted during hospitalization. It can also be hypothesized that chronically insulin-resistant horses grazing on pasture experience minor

intestinal events resulting in endotoxemia that exacerbates IR and contributes to the development of laminitis. Although the single endotoxin dose used in this study was sufficient to exacerbate IR, none of the treated horses developed laminitis. However, a bolus infusion is only a crude approximation of the clinical condition because endotoxemia is more severe and persists for longer in affected animals. Further studies are required to explain why L-T_4 treatment failed to prevent the decrease in SI that occurred across the pretreatment period, yet protected horses against endotoxin-induced IR.

CHAPTER 6

Effects of endotoxemia on the development of laminitis and glucose and insulin dynamics in horses

6.1 Introduction

Endotoxemia, carbohydrate overload, and insulin resistance (**IR**) are important risk factors for laminitis in horses and these conditions can occur concurrently (Garner *et al.* 1975; Parsons *et al.* 2007; Treiber *et al.* 2006b; van Eps and Pollitt 2006). An association between carbohydrate overload, endotoxemia, and laminitis was first described by Sprouse *et al.* in 1987 (Sprouse *et al.* 1987). This group detected elevated plasma endotoxin concentrations in 85% of horses that developed laminitis following carbohydrate overload (Sprouse *et al.* 1987). Alterations in the mucosal barrier, occurring after carbohydrate overload may enable endotoxin absorption during the prodromal stages of laminitis (Weiss *et al.* 1998). An association between endotoxemia and IR has recently been established, based upon results of two independently conducted studies in which endotoxemia was experimentally induced in healthy horses (Toth *et al.* 2008; Vick *et al.* 2008). Insulin sensitivity significantly decreased 24 h after intravenous administration of exogenous endotoxin to healthy horses in both reports, suggesting that this disturbance is a component of the systemic inflammatory response induced by endotoxemia. Results of these studies suggest that intestinal carbohydrate overload leads to endotoxemia, which induces IR. However, no studies have been performed to date to determine whether pre-existing endotoxemia increases the likelihood or severity of experimentally-induced laminitis in horses.

Endotoxemia frequently develops in horses suffering from gastrointestinal disturbances, metritis, pleuropneumonia or Gram-negative sepsis (Morris 1991; Werners *et al.* 2005). The absorption of lipopolysaccharide (**LPS**) into circulation is a prolonged process in most clinical cases of endotoxemia, yet most experimental models involve the administration of a single bolus infusion of LPS with dosages ranging from 0.02 μg/kg to 200 μg/kg (Barton *et al.* 2004; Bottoms *et al.* 1981; Doherty *et al.* 2003; Toth *et al.*

2008). There are only few reports describing the administration of exogenous endotoxin to horses as a continuous rate infusion (**CRI**) (Duncan *et al.* 1985; Kindahl *et al.* 1991; MacKay *et al.* 1991). Infusion rates ranged from 0.03 μg/kg/h to 1 μg/kg/h in these studies, resulting in cumulative doses that exceed the amount administered as a bolus infusion in other studies. In humans, however, 100 times lower dosages of 0.75 ng/kg/h LPS have been used to model low-grade inflammation (Taudorf *et al.* 2007). Lower LPS dosages may therefore represent a model for endotoxemia associated with minor intestinal events if they occur when horses and ponies are grazing on pasture grass rich in soluble carbohydrate.

The association between pasture-associated laminitis and IR is supported by results of a study by Treiber *et al*. (2006b). This group demonstrated that ponies suffering from prelaminitic metabolic syndrome, a condition characterized by lower resting insulin sensitivity, have an odds ratio of 10 to develop clinical laminitis. Laminitis became apparent in these ponies in the month of May when pasture grass contained significantly more starch than in March (Treiber *et al.* 2006b). When starches are abundant, the digestive capacity of the equine small intestine is exceeded and this carbohydrate enters the hindgut and undergoes rapid bacterial fermentation, resulting in the proliferation of lactic acid-producing bacteria, and a concomitant decrease in Gram-negative bacteria (Garner *et al.* 1978; Longland and Byrd 2006). Analysis of hindgut bacterial populations during the development of carbohydrate-induced laminitis revealed that bacteria belonging to the *Streptococcus bovis/equinus* complex, predominantly *Streptococcus lutetiensis* (formerly known *Streptococcus infantarius* ssp. *coli*) proliferate (Milinovich *et al.* 2007; Milinovich *et al.* 2006). This alteration precedes the development of lameness, which implicates these specific microorganisms in the pathogenesis of alimentary laminitis. Additionally, damage to the intestinal mucosa, triggered by high intraluminal levels of lactic acid and endotoxin, may compromise the mucosal barrier, and thereby facilitate absorption of endotoxin (Garner *et al.* 1978). If these intestinal events occur at a minor level, subclinical endotoxemia may contribute to the development of clinical laminitis in horses and ponies grazing on pasture. This could occur through exacerbation of preexisting IR or as a result of sustained inflammatory

reaction (Toth *et al.* 2008). The significance of endotoxemia in the development of laminitis has been further emphasized by the work of Parsons *et al.* (2007). This group demonstrated that hospitalized horses suffering from clinical endotoxemia have 5-fold higher odds of developing laminitis than horses without endotoxemia.

One method for experimentally inducing laminitis in horses involves the administration of oligofructose (**OF**) at dosages ranging from 5 g/kg to 12.5 g/kg (French and Pollitt 2004b; Kalck *et al.* in press; van Eps and Pollitt 2006). In this model, higher OF dosages are associated with more severe laminitis and greater loss of hemidesmosomes between the lamina densa of the basement membrane and the epidermal basal cells (French and Pollitt 2004b). Development of laminitis in the OF model is also associated with altered glucose and insulin dynamics (Kalck *et al.* in press; van Eps and Pollitt 2006) as well as clinical signs suggestive of a systemic inflammatory response (van Eps and Pollitt 2006).

