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THE EFFECT OF PITUITARY PARS INTERMEDIA DYSFUNCTION ON PROTEIN METABOLISM AND INSULIN SENSITIVITY IN AGED HORSES

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THE EFFECT OF PITUITARY PARS INTERMEDIA DYSFUNCTION ON PROTEIN METABOLISM AND INSULIN SENSITIVITY IN AGED HORSES

THESIS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the College of Agriculture at the University of Kentucky

By
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Lexington, Kentucky

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2013

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ABSTRACT OF THESIS

THE EFFECT OF PITUITARY PARS INTERMEDIA DYSFUNCTION ON PROTEIN METABOLISM AND INSULIN SENSITIVITY IN AGED HORSES

Equine pituitary pars intermedia dysfunction (PPID) typically occurs in horses older than 15 years of age and is characterized by hair coat abnormalities, muscle atrophy and decreased insulin sensitivity. The first objective of this research was to compare the rate of whole body protein metabolism and relative abundance of key factors in the signaling pathways associated with muscle protein synthesis and protein breakdown in response to feeding in Control and PPID horses. No differences (P > 0.05) were seen between the PPID and Control groups in whole-body protein metabolism or post-prandial activation of the muscle signaling pathways regulating skeletal muscle protein synthesis and breakdown. The second objective of this research was to determine if aged horses with PPID had reduced insulin sensitivity and alterations in the insulin-mediated signaling pathways in the skeletal muscle when compared to non-PPID, aged Control horses. Measures of insulin sensitivity and the activation of factors associated with protein synthesis and breakdown were similar between the PPID and Control groups (P > 0.05). Overall, insulin sensitivity and protein metabolism are similar between the PPID and Control groups. The studies suggest that abnormalities may exist as a function of advanced age rather than PPID status directly.

KEYWORDS: PPID, insulin sensitivity, protein metabolism, mTOR, horse

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CHAPTER I.
*The literature review*

1.1 INTRODUCTION

The focus of this review is to examine the literature pertaining to the current knowledge of pituitary pars intermedia dysfunction (PPID) in aged horses, specifically as it relates to changes in insulin sensitivity and protein metabolism. A summary of the prevalence, physiology, and clinical signs associated with PPID will be followed by a brief overview of the disease as seen in other species. Next, diagnostic methods and treatments for PPID will be discussed. This will be followed by a synopsis of insulin resistance (IR) in the horse including the role of insulin especially as it relates to aged horses, current techniques that are being used in the assessment and diagnosis of IR, and other conditions that may cause IR will be given. Finally, protein metabolism in the horse and other relevant species will be reviewed, including an overview of the factors that regulate whole-body protein synthesis and breakdown, the signaling pathways that regulate muscle protein synthesis and degradation, a brief overview of the methods used to study whole-body and muscle protein metabolism, and the effects of old age on protein metabolism.

1.2 PITUITARY PARS INTERMEDIA DYSFUNCTION

Pituitary pars intermedia dysfunction (PPID), commonly known as Cushing’s disease, is estimated to affect 15% to 30% of horses older than 15 years of age (1, 2). However, estimates of its prevalence vary greatly likely due to lack of a set of universally accepted diagnostic criteria (3). In an Australian survey of aged horses older than 15
years of age, only 1.7% of owners reported having a horse with Equine Cushing’s syndrome, but 22% of owners reported that their horse had a change in hair coat, a common clinical sign of PPID (4). The onset of PPID usually occurs in horses 18 to 20 years old and very rarely occurs in horses younger than 10 years of age (5). Despite initial reports, it does not appear that the disease has a greater prevalence based on breed (6) or sex (7) of the horse.

1.2.1 Physiology of PPID

The equine pituitary gland is made up of four different regions: pars distalis, pars intermedia, pars tuberalis and pars nervosa (Figure 1.1). PPID occurs when an adenoma, hypertrophy, or hyperplasia is present on the pars intermedia of the horse’s pituitary gland.

In both the pars distalis and the pars intermedia, pro-opiomelanocortin (POMC) is produced by corticotropes and melanotropes, respectively. POMC is further processed to yield adrenocorticotropic hormone (ACTH) and β-lipotropin in the pars distalis by prohormone convertase 1 (PC1). PC1 is expressed in both corticotropes and melanotropes, whereas prohormone convertase 2 (PC2) is only expressed in melanotropes. PC2 further cleaves ACTH to α-melanocyte-stimulating hormone (α-MSH) and corticotropin-like intermediate lobe peptide (CLIP) and cleaves β-lipotropin to β-endorphin (β-END), lipotrophin and other small peptides, which are released into the bloodstream (Figure 1.2) (8). In a healthy horse, nearly all ACTH is produced in the pars distalis; however, in a horse affected by PPID, ACTH is also produced in the pars intermedia. Therefore, horses with PPID have increased levels of anti-inflammatory
hormones, ACTH and α-MSH. A study of hormonal changes in these horses found that
ACTH was elevated to a similar level to that of human Cushing’s disease; however, α-
MSH, β-MSH, CLIP and β-END were found to be disproportionately increased (9).

