

8-8-2019

## Effects of Adrenergic or A Combination of Adrenergic and Opioid Drugs on Assessments of Insulin Sensitivity in Mares

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EFFECTS OF ADRENERGIC OR A COMBINATION OF ADRENERGIC AND OPIOID  
DRUGS ON ASSESSMENTS OF INSULIN SENSITIVITY IN MARES

A Thesis

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

in

The School of Animal Sciences

by  
Lauren Kerrigan  
B.S., Louisiana State University, 2017  
December 2019

## **ACKNOWLEDGEMENTS**

I would like to begin by thanking my major professor, Dr. Donald L. Thompson, Jr. I would not be here if it weren't for him and I am lucky to have studied under his guidance and extend my sincerest gratitude to him. I'd like to express gratitude toward Dr. Erin L. Oberhaus for mentoring me throughout these experiments and guiding me during this journey. I'd like to thank Dr. Ann M. Chapman, for her expertise in the field and Dr. Cathleen Williams and Dr. Dale Paccamonti for their willingness to serve as advisors on my committee. Thank you to Nicole Arana Valencia for contributing to my success, whether it was advice or teaching me in the lab. Thank you to LSU Equine Health Studies Program within the LSU School of Veterinary Medicine for partial funding of this project. I'd like to thank Kristina Lofrumento for her encouragement and friendship during these two years. I could always count on her for her support, and her love for glucose and insulin to inspire me during these experiments. I would also like to thank my other fellow graduate students, Kaitlyn Wilson, Caroline Camp, and Brianna Whitley, for their help in the very early mornings during the cold winter months, and teaching me to love fast food. I am grateful to Mr. Randy Wright, Mr. Joe Paul, Ms. Sonyja Thomas, Steven Blair, Dalton Bevill, and Cobey Hendry for their unending willingness to help on my projects: my research would not have been possible without their help in moving and caretaking for the horses, and their dedication to the LSU farms. An additional thanks to all the students and student workers for their assistance at the farm and in the lab, these projects would not have run as smoothly or efficiently without their help. Thank you to my Mom, Dad, and sister, Kristi for their unconditional love and encouragement. Finally I'd like to thank my grandmother, "Sittee", and godmother, "Aunt Terry", who both passed away during my degree.

Although they are not here with me to see me finish, their unequivocal strength as women, kindness, and selflessness, taught me how to treat others and to be the woman I am today.

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## ABSTRACT

Excitement is a problem when conducting endocrine tests on fractious horses. Epinephrine (EPI), for example, was shown to obliterate the insulin-induced decrease in blood glucose concentrations. Sedation may be a solution; however, perturbation of results may preclude useful information. The objectives of the four experiments presented herein were 1) to determine the effects of EPI on insulin response to glucose infusion (IR2G), 2) to assess the effects of the  $\alpha$ -adrenergic sedative, detomidine (DET), alone or in combination with the opioid agonist, butorphanol (BUT), on IR2G and glucose response to insulin (GR2I), and 3) to assess the effects of BUT alone on IR2G. In Experiment 1, mares were administered saline or EPI (5 ug/kg, intravenously) immediately before infusion of glucose (100 mg/kg BW). Glucose caused peak rise ( $P < .05$ ) in plasma insulin in controls at 5 minutes that persisted through 30 minutes; insulin was suppressed ( $P < .05$ ) by EPI from 5 to 15 minutes, rising gradually through 30 minutes. Experiments 2 (IR2G) and 3 (GR2I) were carried out as separate triplicated 3 x 3 Latin square designs with the following treatments: saline (SAL), DET, and the combination of DET/BUT, all administered intravenously at .01 mg/kg BW. There was a 6-day washout period between phases. Blood samples from indwelling catheters were collected at -10 and 0 minutes before treatment, and continued every 5 minutes for 40 minutes. Infusion of glucose occurred 10 minutes after sedation. Glucose caused an immediate rise ( $P < .05$ ) in plasma insulin that persisted through 30 minutes in SAL mares; DET and DET/BUT severely suppressed ( $P < .0001$ ) the IR2G. To assess the GR2I in Experiment 3, blood glucose was measured after treatment with recombinant human insulin (50 mIU/kg BW, intravenously). Sedation did not affect resting blood glucose and had inconsistent effects on the GR2I. The administration of BUT alone in Experiment 4 had no effect on IR2G. In conclusion, adrenergic agonists severely

suppress the IR2G and cannot be used for sedation for this test. The use of DET did not alter the GR2I, and therefore may be useful for conducting this test in fractious horses.

## CHAPTER 1. INTRODUCTION

The most common form of insulin dysregulation in horses is compensated insulin resistance [1,2]. Whatever the cause, the long term resistance of peripheral tissues to insulin secreted by the pancreatic beta ( $\beta$ ) cells necessitates the hypersecretion of insulin in order to maintain normal blood glucose concentrations. Thus, both resting (nonfed, feed deprived, or between meals) insulin concentrations as well as postprandial insulin responses are greater in resistant horses compared to horses of normal insulin sensitivity [1-5]. Given the complexity of the two classical methods of assessing insensitivity in horses (the hyperinsulinemic-euglycemic clamp [6,7] and the minimal modeling of the modified glucose tolerance test [8,9]), Caltibilota et al [10] developed and tested a simple insulin challenge method based on intravenous injection of recombinant human insulin (50 mIU/kg of body weight to start) and glucose measurement with a hand held glucometer. In that report, and in several subsequent reports of follow-up research [4,11-13], it was demonstrated that this method (hereafter referred to as the GR2I) produced repeatable estimates of insulin sensitivity and could be used in lieu of the two more complex methods for assessing effects of various conditions (such as obesity), treatments (such as dexamethasone injection), and feeding regimens (e.g., fed versus feed deprived).

In reports published subsequent to Caltibilota et al [10], the insulin response to glucose infusion (hereafter referred to as IR2G) was also assessed [4,13]. Cartmill et al [5] first reported that hyperleptinemic horses had exaggerated IR2G. Those hyperleptinemic horses were subsequently found to be insulin resistant [14], and it was concluded that the long term hyperinsulinemia was driving the hyperleptinemia. The difference between the insulin responses of horses selected for insulin resistance and those selected for high insulin sensitivity are very revealing: the pre-glucose resting insulin concentrations in plasma are typically twice as high



compared to normal horses, and the immediate rise in insulin concentrations after glucose infusion (net change in concentrations) also double or triple, depending on the experiment [4,11,13].

One factor known to perturb the assessment of insulin sensitivity by the classical methods is excitement. Apparently epinephrine (EPI) from the adrenal glands in spooked or fractious horses acts on insulin sensitive tissues and interferes with the action of insulin. In fact, Earl et al [11] reported that administration of EPI at 5 ug/kg of body weight (BW) totally obliterated the glucose response (decrease) to injected insulin; in fact, blood glucose increased in the face of high insulin, apparently due to liver glycogenolysis. Thus, the question that arises is “how does one assess insulin sensitivity in a fractious horse?” Sedatives are typically used to perform various simple chores on fractious horses; however, their effects on GR2I or IR2G are not known.