It was therefore hypothesized that the IR associated with alimentary carbohydrate overload would be significantly greater in horses pretreated with endotoxin, and that the severity of laminitis would increase when endotoxin and OF treatments were combined. Specific aims of this study were (1) to use low-dose endotoxin CRI as an experimental model for minor intestinal events occurring in horses grazing on pasture, (2) to determine whether LPS infusion alone induced IR and/or laminitis, and (3) to investigate whether pre-treatment with LPS would accentuate IR and/or laminitis induced by intestinal carbohydrate overload. An OF dosage of 5g/kg bwt was selected for this study because our research group has recently determined that this dosage induces mild laminitis that horses can successfully recover from (Kalck *et al.* in press)

6.2 Materials and methods

Horses – Twenty-four adult mares from the University of Tennessee research and teaching herd were included in the study. Mean ± SD bodyweight (**bwt**) was 495 ± 43 kg (range from 403 – 559 kg) with the body condition score ranging from 4 to 6 (Henneke *et al.* 1983). Mean ± SD age of the horses were 11.2 ± 3.8 (range: 6 – 16); breeds included

Quarter Horse/Tennessee Walking horse crossbred (n = 8), Quarter Horse (n = 11), Tennessee Walking Horse (n = 4), and Thoroughbred (n = 1).

Experimental design – Procedures were performed between the months of January and June 2008. Three treatments were compared using a completely randomized design with eight repetitions (horses) per treatment. Treatment groups consisted of:

LPS group (n = 8)	Endotoxin CRI for 8h followed by administration of 4L water via nasogastric intubation
OF group (n = 8)	Saline (0.9% NaCl) CRI for 8h followed by 5g/kg bwt OF dissolved in 4L water
LPS/OF group (n = 8)	Endotoxin CRI followed by 5g/kg bwt OF dissolved in 4L water.

Horses were brought into the teaching hospital in groups of three and one horse was assigned to each treatment group. Each horse was individually housed in a 3.7-m ×3.7-m stall and fed grass hay in amounts equivalent to 2% of bwt and *ad libitum* water.

Upon admission (Friday), horses were weighed and physical examinations were performed. An intravenous catheter was inserted into the jugular vein 24h prior to the baseline frequently sampled intravenous glucose tolerance test (**FSIGTT**). The baseline FSIGTT was then performed at 0800 on day four (Monday). A CRI of either LPS or 0.9% NaCl solution was initiated at 0800 on day five (Tuesday) and continued until 1600. At 0800 on day six (Wednesday; 16h after completion of the CRI), each horse received either 5 g/kg OF dissolved in 4L of water or 4L water alone via nasogastric intubation. A post-treatment FSIGTT was performed 24h later, starting at 0800 on day seven (Thursday). Horses were evaluated for the presence of laminitis for 48h after the administration of water or OF using the Obel grading system (Taylor *et al.* 2002). Horses that developed laminitis remained hospitalized and were treated with phenylbutazone until clinical signs of laminitis resolved or euthanasia was performed. The study protocol was approved by the University of Tennessee Institutional Animal Care and Use Committee.

Lipopolysaccharide administration – *Escherichia coli* O55:B5 LPS[1] was mixed with 1L 0.9% NaCl with the investigator wearing protective attire. Lipopolysaccharide solution was infused intravenously at a dosage of 7.5 ng/kg/h for 8h using an infusion pump set at a rate of 100 mL/h. Horses enrolled in the OF group received 800 mL 0.9% NaCl intravenously at the same rate. Horses were observed for signs of colic and physical examination parameters were monitored, including rectal temperature, heart rate and respiratory rate, for 72h after the initiation of the CRI. Data were recorded hourly during the 8-hour CRI, then every 2h for 8h, and finally every 4h for 56h.

Oligofructose administration – Oligofructose[2] was administered to horses at a dosage of 5 g/kg bwt. Powder was mixed with 4L warm water until dissolved and then administered via nasogastric intubation. Horses in the LPS group received 4L water alone by the same route.

Complete blood count (CBC) analysis – To evaluate the individual response to endotoxin, CBC analysis was performed in each horse on three occasions. Blood was collected from the indwelling jugular catheter into tubes containing EDTA before the CRI was initiated, 2 hours after initiation of the CRI, and finally at the end of the 8-hour infusion. Samples were immediately transported to the clinical pathology laboratory within the teaching hospital for CBC analysis.

FSIGTT procedure – A 14-gauge polypropylene catheter[3] was inserted into the left jugular vein the day before procedures were initiated and an injection cap and infusion set[4] (length, 30 cm; internal diameter, 0.014 cm) were attached to the catheter during each test. The FSIGTT first described by Hoffman (Hoffman *et al.* 2003) and further refined by our research group (Chapter 4) was used. Briefly, a rapid (< 1 min) bolus infusion (100 mg of glucose/kg) of 50% (wt/vol) dextrose solution[5] was administered to

[1] Sigma Chemical Co., St Louis, MO,
[2] Orafti P95 BENEO-Orafti Inc. 2740 Route 10 West, Morris Plains, NJ 07950
[3] Abbocath-T 14G X 140mm, Abbott Laboratories, North Chicago, IL.
[4] Butterfly, Abbott Laboratories, North Chicago, IL.
[5] Dextrose 50% injection, Abbott Laboratories, North Chicago, IL.

each horse via the infusion line and catheter, followed by 20 mL infusion of saline solution containing heparin (4 U/mL). Blood samples were then collected via the catheter 10, 5, and 1 min prior to, and 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, and 19 min after infusion of dextrose. At 20 min, regular insulin[6] (20 mU/kg) was infused, followed by another infusion of 20 mL saline solution containing heparin. Blood samples were subsequently collected via the catheter at 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150, and 180 min relative to the dextrose bolus infusion. At each time point, 3 mL of blood was withdrawn from the infusion line and discarded. A 6 mL blood sample was then collected, followed by infusion of 5 mL of saline solution containing heparin. Half of the volume of blood was transferred to a tube containing sodium heparin, which was immediately cooled on ice and then refrigerated. The remaining blood was transferred to a tube containing no anticoagulant. These samples were allowed to clot at 22°C for 1h and then serum was harvested via low-speed (1,000 × *g*) centrifugation. Plasma and serum samples were stored at –20°C until further analyzed.

Laminitis scoring – Obel grading was performed as previously described (Taylor *et al.* 2002) and horses were assessed before LPS or saline was infused intravenously on day four. Obel grades were also determined every 4h for 56h after OF or water was administered. Horses that developed laminitis were subsequently monitored every 12h until lameness resolved (n = 6) or euthanasia was performed (n = 1).