Dopamine is known to negatively regulate POMC production in the horse by
inhibiting prohormone convertase activity in the pars intermedia. In horses with PPID, an
adenoma on the pars intermedia causes dopamine production to decrease leading to an
increase in POMC-derived peptides. Specifically, a study of pars intermedia tissue found
that horses with PPID had 8-fold less dopamine concentration than the tissue from aged
matched controls (10). Additionally, another study found that horses with PPID had a 6-
fold reduction in dopaminergic nerve terminals (11). Prohormone convertase is
inhibited by dopamine and in a study of mice lacking a dopamine receptor PC1 activity
increased 4-5 fold and PC2 activity increased 2-3 fold (12). Similar changes are believed
to occur in horses with PPID, since PPID affects dopamine receptors in the pars
intermedia (9). These changes cause ACTH to be produced at a faster rate than it can be
cleaved and thus net ACTH production occurs in the pars intermedia, as well as the pars
distalis.

In a study of horses injected with a dose of ACTH twice a day for four days,
ACTH increased plasma cortisol concentrations (13). Additionally, glucose
concentrations were higher in horses receiving ACTH and a transient increase in insulin
concentration was seen. Horses with PPID have been shown to have elevated ACTH and
α-MSH; however an increase in plasma cortisol has not been seen (14, 15). The
mechanism that would explain this phenomenon is currently unclear. Ultimately, the
physiological changes that occur as a result of PPID lead to a variety of clinical signs.
1.2.2 Clinical Signs

There are many clinical signs associated with PPID and horses can be highly variable in their presentation of these clinical signs (Table 1.1). In the early stages of PPID, it is possible to have very few or no clinical signs present. The classical sign of PPID is hypertrichosis, characterized by a long, curly hair coat that fails to shed, as well, delayed shedding, incomplete shedding, and lightening of the hair coat may be present. Pathogenesis of this clinical sign is unknown although it has been hypothesized that it may occur as a consequence of chronic elevations in MSH peptides due to their known role on skin cells (14). Due in part to their long hair coat, horses with PPID have been reported to have an increased incidence of hyperhidrosis or excessive sweating. However, excessive sweating has been reported even in a cool environment or when the horse is body clipped, suggesting that hyperhidrosis may be caused by other factors in addition to the long hair coats (5).

Weight loss and muscle atrophy, primarily in the epaxial and gluteal musculature, has also been reported to be associated with PPID (8). Studies of muscle atrophy in horses with PPID revealed atrophy of type 2 fibers, sarcoplasmic lipid accumulation, increased myofiber size variation, and increased proteolysis which is consistent with increased glucocorticoid production as seen in other species (16, 17). Combined with muscle loss, horses with PPID may also have abnormal distribution of fat along the crest of the neck, over the tail head and behind the eyes. A proposed mechanism of this occurrence is that hyperinsulinemia seen in many horses with PPID, may contribute to fat deposition by inhibiting lipolysis (5).
Insulin resistance is also commonly reported in association with PPID; however, it is unclear if it is directly associated with PPID. Two studies have been published comparing insulin sensitivity in horses with PPID to younger healthy horses. In both of these studies, the horses with PPID were found to be less insulin sensitive than their younger counterparts (18, 19), when an intravenous glucose tolerance test and euglycemic hyperinsulinemic clamp were used to assess insulin sensitivity. Interestingly, a study compared aged (>15 years old) horses with confirmed PPID (positive dexamethasone suppression test (DST), hirsutism, and at least one other clinical sign), horses with unconfirmed PPID (positive DST, but no hirsutism) and healthy controls (negative DST and no hirsutism) (20). The study found that horses with confirmed PPID had higher basal levels of plasma glucose and insulin compared to both the unconfirmed PPID and control group. It has been suggested that high serum insulin concentrations, may be the cause of laminitis in horses (21, 22). In a study of 40 horses with laminitis, 70% were found to have PPID as defined by a plasma ACTH concentration greater than 35 pg/mL. Laminitis is the major clinical complication associated with PPID and is often the reason for euthanasia in these horses (7).

Other clinical signs of PPID include, polyuria and polydipsia, increased opportunistic infections, immunosuppression, behavior abnormalities and neurologic disease (8). It is important to note that while all of the clinical signs discussed are believed to be associated with PPID, the prevalence of very few of the clinical signs have been compared with healthy aged horses. In a study of 165 Thoroughbreds looking at the ACTH-immunoactive cells of the pars intermedia, 5 out of the 165 mares had an enlarged pituitary gland consistent with PPID although none of the horses in this study exhibited
clinical signs (23). While many clinical signs have been reported to be associated with PPID, the mechanistic cause of most of these signs and the interrelationships between these symptoms remains unknown.

1.2.3 Cushing’s disease in other species

As previously mentioned, PPID was referred to as Equine Cushing’s disease (ECD) or Equine Cushing’s syndrome (ECS) in most scientific literature up until the late 1990s and these terms are still seen in some of the recent literature, particularly in studies published outside of the United States or in Veterinary Medicine journals. Additionally, popular press articles, veterinarians, and other people in the equine community, generally still refer to PPID as ECD.