Given these facts, the four experiments described herein were conducted to empirically determine 1) the effect of EPI pretreatment on the IR2G, as a follow-up to the data of Earl et al [11], 2) the effects of two common methods of sedation used in horses [15]; detomidine alone versus detomidine plus butorphanol) on the results of the GR2I and the IR2G relative to the nonsedated (control) responses, and 3) the effects of BUT alone on IR2G.

## CHAPTER 2. REVIEW OF LITERATURE

### 2.1 Insulin secretion

Insulin is produced within the body by the pancreas, which is a glandular organ that plays a vital role in the digestive system as an exocrine organ and an endocrine gland [16]. The exocrine functions of the pancreas include neutralizing gastric acid to prevent damage to duodenal mucosa, and breaking down proteins, fats, and starch in order for their constituents to be absorbed through the mucosa into blood. As an endocrine organ, the pancreas' role is primarily carbohydrate and lipid metabolism via the secretion of insulin and glucagon, which aid in maintaining normal blood glucose concentrations [16].

Within the pancreas, there is a collection of cells clustered into islands, known as the Islets of Langerhans [16]. The islets of Langerhans are essential for maintaining normal glycemia. The predominant cells (90% by number) within these islets consist of two types, the alpha ( $\alpha$ ) and beta ( $\beta$ ) cells. Beta cells release insulin, while  $\alpha$  cells release glucagon. Following a meal, the hyperglycemia induced postprandially causes islet  $\beta$ -cells to release insulin which stimulates insulin-sensitive cells in the body (primarily striated muscle, liver, and fat) to readily absorb glucose [17]. When hypoglycemia occurs between meals, the  $\alpha$ -cells secrete glucagon, the major blood glucose-elevating hormone [17]. Insulin secretion is stimulated when blood glucose is higher than necessary, causing cells to take up excess glucose from the blood and convert it to stored glycogen and triacylglycerols (fat). Glucagon secretion is stimulated when blood glucose is lower than normal, causing tissues, like the liver, to breakdown glycogen to produce glucose (glycogenolysis), produce new glucose (gluconeogenesis), and to oxidize fats to reduce the need for glucose [18].

The  $\beta$ -cells store insulin in secretory vesicles, and in order to release insulin outside the cell, calcium must be present, therefore calcium receptors exist on these secretory vesicles [19]. Another important component of  $\beta$ -cells is the presence of potassium channels. These potassium channels allow  $K^+$  to leave the  $\beta$ -cells through facilitated diffusion [20]. When the cell is unstimulated, there are more  $K^+$  in the extracellular fluid surrounding cells than inside the cells. This prevents the  $\beta$ -cells from depolarizing. When glucose concentrations increase inside the  $\beta$ -cell,  $K^+$  levels rise, eventually resulting in a depolarization of the cells [18]. When the  $\beta$ -cell membrane depolarizes, calcium channels present in the cell membrane open and allow  $Ca^{++}$  ions into the cytoplasm, resulting in vesicle fusion with the cytoplasmic membrane and exocytosis of stored insulin [16].

## **2.2 Insulin action**

Once insulin is secreted, it must bind to its specific receptors in target tissues in order to transmit its signal [16]. These receptors are known to be members of a larger family of plasma membrane receptors that possess inherent protein kinase activity; these are fundamentally different than G-protein coupled receptors (later discussed) activity when transmitting its extracellular signal [21]. Tyrosine kinases have unique domains that phosphorylate tyrosine residues in specific target proteins inside the cell [21]. Insulin does not enter the cell, but instead initiates its signal at the plasma membrane level via specific receptors that transmit the signal to insulin-sensitive second messenger systems in the cytosol. The insulin receptor contains two  $\alpha$  subunits and two  $\beta$  subunits. The alpha subunits contain the insulin-binding domain, while the beta subunits contain the tyrosine kinase activity that is responsible for transferring a phosphoryl group from ATP to the hydroxyl group of the tyrosine residues in the target protein [21]. The

action of insulin inside the cell is well described in detail by Krahl [22] and graphically depicted in detail by Rhodes [22].

### **2.3 Glucose metabolism**

Every cell in the body needs energy to survive and perform different functions. Energy is needed to stimulate other cells to send signals and messages throughout the body. For example, muscle cells need energy to contract and perform basic functions and the primary source of energy is glucose [18]. Glucose is an abundant monosaccharide contained in the disaccharides and starches of various plants that are consumed by animals. In animals, in order to produce glucose, glycogen must be broken down through the process of glycogenolysis [18]. Glucose is also synthesized in the liver and kidneys from non-carbohydrate intermediates (i.e. glycerol, amino acids, lactate, and propionate) through gluconeogenesis [18].

Once absorbed or made, glucose is delivered through portal venous blood to many target tissues that require energy. When a cell needs energy, glucose is taken up into the cell, which must be assisted by the hormone, insulin. Insulin receptors exist on the cell membrane of many target tissues, such as skeletal muscle, adipose tissue, and the liver [22]. Insulin must bind to the insulin receptors to allow glucose to be taken up by the cell. This process however can be disrupted if the body cannot produce insulin (from the pancreas), therefore there won't be insulin to bind to the insulin receptors, and therefore, glucose will not be allowed to enter into the cell. This is known as Type I diabetes mellitus in humans [16]. Another disruption that commonly occurs is a lack of insulin action in target cells. In this case, there is the presence of insulin, yet glucose is not taken into the cell in a normal fashion. This is known as type II diabetes mellitus in humans [16]. In addition to a paucity of glucose available for tissue function in type II diabetics, there are other dysregulations that can lead to illness and possibly death if untreated,

such as kidney disease, hyperglycemia, dyslipidemia, hyperlipidemia, and eventual arteriosclerosis [18].

## **2.4 Insulin sensitivity and resistance in horses**

Insulin sensitivity is an interesting phenomenon in horses compared to humans because of their ability to withstand higher levels of insulin secretion from pancreatic  $\beta$  cells for longer periods of time. Insulin resistance in horses is a metabolic condition, like type II diabetes in humans, in which the body's peripheral tissues show a reduction in insulin sensitivity or inability to respond, despite having normal or increased insulin concentrations in the blood [1].

A main difference between horses and humans relative to insulin resistance is that horses can tolerate hyperinsulinemia without experiencing pancreatic exhaustion, which could ultimately lead to type I diabetes mellitus in humans [18]. Horses with insulin resistance compensate for the reduced action of insulin at the target tissue level by hypersecreting insulin virtually 24 hours a day [1], hence the term coined by Kronfeld and others [1,2] “compensated insulin resistance” for the type II diabetic situation in horses. Insulin resistant horses display resting (basal, feed-restricted, or between meal) blood insulin concentrations about twice those observed in normal horses [5,25]. Moreover, the insulin response to a meal or to glucose infusion is also greatly exaggerated [4,5,25].