Phenylbutazone administration – Treatment for laminitis was initiated as soon as Obel grade ≥ 2 laminitis was detected. Phenylbutazone[7] was administered at an initial dosage of 4.4 mg/kg IV or PO q12h. The dosage was then gradually reduced over time, according to the response shown by each individual horse.

Plasma glucose and serum insulin concentrations – Plasma glucose concentrations were measured by use of a colorimetric assay[8] on an automated discrete analyzer[9].

[6] Humulin R, Eli Lilly and Co, Indianapolis, IN.
[7] Equi-Phar, Vedco, Inc., St. Joseph, MO.
[8] Glucose, Roche Diagnostic Systems Inc, Somerville, NJ.

Serum insulin concentrations were determined by use of a radioimmunoassay[10] that has previously been validated for use in horses (Freestone *et al.* 1991). Each sample was assayed in duplicate and intra-assay coefficients of variation < 5% or < 10% were required for acceptance of glucose and insulin assay results, respectively.

Interpretation of FSIGTT data by use of the minimal model – Values of insulin sensitivity (**SI**), glucose effectiveness (**Sg**), acute insulin response to glucose (**AIRg**), and disposition index (**DI**) were calculated for each FSIGTT in accordance with the minimal model (Bergman *et al.* 1981) by use of commercially available software[11,12] and previously described methods (Hoffman *et al.* 2003). Disposition index was calculated via multiplication of AIRg by SI.

Statistical analysis – White blood cell, neutrophil and lymphocyte counts obtained at 0, 2h, and 8h relative to the initiation of the LPS infusion were compared between horses receiving endotoxin (LPS and LPS/OF) versus saline control (OF group) using a mixed model analysis of variance with repeated measures. Heart rate, respiratory rate and rectal temperature measurements were compared among treatment groups using mixed model analysis of variance with repeated measures. Fisher's Exact Test was used to evaluate the relationship between development of laminitis and treatment groups. In each horse, area under the curve for glucose (**AUCg**) and for insulin (**AUCi**) were calculated for both FSIGTT procedures (baseline, post-treatment) from the glucose and insulin concentrations measured, by use of the trapezoidal method, using commercially available computer software.[13] To determine the effects of treatments and time (baseline, post-treatment) on Sg, SI, AIRg, DI, AUCi and AUCg mixed model analysis of variance with repeated measures was used. Two outliers, an SI value with a studentized residual of 4.79 and an AIRg value with a studentized residual of 3.22, were eliminated from the final analysis. When a significant treatment × time effect was detected, protected Least

[9] Cobas Mira, Roche Diagnostic Systems Inc, Somerville, NJ.
[10] Coat-A-Count insulin, Diagnostic Products Corp, Los Angeles, CA.
[11] MinMod Millenium, version 6.10, Raymond Boston, University of Pennsylvania, Kennet Square, PA.
[12] Stata 9.2, Stata Corporation, College Station, TX.
[13] PROC MIXED, SAS, version 9.1, SAS Institute Inc, Cary, NC

Significant Difference mean separation method was used to compare groups at different time points. Significance was defined at a value of $P < 0.05$.

6.3 Results

Horses that received LPS exhibited signs of abdominal pain, including pawing, stretching and occasional attempts to roll during the first 4h of the LPS infusion. Transient diarrhea was noted 16 to 24h following OF administration when horses received this treatment. At the initiation of the study, none of the horses exhibited signs of laminitis (Obel grade 0). Laminitis developed in 7 horses 28 to 36h following OF administration and all affected animals recovered after treatment with phenylbutazone, with one exception. This horse from the LPS/OF group developed Obel grade 4 laminitis and remained lame despite treatment with phenylbutazone. Euthanasia was performed and pathological examination revealed marked rotation of the third phalanx in all four feet, compatible with severe laminitis.

Significant time × treatment effects ($P < 0.001$) were registered for WBC, neutrophil, and lymphocyte counts (**Table 6.1** and **Figure 6.1**). Two hours following the initiation of the LPS infusion, neutrophil, WBC and lymphocyte counts significantly decreased in LPS and LPS/OF groups relative to baseline values, and compared to horses receiving 0.9 % NaCl treatment (OF group). Neutrophil and WBC counts significantly increased whereas lymphocyte counts further decreased 8 h following the commencement of the LPS infusion in LPS and LPS/OF groups, relative to baseline or control (OF group) values.

With respect to physical examination findings, significant treatment × time effects were registered for rectal temperature ($P < 0.001$), heart rate ($P < 0.001$) and respiratory rate ($P = 0.004$). A biphasic response for rectal temperature and heart rate was observed in horses from the LPS/OF group, with peaks apparent approximately 2 to 8h after commencement of the LPS infusion and again 24h after nasogastric intubation with OF. Single peaks were observed approximately 2 to 8h after the initiation of the LPS infusion and at 24 h after OF administration in the LPS and OF groups, respectively (**Figure 6.2**).

Table 6.1 – Mean ± SD white blood cell, neutrophil, and lymphocyte counts registered at 0, 2 and 8 h relative to the initiation of an 8-hour lipopolysaccharide (LPS and LPS/OF groups) or saline (OF group) continuous rate infusion.

		Treatment		Treatment × time
		LPS and LPS/OF (n = 16)	**OF (n = 8)**	***P***
WBC ($\times 10^3/\mu L$)	0 h	7.32 ± 1.35 [a]	6.98 ± 1.60 [a]	< 0.001
	2 h	3.05 ±1.18 [b]	7.28 ± 1.81 [a]	
	8 h	8.81 ± 2.30 [c]	7.20 ± 1.48 [a]	
Neutrophils ($\times 10^3/\mu L$)	0 h	4.24 ± 1.26 [a]	4.02 ± 1.01 [a]	< 0.001
	2 h	1.54 ± 1.00 [b]	4.17 ± 1.41 [a]	
	8 h	7.89 ± 2.34 [c]	4.09 ± 1.07 [a]	
Lymphocytes ($\times 10^3/\mu L$)	0 h	2.68 ± 0.59 [a]	2.50 ± 0.84 [a]	< 0.001
	2 h	1.39 ± 0.36 [b]	2.63 ± 0.87 [a]	
	8 h	0.80 ± 0.39 [c]	2.66 ± 0.78 [a]	

[a-c] For variables with significant ($P < 0.05$) treatment × time effects, mean values with different superscripts differ significantly, as determined by ANOVA for repeated measures and comparison of least squares mean values by use of a Least Significant Difference adjustment.

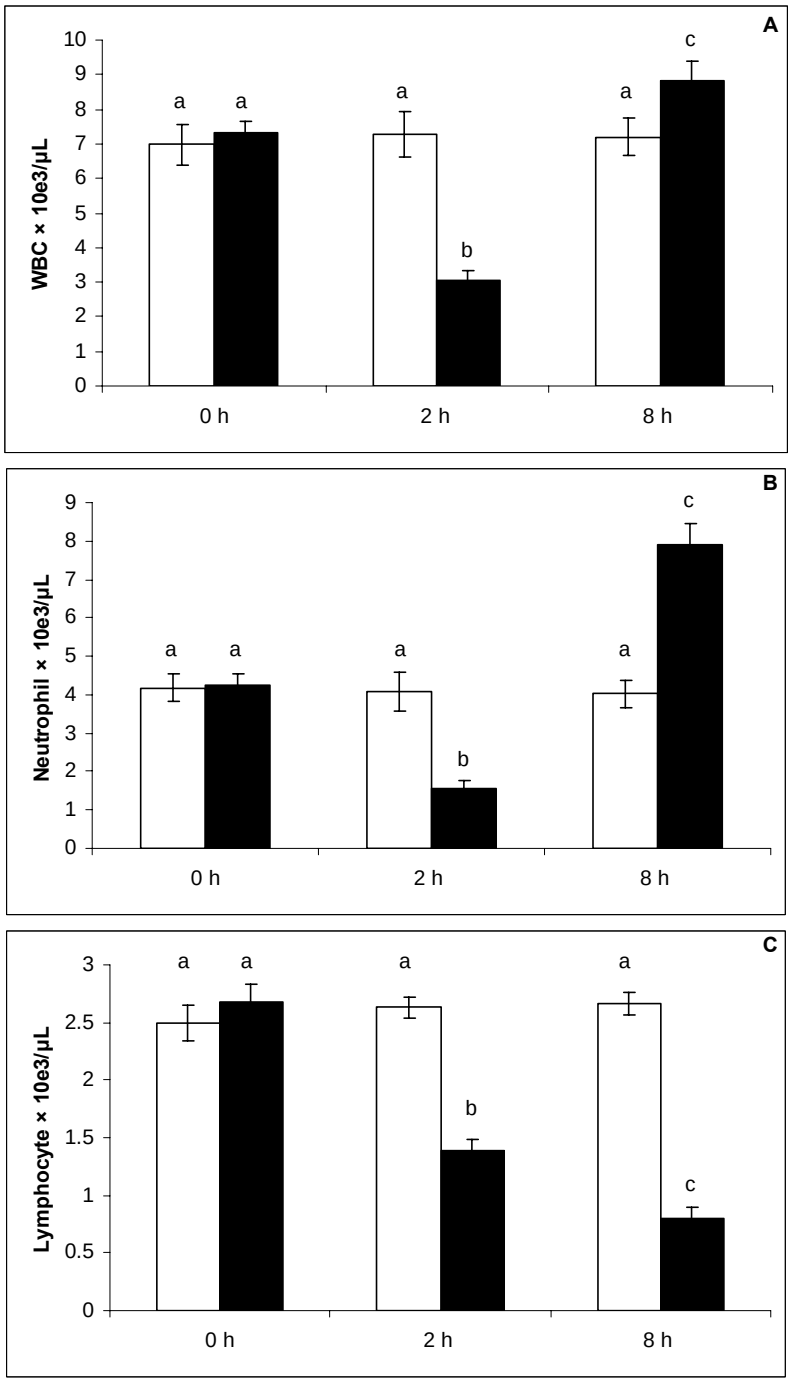

Figure 6.1 – Mean ± SE white blood cell (panel A), neutrophil (panel B), and lymphocyte (panel C) counts registered at 0, 2 and 8 h relative to initiation of the LPS (open columns; LPS and LPS/OF groups) or saline (solid columns; OF group) continuous rate infusion. Columns with different superscripts differ significantly at the $P < 0.05$ level.