The constantly high blood insulin concentrations in resistant horses can cause various dysfunctions in other systems in the body. Insulin resistance is involved in, or at least associated with, the pathogenesis of equine conditions such as pituitary pars intermedia dysfunction (PPID; [25]), equine metabolic syndrome (EMS; [26]), hyperlipidemia, laminitis, endotoxemia, and osteochondrosis (reviewed by Firshman and Valberg [27]). Dysregulation of insulin such as hyperinsulinemia and a decreased sensitivity to insulin are two major causes of EMS, which is a

common endocrinopathy in horses characterized by the persistent high levels of blood glucose and subsequent pancreatic exhaustion, upregulation of inflammatory markers, arterial hypertension, and reduced glycemic control [30]. It may also cause infertility, hyperleptinemia, and hypertriglyceridemia (reviewed by Johnson et al. [30]). Insulin resistance is believed to have a genetic component, at least in pony mares [28], but has been shown to be inducible in horses by high glycemic diets [1,2,29], dexamethasone administration [31-33], and free fatty acid infusion [34].

## **2.5 Methods for testing insulin sensitivity**

The two classical methods of assessing insensitivity in horses are the hyperinsulinemic-euglycemic clamp [6,7] and the minimal modeling of the modified glucose tolerance test [8,9]. The clamp technique involves infusion of insulin at 1 or more fixed doses (only one per trial) and then sufficient glucose to maintain euglycemia [6,7]. The amount of glucose required to counteract the given dose of infused insulin indicates the relative glucose sensitivity of the subject: sensitive horses uptake more glucose than insensitive horses, hence a greater glucose infusion rate is required to establish and maintain euglycemia. The minimal model technique is basically a modified glucose tolerance test that involves the rapid infusion of a high dose of glucose at time 0, and then very frequent blood sampling to assess the rise and subsequent fall of blood glucose concentrations [8,9]. In the modified version, a large dose of insulin is administered intravenously at 20 minutes after glucose infusion. For proper application of either of these techniques, insulin concentrations must be measured in the laboratory to calculate the estimates of insulin sensitivity. The complexity of these techniques and the need for insulin measurement preclude them being useful for on-farm use.

In 2010, Caltibilota and colleagues [10] at LSU, reported on a simple, on-farm friendly alternative to these complicated approaches. Their test involves measuring the glucose response in horses to a standard intravenous injection of recombinant human insulin (50 mIU/kg BW). Blood glucose concentrations are measured before injection (-10 and 0 minutes) by means of a simple hand-held glucometer and then subsequent to insulin injection at 40 and 60 minutes. The post-treatment blood glucose concentrations are compared to the pre-injection average, and the greatest decrease, relative to pre-injection, is calculated as a percentage of the pre-injection average. Horses with normal or high insulin sensitivity will typically have a maximum post-injection decrease of 50% or greater [10-12]. Insulin resistant horses will display post-injection decreases of 0 to 30%. Decreases falling between 30 and 50% are equivocal and would require further study. For the most reliable assessment of insulin sensitivity, Caltibilota et al [10] suggested that three insulin doses should be tested (the standard of 50 mU/kg BW, plus two higher or lower depending on the first response); however, follow-up research showed that the response to the standard dose was very predictive of the results for the three-dose regimen [11].

Although the decrease in blood glucose after a standard injection of insulin (referred to as the glucose response to insulin, or GR2I) was very revealing as to a horse's insulin sensitivity status, subsequent research drew upon the fact that horses with compensated insulin resistance also consistently display an exaggerated insulin response after a meal or after the infusion of glucose [1,2,5]. Thus, subsequent research reports on factors affecting insulin sensitivity utilized both GR2I results as well as results from glucose infusion tests (the insulin responses to glucose, or IR2G). Combined, the results from these two tests give very reliable estimates of the assessment of insulin sensitivity [13,35].

A currently in vogue approach to assessing insulin sensitivity is the oral glucose tolerance test [36,37]. The basis of the test is the administration of a glucose solution, or alternatively, Karo syrup, into the horse's mouth, with subsequent blood samples typically taken 90 minutes later. Due to the need to have insulin concentrations measured in the blood samples, this test is not truly on-farm friendly. Other problems with repeatability of the test have been raised by researchers [37,38].

## **2.6 Adrenergic receptors**

There are two main types of adrenergic receptors:  $\alpha$  and  $\beta$  [39]. These main types are further differentiated pharmacologically into  $\alpha 1$ ,  $\alpha 2$ , and  $\beta 1$ ,  $\beta 2$ , and  $\beta 3$  [39] subtypes. Both the  $\alpha 1$  and  $\alpha 2$  types can in fact be even further differentiated in three sub-subtypes each ( $\alpha 1$  A, B, and D; and  $\alpha 2$  A, B, and C). Differentiations of subtypes and sub-subtypes are based on both the agonists and antagonists that bind and cause action through the receptors, and also on the relative activity of any given agonist (often epinephrine or norepinephrine).

Beta-adrenergic receptors are primarily involved in the effects of stimulation of the sympathetic nervous system [18]. Actions occurring through activation of  $\beta 1$  receptors increase the force or energy of muscular contractions (inotropic effects), increase heart rate, and increased blood pressure via vascular constriction [18]. Activation of  $\beta 2$  receptors induces smooth muscle relaxation in the lungs, gastrointestinal tract, uterus, and various blood vessels [18], as well as vasodilation within cardiac muscle. Beta-3-adrenergic receptors are primarily found on adipose tissue and are involved with fat metabolism and release [18].

Alpha-2-adrenergic receptors ("adrenoceptors") have been localized both presynaptically and postsynaptically [40]. Alpha-2-adrenoceptors have been identified within hypothalamic areas such as the periventricular nucleus and supraoptic nucleus, in which these receptors influence



food intake [41]. Within adipocytes,  $\alpha_2$ -adrenoceptors modulate antilipolytic effects [42]. The activation of both  $\alpha$ - and  $\beta$ -adrenoceptors mediates the sympathoadrenal system and have important functions in regulating blood glucose and insulin secretion [43]. Fagerholm et al. [44] studied  $\alpha_2$  regulation of blood glucose and discovered that receptors can be found on sympathetic nerves and adrenomedullary chromaffin cells where their activation limits sympathoadrenal output. Within the  $\beta$  cells of the islets, activation of  $\alpha_2$ -adrenoceptors leads to hypoinsulinemia and hyperglycemia [45-47].

Alpha-2-adrenoceptors have been reported to be integrally involved in the control of insulin and glucose. An increase in expression of  $\alpha_2A$  receptor gene activity has been associated with a decrease in the insulin response to an injection of glucose, increase in fasted blood glucose, and an increase in the risk of type II diabetes mellitus [44]. This inhibitory effect on insulin from adrenoceptor agonists is not related to vasoconstriction [48], but rather the prevention of cAMP generation from a decrease in adenylate cyclase activation [42,49].