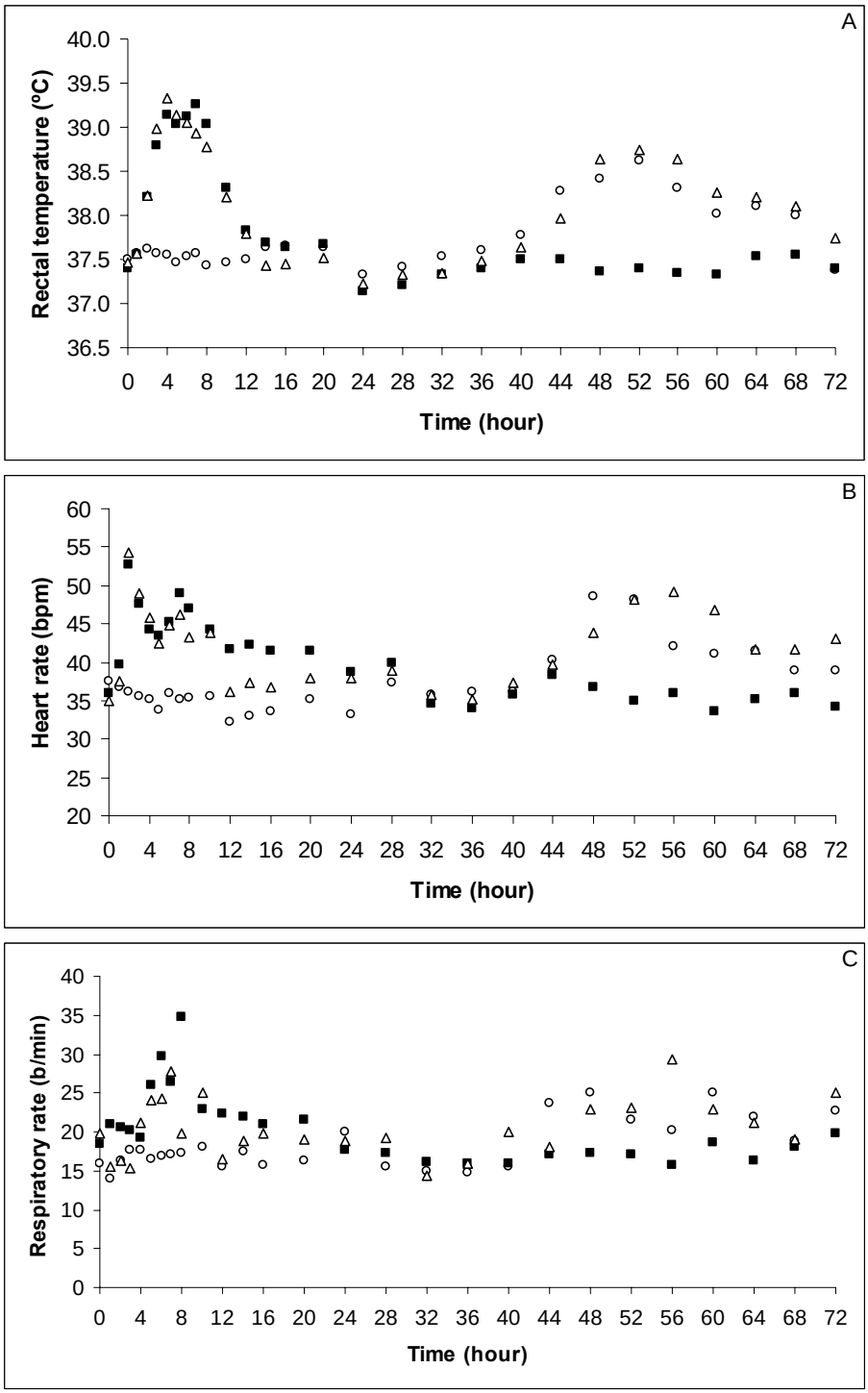

Figure 6.2 – Mean rectal temperature (panel A), heart rate (panel B) and respiratory rate (panel C) registered in horses receiving lipopolysaccharide (LPS; solid squares), oligofructose (OF; open circles), or LPS followed by OF (open triangles).

Mean ± SD Obel laminitis grade was zero, 0.4 ± 0.7, and 1.7 ± 1.5 for LPS, OF and LPS/OF groups, respectively. Laminitis was not observed in the LPS group. Two horses in the OF group developed Obel grade 1 (n = 1) or 2 (n = 1) laminitis and five horses in the LPS/OF group developed Obel grade 2 (n = 2), 2.5 (n = 1), 3 (n = 1), and 4 (n = 1) laminitis. The Fisher's exact test revealed a significant (P = 0.021) association between treatment groups and development of Obel grade $\geq$ 2 laminitis with 0, 12.5 and 62.5 % of horses developing Obel grade $\geq$ 2 laminitis of in LPS, OF and LPS/OF groups, respectively. Individual comparisons between treatment groups using the Fisher's exact test demonstrated that horses in the LPS/OF group were significantly (P = 0.013; one-tailed test) more likely to develop laminitis of Obel grade $\geq$ 2 than horses in the LPS group. A trend (P = 0.060; one-tailed test) towards increased likelihood of developing laminitis of Obel grade $\geq$ 2 in horses receiving both LPS and OF versus OF alone was also detected. It was noted that 4 of 5 cases of laminitis registered in the LPS/OF group occurred during the months of February and March, when horses were kept on pasture and fed primarily hay in round bales.

Although post-treatment SI was significantly decreased over time compared to baseline in LPS (P = 0.006), OF (P < 0.001), and LPS/OF (P = 0.018) groups, the treatment × time effect was not statistically significant (P = 0.224; **Table 6.2**). Conversely, a significant treatment × time effect (P = 0.037) was detected for AIRg, as the pancreatic insulin response increased significantly over time in the LPS (P = 0.005) and LPS/OF (P < 0.001) groups, but remained unchanged (P = 0.104) in the OF group. Disposition index remained unchanged over time for LPS (P = 0.293) and LPS/OF (P = 0.160) groups, but significantly decreased after OF (P = 0.011) administration, which resulted in a significant treatment × time effect (P = 0.022). No significant alterations in Sg were registered.

Mean post-treatment AUCg and AUCi were significantly higher than pre-treatment baseline values in all three groups. A significant treatment × time effect was registered for AUCg (P < 0.001), but not AUCi (P = 0.093).

Table 6.2 – Mean ± SD glucose and insulin variables values registered before (pre) and after (post) treatment in horses from lipopolysaccharide (LPS), oligofructose (OF), and LPS/OF groups.

		Treatment			Treatment × time
		LPS	**OF**	**LPS/OF**	***P***
SI ($L \cdot min^{-1} \cdot mU^{-1}$) $\times 10^{-4}$	Pre	3.59 ± 1.62 [a,b]	5.1 ± 2.28 [a]	3.61 ± 2.09 [a]	0.224
	Post	1.21 ± 0.93 [c]	1.31 ± 0.71 [c]	1.85 ± 2.15 [b,c]	
Sg (min^{-1}) $\times 10^{-2}$	Pre	2.44 ± 0.56	3.24 ± 1.59	2.1 ± 0.9	0.320
	Post	2.09 ± 1.75	2.85 ± 1.45	2.85 ± 1.09	
AIRg ($mU \cdot min \cdot L^{-1}$)	Pre	469 ± 316 [a]	490 ± 386 [a,b]	346 ± 114 [a]	0.038
	Post	861 ± 456 [b,c]	702 ± 449 [a,b,c]	1046 ± 602 [c]	
DI $\times 10^{-2}$	Pre	13.5 ± 4.8 [a,b,c]	18.6 ± 5.9 [a,b]	15.4 ± 9.8 [a,b,c]	0.023
	Post	9.6 ± 7.0 [b,c]	8.2 ± 6.0 [c]	20.8 ± 18.2 [a]	
AUCi (mU/mL•min)	Pre	6.29 ± 3.01 [a,b,c]	5.66 ± 2.64 [a,b]	4.46 ± 1.43 [a]	0.093
	Post	12.18 ± 3.80 [d]	8.04 ±3.08 [c]	8.11 ± 3.52 [b,c]	
AUCg (g/dL•min)	Pre	16.00 ± 1.41 [a]	16.43 ± 1.40 [a,b]	16.18 ± 1.14 [a]	< 0.001
	Post	18.25 ± 2.64 [b,c]	23.36 ± 2.67 [d]	19.92 ± 1.67 [c]	

[a-c] For variables with significant ($P < 0.05$) treatment × time effects, mean values with different superscripts differ significantly, as determined by ANOVA for repeated measures and comparison of least squares mean values by use of a Least Significant Difference adjustment.