## **2.7 G-protein coupled receptors**

All types and subtypes of adrenoceptors are members of a larger class of membrane receptors called G-protein coupled receptors (GPCR; [39]). This is a large and diverse family of cell surface receptors that respond to many external signals [21], are only found in eukaryotes, and represent the largest class of membrane receptors [50]. An essential and unique characteristic of GPCR is their 7-transmembrane  $\alpha$  helices [21]. The G-protein coupled receptors combine with heterotrimeric G-proteins that bind both guanosine triphosphate (GTP) and guanosine diphosphate (GDP). Each G-protein has three subunits;  $\alpha$ ,  $\beta$ , and  $\gamma$ , that are held to the cell membrane via lipid anchors [51]. Detailed graphics showing how GPCR are involved with

various responses inside the cell are presented in Dorsam and Gutkind [51] as well as in Neumann et al [52].

## **2.8 Detomidine, an $\alpha_2$ -agonist**

Detomidine is a sedative analgesic used to quiet animals for veterinary procedures [53] and is widely used in the horse for standing chemical restraint that produces effects centrally [15]. Common effects observed using detomidine in the horse are lowering of the head, lower lip drooping, slight eye closure, occasional muscle twitches, slight head jerks, leaning on barn walls or doors for stability and support, diuresis, and ataxia. The degree of ataxia from detomidine has been discovered to be dose-dependent: the higher the dose the more severe the effects for a short time [53]. Detomidine has also been reported to cause hypothermia, mydriasis, and hypotension [40].

Detomidine analgesia is believed to result from the interaction of the agonist with  $\alpha_2$ -adrenoceptors located presynaptically on afferents and  $\alpha_2$ -adrenoceptors located postsynaptically on projection neurons [54]. On these projection neurons, the agonists increase K<sup>+</sup> flow through inhibitory G-coupled potassium channels, thereby causing the cell to hyperpolarize [54]. The sedative effect of agonists is also dose-dependent at supraspinal sites, in which the agonists are believed to act in a similar manner as the analgesic response, mediated through postsynaptic  $\alpha_2$ -adrenoceptors and inhibitory G proteins [54].

## **2.9 Butorphanol, an opioid agonist**

Opioids are commonly used in the veterinary world to treat acute and chronic pain. In order to improve and hasten the patient's recovery, it is important to find a balance between sedative drugs that bind to different targets in different locations, or by different pain pathway mechanisms. This helps to improve the analgesic effect, reduces adverse side effects, and

reduces costs [55]. This balance is important when opioids are often used as adjuncts with  $\alpha 2$ -agonists during procedures. This multimodal approach enables veterinarians to use smaller doses, therefore reducing the side effects. Combining opioids with  $\alpha 2$ -agonists can reduce and abolish a horse's response to specific stimuli, permitting veterinarians and clinicians to perform certain diagnostic and therapeutic procedures [55].

Butorphanol (commercially available as the tartrate salt, Torbugesic, Zoetis US, Parsippany, NJ) is a short acting (3 to 4 hours when administered intravenously) synthetic narcotic analgesic, which acts centrally and must be administered by or on the order of a licensed veterinarian [56]. Butorphanol has been approved for use in the horse in the United States, and can be administered IV, IM, and SC. It is 4-30 times more potent than morphine and pentazocine in animals. Some common side effects include respiratory depression and gastrointestinal stasis [55]. When comparing the analgesic contribution between opioids and  $\alpha 2$ -agonists,  $\alpha 2$ -agonists alone have been found to provide more analgesia and significantly more sedation; opioids contribute little to the effects seen by the combination of opioid and  $\alpha 2$ -agonist [57,58]. Jochle et al. [57] studied horses presenting with abdominal pain and compared the effects of detomidine or butorphanol (among other sedative agents) and found that detomidine (at 20 or 40 ug/kg BW) caused a bigger depression of pulse rate and respiratory rate than butorphanol (.1 mg/kg BW). There were also significant differences in clinical signs between butorphanol and either dose of detomidine, such as sweating, kicking, pawing, head and body movement, attitude, pulse rate, and respiratory rate [57]. Another study [59] found that when comparing detomidine in combination with other opioids, detomidine and butorphanol produced the most reliable response to sedation (compared to other opioids: morphine, methadone, pethidine).

## 2.10 Epinephrine

Epinephrine (EPI), sometimes referred to as adrenaline, is a naturally occurring catecholamine and neurotransmitter of the sympathetic nervous system found in the body [18]. Epinephrine has both  $\alpha$ - and  $\beta$ -adrenergic activities, although it has a higher affinity for the  $\beta$ -adrenergic receptors than the  $\alpha$ -adrenoceptors [21]. Within the sympathetic nervous system, EPI is the neurotransmitter found in postsynaptic, sympathetic efferents, and is also produced and secreted directly into the blood from the adrenal medulla [18]. When faced with a fight-or-flight situation, sympathetic mass discharge results in activation of both systems resulting in the characteristic increase in heart rate, constriction of peripheral blood vessels, increased respiratory rate, and often subsequent perspiration [18]. Other actions of EPI in the body include increased blood glucose concentration due to muscle and liver glycogenolysis, increased energy-yielding metabolism in muscle, liver, and adipose tissue, and muscle quiver [21].

Epinephrine administration has been used to assess various systems within animal research, including glucose metabolism [60-63] and lipid metabolism [64-66]. Exogenous EPI increases blood glucose concentrations via increased liver and muscle glycogenolysis [18] and also stimulates blood nonesterified fatty acid concentration via increased activity of hormone sensitive lipase on adipose tissue [18]. Altered release of glucose or fatty acids in response to EPI may reveal metabolic alterations in the body that would not be noticeable otherwise [60-66].

Clinicians applying the hyperinsulinemic-euglycemic clamp and the minimal model technique have known for years that subjects must remain calm, and that excitement during testing will invalidate the test results. The endogenous release of epinephrine following stress results in impaired glucose tolerance due to epinephrine suppressing insulin secretion [67]. Earl et al [11] showed that EPI administration before a GR2I test totally obliterated the normal

insulin-induced decrease in blood glucose concentrations, and in fact, concentrations actually increased due to glucose mobilization. Epinephrine has also been shown to inhibit the glucose-stimulated insulin release in other species like the rat [68], however this has not yet been reported for the horse.

### **2.11 Rationale for present experiments**

Endocrine diseases like PPID, EMS, and insulin dysregulation are difficult diseases to recognize and diagnose. Researchers have shown that two important tests to evaluate these disorders relating to insulin and glucose metabolism are the IR2G and GR2I. However, in order to evaluate these responses, horses need to be calm; therefore, fractious horses can be a problem when performing these tests. Standing chemical restraint in horses is a common practice used to avoid complications associated with excited or unruly horses. The drug combination used herein (an  $\alpha$ 2-adrenergic agonist in combination with an opioid) was suggested as a commonly used protocol for providing sedation and analgesia in horses.