6.4 Discussion

Administration of LPS, OF, or both treatments lowered insulin sensitivity, but the magnitude of change did not differ between groups, so our hypothesis that pretreatment with LPS would exacerbate the insulin resistance induced by OF was not supported. Nevertheless, significant association between treatments and the incidence of Obel grade $\geq$ 2 laminitis was demonstrated, with the highest incidence of laminitis observed in the LPS/OF group. Results suggest that endotoxemia is an important determinant of laminitis severity in the OF experimental model, but lipopolysaccharide does not induce the condition when administered alone for 8h. Furthermore, physical examination and complete blood count results indicate that continuous infusion of LPS at a rate of 7.5 ng/kg/h is sufficient to induce a systemic inflammatory response. This provides an experimental model for systemic inflammation and results help to explain why endotoxemia is an important risk factor for laminitis in horses.

Alterations in WBC, neutrophil, and lymphocyte counts were induced by endotoxemia in this study. Previous studies investigating the effects of endotoxemia on blood leukocytes in horses have yielded variable results (Kelmer *et al.* 2008; MacKay *et al.* 1999; Morris *et al.* 1990). Intravenous administration of 30 ng/kg *Escherichia coli* O55:B5 endotoxin significantly decreased the WBC count between 60 and 300 min after endotoxin infusion (MacKay *et al.* 1999). Morris *et al.* (1990) used the same endotoxin dosage and detected a significantly lower WBC count 1.5 and 2 h following endotoxin infusion, with counts returning to baseline 2.5 to 8h after LPS administration. In the study reported here, the initial leukopenia registered at 2 h was followed by a rebound leukocytosis recorded at 8 h, with the WBC count significantly higher than baseline. The rebound leukocytosis was most likely due to the significant increase in neutrophil count registered 8 h after initiation of the LPS infusion. This latter finding is consistent with results reported by Kelmer *et al*. (2008). This group detected a significantly higher mean polymorphonuclear cell count 6h after 0.2 μg/kg LPS was administered intravenously to horses. The higher neutrophil count detected 8h after initiation of the endotoxin CRI in our study is likely to be a consequence of enhanced granulocyte production and release from the bone marrow (Morris 1991). This was likely to have been triggered by

endotoxin-induced secretion of granulocyte colony stimulating factor, keratinocyte-derived chemokine, and macrophage inflammatory protein-2 (Morris 1991; Zhang *et al.* 2005). Elevated plasma cortisol concentrations have been detected in humans following endotoxin administration and this would provide an explanation for the progressive lymphopenia registered during the endotoxin CRI if the same alteration occurs in horses (Richardson *et al.* 1989). Unfortunately, blood cortisol concentrations were not measured in this study.

Alterations in physical examination parameters have previously been associated with exogenous endotoxin administration in horses (Barton *et al.* 2004; Clark and Moore 1989; Kelmer *et al.* 2008; Moore *et al.* 2007). Administration of LPS to horses at different dosages (20 to 200 ng/kg) significantly increased heart rate and rectal temperature with peak values registered between 60 – 120 min and 120 – 180 min after bolus infusion, respectively (Barton *et al.* 2004; Kelmer *et al.* 2008; Morris *et al.* 1990). In horses, physical examination findings have also been significantly altered by OF administration (Kalck *et al.* in press; van Eps and Pollitt 2006). Administration of 10g OF/kg bwt significantly increased rectal temperature and heart rate, with the highest values demonstrated at 20 and 32 h relative to OF administration (van Eps and Pollitt 2006). These findings correspond well with the alterations in physical examination parameters detected in this study, and provide an explanation for the biphasic response observed in horses receiving OF sixteen hours after completion of the LPS infusion (LPS/OF group).

Endotoxemia has previously been shown to influence glucose and insulin dynamics in horses. Our research group demonstrated that intravenous administration of 20 ng/kg *Escherichia coli* O55:B5 lipopolysaccharide significantly lowered SI and DI, and raised AIRg when administered to healthy horses (Toth *et al.* 2008). Endotoxemia-induced IR has also been detected in horses by Vick *et al*. (2008). This group used the euglycemic hyperinsulinemic clamp and showed that insulin sensitivity significantly decreased 24h after the administration of 0.045 μg/kg LPS. These findings correspond well with results of the current study, which also show that SI significantly decreased from the baseline mean value of 3.59×10^{-4} $L \bullet min^{-1} \bullet mU^{-1}$ to the post-treatment value of

1.21×10^{-4} L•min^{-1}•mU^{-1} in horses receiving LPS. Additionally, the significantly higher AUCg and AUCi detected in horses treated with LPS provides further evidence of reduced insulin sensitivity.

It has previously been shown that administration of 7.5 to 12.5 mg/kg OF via nasogastric intubation alters blood glucose and insulin concentrations in horses (van Eps and Pollitt 2006). Nasogastric intubation with 10 g/kg OF significantly increased plasma glucose concentrations from 12 to 48 h after its administration, relative to saline treated controls. A similar trend was noted for serum insulin concentrations as well, although significant differences were only registered at 12, 40, and 44h (van Eps and Pollitt 2006). Results of a recent study performed by our research group indicate that intestinal carbohydrate overload induced by OF significantly alters glucose dynamics in horses. Higher AUCg values for the FSIGTT were detected 24 and 48 h after administration of 5 g OF/kg bwt, when compared with controls (Kalck *et al.* in press). Minimal model analysis results from the same study indicated lower insulin sensitivity 24 and 48 h after OF administration relative to controls, but this difference was not statistically significant because of wide variability in results between and within horses.