Given these facts, the four experiments described herein were conducted to empirically determine 1) the effect of EPI pretreatment on the IR2G, as a follow-up to the data of Earl et al [11], 2) the effects of two common methods of sedation used in horses ([15]; detomidine alone versus detomidine plus butorphanol) on the results of the GR2I and the IR2G relative to the unsedated (control) responses, and 3) the effects of BUT alone on IR2G.

## **CHAPTER 3. MATERIALS AND METHODS**

### **3.1 Experiment 1. Epinephrine effects on IR2G**

The LSU AgCenter Institutional Animal Care and Use Committee approved the procedures used in this and the subsequent two experiments. Ten light horse mares (primarily Quarter horse and thoroughbreds) housed at the LSU AgCenter Central Station horse unit previously assessed as insulin sensitive [10] were used. They ranged in age from 3 to 12 years, weighed between 385 and 519 kg, and had body condition scores (BCS; Henneke et al [69]) between 4.5 and 7. Mares were maintained outdoors on native grass pasture for the duration of the experiment. This experiment was performed in June of 2018.

The experiment was performed as a completely random design with a single switchback. That is, on the first day of treatment, five mares were treated with EPI prior to infusion of glucose, and five mares received saline prior to glucose infusion. After a week of rest (no treatments), the experiment was repeated with the treatment groups reversed.

In the evening before each treatment day, mares were brought in from pasture and housed in stalls in a barn overnight with free access to water but no feed. The next morning at approximately 0700 each mare was fitted with a 14-gauge indwelling jugular, which was affixed in place with cyanoacrylate glue. A .05% sodium citrate solution was used to keep the catheter patent. Once all catheters were in place, the mares were left unperturbed for 1 hour.

After the hour of rest, blood samples were drawn at -10 and 0 minutes relative to treatment. Mares receiving EPI were injected through the catheter with 5  $\mu\text{g/kg}$  BW of EPI (as a 1 mg/mL solution; Sigma Chem. Co., St. Louis, MO) in saline. Five control mares received saline at .005 mL/kg BW. Within 30 seconds after the first injection, each mare was infused with glucose (100 mg/kg BW as a 50% solution; Durvet Inc., Blue Springs, MO) through the

jugular catheter. Infusion took about 1 minute to complete. Blood samples were collected at 5, 10, 15, 20, 25, and 30 minutes relative to onset of glucose infusion. Blood samples were placed into 6-mL evacuated plastic tube containing K<sub>3</sub>EDTA (Vacuette, Greiner Bio-One, Monroe, NC, USA). All samples were placed immediately in an ice bath until centrifugation at 1200 x g for 15 minutes at 5°C. Plasma was harvested and stored at -20°C.

Once all blood samples had been collected on the first day, the mares were returned to pasture until the following treatment day one week later. Procedures from the first treatment day were followed on the second treatment day, except the treatments were reversed.

All plasma samples were analyzed for insulin by immunoradiometric assay (Immuno-Biological Laboratories, Inc., Minneapolis, MN). Lower limit of detection of the insulin assay was < 1 mIU/L. All samples were estimated in one assay; intra-assay coefficient of variation was 8.8%.

Insulin data were analyzed in an analysis of variance (ANOVA) with the General Linear Model procedure of SAS software (SAS Inst., Cary NC) in a replicated Latin square (5 replicates of a 2 x 2 Latin square; Steel et al [70]); square was ignored in the analysis because mares were not paired per se and all were treated at the same time. The repetitive nature of the sampling was accounted for by use of a split-plot design [70]. Post analysis mean separation was performed across time periods with the SLICE command of SAS.

### **3.2 Experiment 2. Detomidine and butorphanol effects on the IR2G**

This experiment was conducted from October 22 through November 5, 2018. Mares of light horse types were used. They ranged in age from 5 to 22 years, weighed between 427 and 528 kg, and had BCS between 5 and 8. Mares were maintained outdoors on native grass pasture

for the duration of the experiment. Alicia Bermuda grass hay was supplemented when pasture grasses diminished in quantity and quality.

The experiment was designed as a triplicated 3 x 3 Latin square design with 9 mares. On the second day of treatment, one mare was discovered to be totally intractable; it was obvious that she would not be able to continue in the experiment and was dropped. The remaining mares were allotted to three treatment groups with three mares per group (except for one), and each mare received each treatment over the three days of treatment. The treatments consisted of saline (SAL, .01 mL/kg BW), detomidine alone (DET, .01 mg/kg BW, Dormosedan, Zoetis, Parsippany, NJ), or a combination of detomidine and butorphanol (DET/BUT, .01 mg/kg detomidine plus .01 mg/kg BW butorphanol; Torbugesic, Zoetis, Parsippany, NJ). All treatments were administered intravenously. A 6-day “washout” period between the three treatment days was used to prevent any effects of one treatment from altering the subsequent treatment response(s).

The night before each treatment day, mares were brought in from pasture and were feed-deprived overnight in stalls; water was available at all times. In the morning (0530), a 14-gauge catheter was aseptically placed into the left jugular vein. One hour of rest (inactivity) was allowed for acclimation to avoid any stress-associated artifacts. At the start of the experiment, baseline blood samples (5-mL) were collected via the catheter at -10 and 0 minutes relative to treatment (SAL, DET, or DET/BUT). Once the treatments had been administered, blood was again collected at 5 and 10 minutes post-injection to assess immediate treatment effects.

Following the 10-minute sample post-treatment, a standard dose of glucose (100 mg/kg BW, 50% aqueous solution; Durvet) was administered through the jugular catheter over approximately a 1-minute period. Subsequent blood samples were collected at 15, 20, 25, 30, 35,



40, and 45 minutes relative to the original treatments (5, 10, 15, 20, 25, 30, and 35 minutes relative to onset of glucose infusion). Blood samples were processed and stored as described for Experiment 1.

After recovery from treatments, mares were returned to pasture until the next treatment day (one week later). Once all plasma samples from the three treatment days had been collected, the samples were analyzed for insulin as described in Experiment 1. Insulin data were analyzed by ANOVA with SAS software for a triplicated 3 x 3 Latin square [70] with repeated measures. Factors in the analysis included square, horse within square, day, treatment, and time (minutes). The interaction of treatment and time was also assessed, and any differences among treatment groups for specific times were tested via the SLICE option in SAS.

### **3.3 Experiment 3. Detomidine and butorphanol effects on the GR2I**

This experiment was basically a repetition of Experiment 2 except that the effects of treatment were assessed for the GR2I. It was performed between November 12 and 26, 2018. The general procedures regarding the schedule of events on treatment days followed in Experiment 2 were repeated. The same mares were used, although a different day-treatment grid was used for the assignment of treatment sequences.

On treatment days, mares were loosely tethered at approximately 0700 in stalls in a barn and two samples of jugular blood were collected 10 minutes apart using a tuberculin syringe fitted with a 21-gauge, 1-inch long needle (-10 and 0 minutes samples). Blood glucose concentrations were assessed in each sample (and all subsequent samples) immediately after withdrawal using a hand held glucometer (OneTouch UltraMini, LifeScan, Inc., Milpitas, CA). If blood glucose concentrations agreed to within 10% for the two samples, the mare was administered its respective treatment. If the samples varied more than 10%, subsequent samples

were drawn to establish a constant baseline before proceeding. Treatments were then administered intravenously as described for Experiment 2.