In the study reported here, horses in the LPS, OF and LPS/OF groups demonstrated a significant decrease in SI, accompanied by significant increases in AUCg and AUCi. Changes in AUCg were greatest in horses receiving the OF treatment alone, indicated by the significant time × treatment interaction. Consistent with these findings Kalck *et al* (in press) also demonstrated that AUCg values increased 34 and 32% when measured 24 and 48h, respectively after oral administration of 5g/kg OF to horses. Higher AUCg values registered following OF administration are more likely to be a consequence of decreased insulin sensitivity than fructan absorption from the small intestine, because only a small amount of this carbohydrate is absorbed in the proximal gastrointestinal tract of the horse (Bailey *et al.* 2007). Administration of LPS followed by OF had no additive effect on SI or AUCi in this study and AUCg was more profoundly affected when OF was administered alone. This suggests that prior exposure to endotoxin reduces the effect of intestinal carbohydrate on glucose metabolism. Development of early endotoxin tolerance, characteristic of repeated endotoxin exposure,

may explain the absence of an additive effect of LPS/OF treatment on glucose and insulin dynamics. Higher maximum TNFα concentrations and earlier, more sustained serum TNFα activity was detected following the first infusion when two endotoxin infusions were administered to horses 24h apart (Allen *et al.* 1996), indicating that early phase endotoxin tolerance develops in horses. Nevertheless, the biphasic response detected for physical examination variables is not consistent with endotoxin tolerance. Alternatively, LPS infusion alone may have maximally lowered SI in the LPS/OF group.

Increased AIRg successfully compensated for lower SI in horses receiving LPS and LPS/OF treatments, as demonstrated by DI values being statistically the same before and after treatment in these groups. It is thought that pancreatic insulin secretion increases to sustain insulin-mediated glucose disposal when insulin sensitivity decreases, and this represents an efficient compensatory mechanism in the horse (Treiber *et al.* 2006b). In contrast, humans with IR more commonly develop pancreatic exhaustion in association with type II diabetes mellitus (Brunton *et al.* 2006; Kaiser *et al.* 2003). Equine pancreatic β-cells maintain high basal insulin concentrations and euglycemia, which makes type II DM a rare condition in the horse (Treiber *et al.* 2005b). Nevertheless, excessive compensation characterized by elevated circulating insulin concentrations may be a precursor of metabolic disorders, such as laminitis (Treiber *et al.* 2005a). Horses in the OF group, however, suffered from uncompensated IR, as indicated by the lower post-treatment mean DI value. Decompensation develops when increased insulin secretion can no longer be sustained. Decompensated insulin resistance characterized by hyperglycemia has been described in horses suffering from laminitis (Treiber *et al.* 2006b).

In the study reported here, a significant association existed between treatment and the incidence of laminitis, with 0 of 8, 1 of 8, and 5 of 8 horses developing Obel grade $\geq$ 2 laminitis in the LPS, OF and LPS/OF groups, respectively. The highest incidence of laminitis occurred in the LPS/OF group and this indicates that endotoxemia plays an important role in the development of this condition. This finding is supported by the previous observation that hospitalized horses suffering from clinical endotoxemia have an odds ratio of five to develop laminitis (Parsons *et al.* 2007). Endotoxemia may contribute

to the development of laminitis by inducing digital vasoconstriction mediated by 5-hydroxytryptamine and thromboxane B_2 (Menzies-Gow *et al.* 2004). Alternatively, endotoxemia may enhance cyclooxygenase-2 mRNA expression in vascular smooth muscle cells (Rodgerson *et al.* 2001) or activate the coagulation cascade (Pernerstorfer *et al.* 1999; Weiss *et al.* 1994). It has recently been shown that horses exhibit a strong innate immune response during the developmental stages of laminitis, and a mixture of innate and adaptive immune responses at the onset of lameness, which suggests that lamellar inflammation plays an important role in laminitis (Belknap *et al.* 2007). Although measurements of inflammatory cytokines were not performed in the study reported here, clinical examination and CBC findings were suggestive of a systemic inflammatory response following the lipopolysaccharide CRI and again after administration of OF. These inflammatory responses provide a plausible explanation for the high incidence of laminitis in horses that received LPS followed by OF.

Although endotoxemia has long been suspected to contribute to the development of laminitis (Elliott and Bailey 2006) and LPS has been demonstrated in the blood of horses subjected to carbohydrate overload to induce laminitis (Sprouse *et al.* 1987), convincing evidence connecting these two conditions has been lacking. Therefore, the ability of endotoxin pretreatment to increase the incidence of Obel grade ≥ 2 laminitis, demonstrated in this study, is of great clinical importance. Our findings indicate that endotoxemia associated with colitis, strangulating intestinal obstruction, metritis or pleuropneumonia (MacKay 2001; Morris 1991) requires aggressive treatment in order to prevent laminitis. Furthermore, if minor intestinal events occur in horses grazing on pasture (Longland and Byrd 2006), they may result in subclinical endotoxemia that could trigger laminitis. Ponies suffering from IR are predisposed to pasture-associated laminitis (Carter *et al.* 2009; Treiber *et al.* 2006b), so endotoxin-mediated exacerbation of preexisting IR may be an important trigger for laminitis when susceptible horses and ponies are grazing on pasture. Lower insulin sensitivity associated with endotoxemia (Toth *et al.* 2008; Vick *et al.* 2008) may contribute to the development of laminitis through the induction of vasoconstriction (Kim *et al.* 2006) or activation of matrix metalloproteinases (Treiber 2005).

At the same time, it is important to note that none of the horses receiving the LPS infusion as the only treatment (LPS group) developed laminitis. This finding is in agreement with results of previous studies in which laminitis failed to develop after experimental administration of endotoxin alone (Barton *et al.* 2004; Kelmer *et al.* 2008; MacKay *et al.* 1999). Only one study reports the development of clinical signs consistent with laminitis in 6 of 7 horses following endotoxin administration (Duncan *et al.* 1985). This study by Duncan *et al.* (1985) involved 24-hour infusion of endotoxin into the portal vein at a rate of 1 μg/kg/h.

Laminitis of Obel grade 1 or 2 developed in two horses from the OF group that did not receive LPS beforehand. Nasogastric intubation with OF, using dosages ranging from 5.0 to 12.5 g/kg has been successfully used to induce laminitis in previous studies (Croser and Pollitt 2006; French and Pollitt 2004b; Kalck *et al.* in press; van Eps and Pollitt 2006). Kalck *et al.* (in press) recently demonstrated that administration of 5 g/kg OF induced clinically apparent laminitis in 3 of 8 horses, which corresponds well with our findings. A temporal progression of histopathologic changes consistent with laminitis has previously been demonstrated when hoof biopsy samples were collected every 6h for 36h following administration of 10 g/kg OF (Croser and Pollitt 2006). Histopathologic examination of lamellar basal cells harvested from horses 48 h after nasogastric intubation with 7.5, 10, and 12.5 g/kg OF revealed that the severity of pathologic changes, including loss of hemidesmosomes, rounding of basal cell nuclei and failure of the cytoskeleton, positively correlated with the OF dosage used (French and Pollitt 2004b). The authors hypothesized that OF administration induces laminitis through activation of matrix metalloproteinases and glucose deprivation of basal cells found within secondary epidermal lamellae (French and Pollitt 2004b). Nasogastric intubation with 10 g/kg OF also induces profound changes in the hindgut bacterial population by causing a shift from the predominantly Gram-negative flora to one dominated by Gram-positive bacteria (Milinovich *et al.* 2006). Initial overgrowth of bacteria belonging to the *Streptococcus bovis/equinus* complex, predominantly *Streptococcus lutetiensis* followed by a rapid population decline were consistently observed in horses developing laminitis after OF administration. These findings suggest

the involvement of streptococci in the development of laminitis induced by OF overload (Milinovich *et al.* 2007; Milinovich *et al.* 2006).