A 5- and 10-minute post-treatment blood sample was collected before insulin was injected to assess any effects on basal blood glucose concentrations. Subsequently, recombinant human insulin (Sigma) in sterile saline was administered intravenously into the left jugular vein at 50 mIU/kg BW. Blood samples were then collected at 40 and 60 minutes after insulin injection and were assessed for blood glucose concentration. After recovery from treatments, mares were returned to pasture until the next treatment day (one week later).

Once all blood glucose concentrations had been obtained from the three treatment days, the percent decreases in blood glucose concentrations from the pre-insulin concentration (the 10 minute post-treatment sample) were calculated for the 40 and 60 minute samples [10]; the largest decrease of the two estimates was used as the maximum percent decrease for that mare on that day. Endpoints calculated from the raw blood glucose concentrations included mean pre-treatment average (mean of the -10 and 0 minute samples), mean post-treatment concentration (the sample collected 10 minutes after treatments were administered), net change in blood glucose due to treatment (the post-treatment sample minus the pre-treatment average), and the maximum decrease in blood glucose after insulin injection.

Data from Experiment 3 were analyzed by ANOVA with SAS software for a triplicated 3 x 3 Latin square [70]. Differences among treatment groups were tested via the PDIFF option in SAS with Dunnett adjustment (each treatment versus the control).

### **3.4 Experiment 4. Butorphanol effect on IR2G**

Given the effects of DET and DET/BUT on IR2G in Experiment 2, the effect of BUT alone was assessed in Experiment 4. Eight light horse mares were used in a single switchback

design conducted during February, 2019. Three of the 8 mares had been used in Experiments 2 and 3. All mares were housed maintained as described for those previous experiments.

On the first treatment day, four mares selected at random were administered SAL (.01 mL/kg BW) and four were administered BUT (.01 mg/kg BW) intravenously. On the second treatment day, the treatment assignments were reversed. Pre- and post-treatment blood sampling times were identical to those in Experiment 1, as was the protocol for infusion of glucose, blood sample processing, insulin measurement, and statistical analysis.

## CHAPTER 4. RESULTS

### 4.1 Experiment 1. Epinephrine effect on IR2G

Mean responses of plasma insulin concentrations to glucose infusion following saline versus EPI pretreatment are presented in Figure 4.1. Glucose infusion resulted in an immediate rise ( $P < .05$ ) in plasma insulin concentrations when mares received SAL; this increase persisted through 30 minutes after infusion. A treatment by time interaction in the ANOVA ( $P < .05$ ) was observed for insulin concentrations, which were suppressed ( $P < .05$ ) by EPI at 5 minutes, but then gradually increased through the 30-minute sampling period to equal those in mares administered SAL.

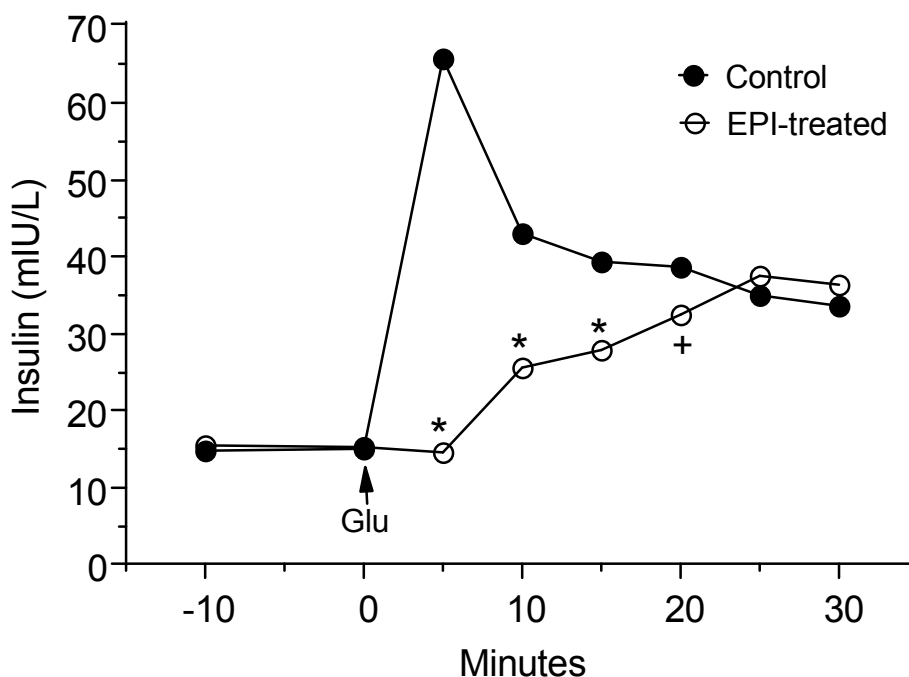


Figure 4.1. Mean plasma insulin concentrations in mares when injected with SAL (Control) or epinephrine (EPI; 5  $\mu\text{g/kg}$  BW) just prior to i.v. infusion of glucose (Glu; 100 mg/kg BW) at time 0. Differences between groups for specified time periods are indicated by asterisks (\*;  $P < 0.05$ ) or a plus sign (+;  $P < .10$ ). The pooled SEM was 7.9 mIU/L.

## 4.2 Experiment 2. Detomidine and butorphanol effects on the IR2G

In order to better evaluate the effects of treatment on plasma insulin concentrations before glucose infusion as opposed to after glucose infusion, two separate analyses were performed. Analysis of the data from -10 minutes through 10 minutes after SAL, DET, or DET/BUT administration revealed that treatment affected resting insulin concentrations (Figure 4.2; all means are presented). Mean insulin concentration was reduced ( $P < .05$ ) from approximately 8.5 mIU/L at time 0 in all mares to 5.1 mIU/L in the DET and DET/BUT treated mares (versus 8.7 mIU/L in SAL mares) 10 minutes later. Once glucose was administered,

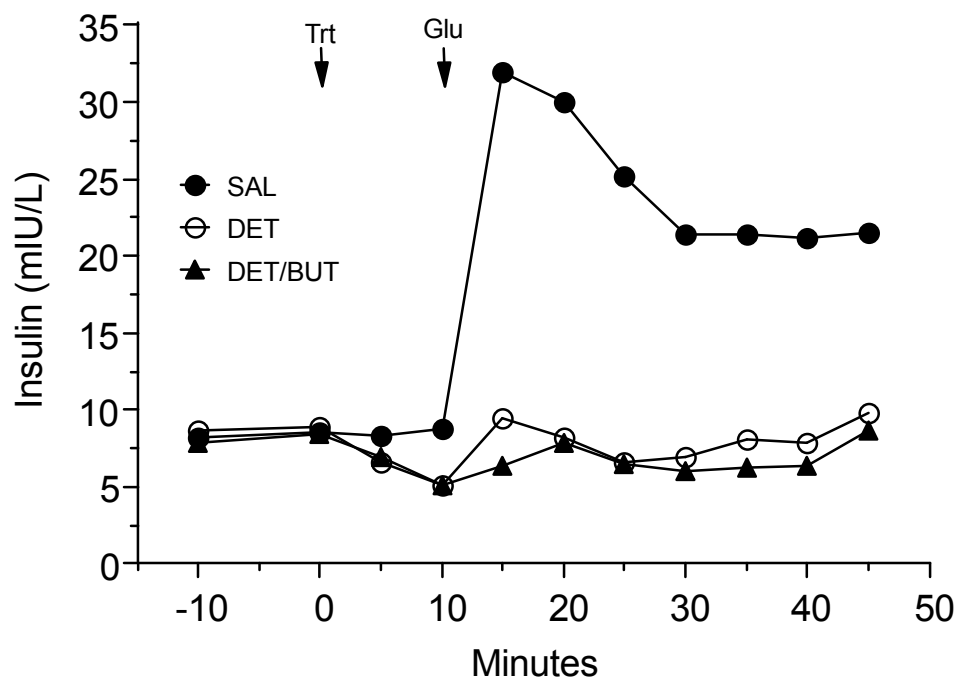


Figure 4.2 Mean plasma insulin concentrations in mares treated (Trt) with SAL, DET, or DET/BUT at time 0 prior to i.v. infusion of glucose (Glu; 100 mg/kg BW) 10 minutes later. Treatment (DET) and (DET/BUT) suppressed ( $P < .05$ ) insulin concentrations before (at 10 minutes) and at all times after glucose administration compared to control (SAL). Pooled SEM was 1.2 mIU/L for the first analysis (-10 through 10 minutes) and 5.2 mIU/L for the second analysis (15 through 45 minutes).

insulin concentrations increased ( $P < .01$ ) in mares administered SAL, but remained suppressed ( $P < .01$ ) in mares administered DET or DET/BUT (Fig. 2) through the last blood sample collected that day.

### 4.3 Experiment 3. Detomidine and butorphanol effects on the GR2I

Summaries of data collected from the GR2I challenges are presented in Table 4.1. Before any treatments were initiated (sampling times -10 and 0 minutes), mean blood glucose concentrations were equivalent across the three treatment groups. At 10 minutes after the SAL, DET, and DET/BUT treatments were administered, mean blood glucose concentrations were again equivalent across the three treatment groups, which was confirmed by the lack of

**Table 4.1. Glucose characteristics before and after treatment and after injection of recombinant human insulin in mares in Experiment 3.**

Treatment <sup>a</sup>	Mean pre-treatment blood glucose (mg/dL) <sup>b</sup>	Mean 10-min blood glucose (mg/dL)	$\Delta$ Glucose after treatment (mg/dL) <sup>c</sup>	Maximum decrease in blood glucose (%) <sup>d</sup>
SAL	101.9	101.9	0	34.8
DET	104.3	101.4	-2.8	25.4
DET/BUT	100.9	99.7	-1.2	35.0
SEM <sup>e</sup>	1.7	2.7	2.7	4.8
P-value <sup>f</sup>	0.20	0.76	0.51	0.11

<sup>a</sup>Treatments were saline (SAL; 0.01 mL/kg BW), detomidine (DET; 0.01 mg/kg BW), and DET plus butorphanol (DET/BUT; 0.01 mg/kg BW each).

<sup>b</sup>Mean of the -10 and 0 minute time samples.

<sup>c</sup>Mean net difference between the 10 minute sample and the mean pre-treatment average.

<sup>d</sup>Largest decrease in blood glucose from time 10 minute sample at either 40 or 60 minutes after insulin injection.

<sup>e</sup>Standard error of the means.

<sup>f</sup>P-value for the treatment effect in the ANOVA.

differences in the net change in concentrations from pretreatment to 10 minutes after treatment ( $\Delta$  glucose, Table 4.1). Finally, the maximum percent decrease in blood glucose concentrations after insulin injection was not affected overall by treatment ( $P = .11$ ), although the PDIFF procedure indicated that the response after DET sedation tended ( $P = .08$ ) to be lower than after SAL treatment.

#### 4.4 Experiment 4. Butorphanol effect on IR2G

Mean plasma insulin concentrations before and after BUT treatment and following glucose infusion are presented in Fig. 4.3. Administration of BUT had no effect ( $P > .1$ ) on resting insulin concentrations (from 0 to +10 minutes); nor did BUT administration alter the glucose-induced rise ( $P < .001$ ) in insulin concentrations over time.

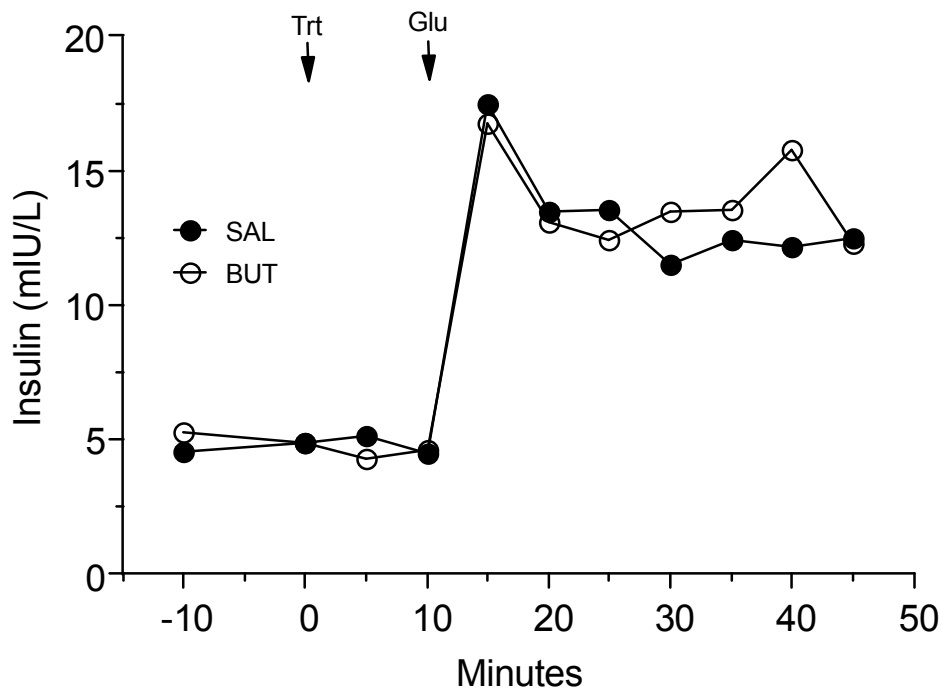


Figure 4.3. Mean plasma insulin concentrations in mares treated (Trt) with SAL or BUT at time 0 prior to intravenous infusion of glucose (Glu; 100 mg/kg BW) 10 minutes later. Treatment had no effect on insulin concentrations before (at 10 minutes) or at any time after glucose administration compared to control (SAL). Pooled SEM was 1.7 mIU/L.