In this study, the incidence of laminitis decreased during the months of April and May when pastures were growing rapidly. It has been shown that the storage carbohydrate content of pasture grass is highest during late spring (Longland and Byrd 2006; Treiber *et al.* 2006b), therefore adaptation of the intestinal flora to this higher carbohydrate load may have prevented acute digestive disturbances associated with OF administration. Our results therefore indicate that horses are at greatest risk for developing carbohydrate overload and laminitis when they are suddenly turned out on pasture, instead of being gradually introduced to the new environment. Further studies are required to determine whether adaptation to a high soluble carbohydrate diet helps prevent laminitis induced by carbohydrate overload. For the purpose of the experimental model, horses should be removed from pasture and fed grass hay for 4 weeks in order to prevent adaptation.

In conclusion, findings of the study reported here indicate that endotoxemia is an important determinant of laminitis severity. Laminitis developed in a greater number of horses and was more severe when LPS was administered before induction of intestinal carbohydrate overload. Results suggest that endotoxemia should be intensively managed in horses suffering from colitis or strangulating obstructions to prevent the development of laminitis. Minor intestinal events associated with grazing on pasture may also result in endotoxemia, which would be expected to raise the risk of laminitis in pastured horses and ponies.

CHAPTER 7
General Summary and Future Directions

Studies reported here aimed to investigate glucose and insulin dynamics in horses and their relationship to endotoxemia and laminitis.

Our results provide substantial evidence of an association between endotoxemia and insulin resistance (**IR**) in horses. However, the specific alterations in insulin signal transduction induced by endotoxemia must still be elucidated in the horse. Accurate characterization of mediators involved in the development of endotoxin-mediated IR may provide new targets for pharmacological interventions aimed to restore normal insulin sensitivity. Results of our study examining the effects of resting insulin sensitivity on the magnitude of IR induced by endotoxemia suggest that pretreatment with levothyroxine sodium days prevents the reduction in insulin sensitivity triggered by endotoxemia. Studies must be performed in the future to determine the minimal duration of treatment required to achieve this protection and investigate the exact mechanisms responsible for this protective effect. This work may lead to the development of new drugs for preventing or treating IR.

Our observation that endotoxemia predisposes horses to laminitis corresponds well with clinical experience, and confirms findings previously reported in a retrospective study (Parsons *et al.* 2007). Nevertheless, the pathophysiological mechanism by which endotoxemia facilitates the development of laminitis must still be identified. The systemic inflammatory response triggered by endotoxemia and IR are likely to play important roles in the development of laminitis (Belknap 2007; Treiber *et al.* 2006a). Induction of IR using a model other than endotoxemia, such as lipid infusion (Sessions *et al.* 2004) or dexamethasone administration (Tiley *et al.* 2007), followed by challenge with 5 g/kg oligofructose would provide a practical method for assessing the role of altered insulin sensitivity in the development of laminitis. If a higher incidence of laminitis were demonstrated in horses with experimentally-induced IR, it would suggest that reduced insulin sensitivity is an independent risk factor for laminitis in horses. Furthermore, mitigation of endotoxin-induced IR by pretreatment of horses with

levothyroxine sodium preceding oligofructose administration would be an alternative method to evaluate the role of endotoxin-induced IR in laminitis development. Temporal measurements of inflammatory mediators in lamellar tissues harvested from horses suffering from clinical endotoxemia with and without laminitis and comparison of these results with healthy horses would help to determine the role of the endotoxin-induced inflammatory response in the development of laminitis.

Minimal model analysis has been used throughout these studies to interpret frequently sampled intravenous glucose tolerance test (**FSIGTT**) data and to assess glucose and insulin homeostasis. This procedure was selected because it not only estimates insulin sensitivity (**SI**), but also acute insulin response to glucose (**AIRg**), glucose effectiveness (**Sg**) and disposition index (**DI**). Technical difficulties associated with the euglycemic hyperinsulinemic clamp (**EHC**) also contributed to the selection of the FSIGTT method. However, our studies also showed that minimal model analysis increased variance, which reduced the likelihood of attaining statistical significance. Thus, to further characterize glucose and insulin dynamics, area under the glucose (**AUCg**) and insulin (**AUCi**) curves were calculated in two studies included in this thesis, and between-horse variability was lower for these values. A study should be performed in the future to investigate the relationship between area under the curve values and minimal model analysis estimates using the statistical method of regression. Area under the curve values alone or AUCg-to-AUCi ratios may provide an alternative method for estimating insulin sensitivity in the future.

C-peptide-to-insulin ratios in horses undergoing the FSIGTT suggest that hepatic insulin extraction decreases following dextrose challenge. Furthermore, the basal C-peptide-to-insulin ratio appeared to be lower in a horse with IR, when compared with horses with normal insulin sensitivity, and this ratio further decreased following dextrose administration during the FSIGTT. This lower C-peptide-to-insulin ratio may indicate that elevated resting insulin concentrations and higher AIRg values registered in horses with IR are a result of reduced hepatic insulin extraction. Further studies are required to compare C-peptide-to-insulin ratios in horses with IR to healthy control animals. Comparing insulin and C-peptide concentrations in blood collected from the portal and

jugular vein in normal and insulin resistant horses would also allow hepatic insulin clearance to be quantified. Development and validation of an equine specific C-peptide radioimmunoassay kit would provide more accurate measurement of C-peptide concentrations and facilitate further evaluation of pancreatic function in horses.

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Vita

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