## CHAPTER 5. DISCUSSION

### 5.1 Experiment 1. Epinephrine effect on IR2G

Epinephrine is a potent adrenergic ligand that binds to a wide spectrum of both  $\alpha$ - and  $\beta$ -adrenergic receptors in the body. As mentioned previously, Earl et al [11] reported that EPI treatment before the injection of insulin in horses resulted in a total obliteration of the expected decrease in blood glucose concentrations relative to saline-treated horses. Moreover, blood glucose concentrations actually increased in EPI-treated animals. The interference of EPI with insulin action on insulin sensitive tissues has been reported for various species [71-73], and it is thought that EPI inhibits insulin-stimulated glucose uptake in skeletal muscle by first stimulating intramuscular glycogenolysis, resulting in high glucose-6-phosphate concentrations that then inhibit glucose phosphorylation [72,73]. It has also been reported that EPI raises blood glucose concentrations via stimulation of glycogenolysis in the liver [74] as well as skeletal muscle [73].

The inhibitory effect of EPI treatment on IR2G presented herein has not been reported previously for horses, although similar effects have been reported for other species [75-77]. The use of specific  $\alpha$ - versus  $\beta$ -adrenergic agonists has shown that the inhibition of insulin secretion is primarily mediated through  $\alpha$ -adrenergic receptors, and in fact, pure  $\beta$ -adrenergic agonists often cause stimulation of insulin secretion, rather than inhibition [77]. Thus, it is assumed that the inhibitory effect of EPI on both resting (unstimulated) and glucose-stimulated insulin secretion in the mares in the present experiment was due to the ability of EPI to bind to  $\alpha$ -adrenergic receptors, and that binding dominated over any possible effect through  $\beta$ -adrenergic receptors that might have occurred at the same time.

The present experiment and that of Earl et al [11] both tested an EPI treatment dose of 5  $\mu\text{g/kg}$  BW on IR2G and GR2I test results. This dose of EPI administered intravenously causes



most horses to sweat within a few minutes and produces minor muscle tremor; its effects are usually short-lived, with recovery occurring within 10 to 15 minutes after injection. How the effects of this dose of EPI compares to those after the acute endogenous release of EPI in response to any given level of stress in horses is unknown. Estimations of the change (rise) in EPI concentrations per se from reports on horses exposed to treadmill exercise (at high Vmax or at exhaustion) vary from 1.35 [78] to 6 [79] to 68.2 [80] nmol/L; the equivalent net (greatest) change after administration of EPI at 5 µg/kg BW intravenously to a 500 kg horse would be approximately 450 nmol/L. Thus, further research is needed to determine the relative minimal dose of exogenous EPI that would alter the various physiologic systems affected (e.g., heart rate, liver glycogenolysis, perturbation of insulin secretion, etc.).

## **5.2 Experiments 2, 3, and 4. Detomidine and Butorphanol effects on IR2G and GR2I**

Detomidine at the dosage routinely used to sedate horses was a powerful inhibitor of insulin secretion, both before stimulation with glucose, as well as after stimulation. Addition of BUT did not further alter that response, and BUT alone (Experiment 3) had no effect on insulin concentrations. Detomidine is known to be a potent  $\alpha$ -adrenergic agonist with apparently little or no  $\beta$ -adrenergic activity [53]. The lack of  $\beta$ -adrenergic activity was in fact confirmed by the results of Experiment 3: the lack of effect of DET on GR2I is in stark contrast to the EPI effect on GR2I reported by Earl et al [11]. Both of these results are consistent with what has been reported for other species. That is,  $\alpha$ -adrenergic agonists consistently suppress insulin secretion from the pancreas and from isolated pancreatic islets [53,81] whereas  $\beta$ -adrenergic agents often stimulate insulin secretion in the absence of  $\alpha$ -adrenergic activity [77,82]. In contrast,  $\beta$ -adrenergic agonists block the action of insulin in insulin-sensitive tissues primarily by blocking the phosphorylation of glucose to glucose-6-phosphate [71], thus eliminating the needed

concentration gradient for glucose transport between the extracellular fluid and intracellular cytoplasm. This suppressive effect of  $\beta$ -adrenergic agonists on glucose uptake is not produced by  $\alpha$ -adrenergic agonists [82,83; results from Experiment 3].

Given the suppressive effect of DET and DET/BUT on IR2G, neither approach to sedation would be useful for assessing insulin sensitivity in horses. However, the lack of effect of DET on the GR2I indicates that this option should be useful for testing fractious horses. The tendency of the lesser response in DET/BUT treated mares may indicate an interaction between the two drugs on GR2I, which would need to be confirmed or rebutted in future experiments.

In conclusion, of the two commonly used assessments of insulin sensitivity in horses, the GR2I and the IR2G, the IR2G is profoundly affected by both EPI and DET, presumably through the  $\alpha$ -adrenergic receptor stimulation. In contrast, the GR2I, previously shown to be severely altered by EPI administration [11], was not affected by DET/BUT sedation. This indicates that this drug combination should be useful for sedating fractious horses for the assessment of insulin sensitivity by this method [10].

## CHAPTER 6. SUMMARY AND CONCLUSIONS

The results from Experiment 1 confirmed that epinephrine administration not only inhibits the insulin-induced uptake of glucose in horses, but interferes with the normal glucose-induced release of insulin from the pancreas. Experiment 2 indicated that the commonly used  $\alpha$ -adrenergic sedative, detomidine, completely precluded the expected insulin response to glucose infusion; that effect was not altered by combining the opioid agonist, butorphanol, with detomidine. In contrast, the combination of the two drugs for sedation had no effect on the expected decrease in blood glucose after insulin administration (Experiment 3); the observed tendency of detomidine to reduce insulin's effect was tenuous and needs further study. Finally, Experiment 4 indicated that butorphanol alone administered to horses did not alter the insulin response to infused glucose.

Taken together, these data indicate that sedation with detomidine, and likely other  $\alpha$ -adrenergic agonists, cannot be used to assess the insulin response to glucose as an assessment of insulin resistance. The combination of detomidine and butorphanol does appear to be a viable alternative for the sedation of horses for measurement of the insulin-induced decrease in blood glucose, which is a proven method of assessing insensitivity in horses.

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## **VITA**

Lauren Elizabeth Kerrigan, daughter of Nancy Barakat and Cary Kerrigan, was born in Chadds Ford, Pennsylvania, in November of 1994. Lauren attended high school at Wilmington Friends School, from which she graduated in 2013. In May of 2017, Lauren received her bachelor of science degree in Animal, Dairy, and Poultry Sciences with a concentration in Animal Science and Technology from Louisiana State University. She then began pursuing a master of science degree from Louisiana State University under the direction of Dr. Donald L. Thompson, Jr., with an interest in equine reproductive physiology and endocrinology. She will receive her master's degree in December of 2019.