The term Cushing’s syndrome is, by definition, the clinical consequence of glucocorticoids acting in excess of physiological requirements. Cushing’s syndrome was first described by Harvey Cushing in human patients. He described the clinical features of hypercortisolism in people with tumors of the pars distalis (PD), which is the most common way people are affected by Cushing’s syndrome (24). Shortly after Harvey Cushing first described Cushing’s disease, Pallaske (25) found a similar disease in horses that became known as Equine Cushing’s disease (cited by Messer IV and Johnson (26)). However, upon further research, it was determined that Cushing’s disease in horses is not associated with elevated plasma cortisol (14, 15, 27), which may warrant an alternate name for the described syndrome.

In humans, Cushing’s disease (CD) is rare. The estimated annual incidences of human CD are in the range of 1.2 to 2.4 new cases per 1,000,000 (28, 29). Other than
pituitary tumors, Cushing’s syndrome in humans can also be caused by ectopic syndrome (ACTH producing tumors), adrenal adenomas, and carcinomas at 12%, 10%, and 8% of cases, respectively (30). Women are more prone to CD than men and may be 3 to 8 times more likely to have the disease (30). As well, Cushing’s disease can occur at any age, but age of diagnosis peaks from 25 to 50 years (30). Clinical signs associated with the disease are truncal obesity where fat is distribution is concentrated in the trunk of the body rather than extremities, facial fullness, glucose intolerance, gonadal dysfunction, hirsutism, hypertension and muscle weakness. Table 1.2 shows the frequency of clinical signs and symptoms associated with human CD as seen in a study by Boscaro et al. (30).

CD also occurs in other species, such as dogs and very rarely in cats. CD in dogs occurs more commonly with 1 to 2 cases per 1,000 dogs diagnosed annually. Although diagnosis is much more prevalent, canine CD is very similar in its clinical presentation to human CD. Dogs with CD show clinical signs such as abdominal obesity, weight gain, fatigue, muscle atrophy and skin changes. It has been proposed that dogs would make a good animal model for the human form of CD (31). A comparison of the clinical signs of CD as seen in humans, dogs and horses is seen in Table 1.3. The table shows the similarities between dogs and human, yet there are few similarities of dogs and humans to horses.

1.2.4 Use of PPID horses as a model for Parkinson’s disease

It has been suggested that horses with PPID may serve as the best animal model for Parkinson’s disease (PD) in humans due to the significant similarities between the two diseases. PD is a leading cause of neurologic disability in the elderly human
population, but research determining the early, initiating factors have been limited due to lack of a suitable animal model. PPID and PD are similar in that they are both spontaneous neuroendocrine diseases that affect aged populations (32).

Additionally, data supports that they are both associated with loss of function dopaminergic periventricular neurons. As previously mentioned, a study by Millington et al. (10) determined that horses with PPID had eight-fold less dopamine and dopamine metabolites in the tissue of the pars intermedia compared with age-matched controls. Current PD research suggests that oxidative stress is responsible for inducing damage, which may be the contributing factor in neuronal dysfunction and death. Similarly, examination of the horse’s pituitary shows increased oxidative stress in the dopaminergic neurons of the pars intermedia with age and more severely with PPID (11). The study found that horses with PPID had 16 times more 3-nitrotyrosine, a marker of oxidative stress, in the pituitary and hypothalamus and aged horses without PPID had 7 times more 3-nitrotyrosine than young horses.

Another similarity between PPID and PD is in the neuronal accumulation and aggregation of α-synuclein in nerve terminal protein. α-synuclein is a natively unfolded, soluble monomeric protein that can improperly fold and aggregate in the nerve terminals and leukocytes. The accumulation of improperly folded α-synuclein disrupts cellular function and will eventually cause cell death (33). The accumulation and aggregation of α-synuclein is known to contribute to PD. As well, horses with PPID have been shown to have 1.7 times more α-synuclein in the pituitary compared with both healthy aged horses and younger controls (33).
Despite their apparent similarities, the diseases do differ in several important aspects. First, in horses with PPID the adenoma occurs on the pars intermedia, but in humans with PD the tumor occurs on the pars distalis. Due to this difference, clinical signs of the two diseases are not the same. The second major difference is the lack of Lewy body formation in the horse, which is a marker of PD (33).

Due to the limitations of the model, it is likely the horse model will be best utilized in investigating the early, systemic events that lead to PD; however, its usage may provide a unique perspective into PD (33).

1.2.5 Diagnosis of pituitary pars intermedia dysfunction

One of the most reliable indicators of PPID is the presence of hypertrichosis, which can positively predict PPID status 90% of the time based on post-mortem evaluation (34); however, hypertrichosis is not present in all horses with PPID, particularly in early stage cases (5). For this reason, laboratory tests are often used to diagnose and confirm PPID status. There are many diagnostic methods and this review will focus on three: plasma ACTH concentration, plasma α-MSH concentration and dexamethasone suppression test (DST). In addition, there are several other tests that have been investigated, including thyroid releasing hormone stimulation test, domperidone response test, serum insulin concentration, cortisol circadian rhythm loss, urinary cortisol/creatinine ratio and use of magnetic resonance imaging; however, these methods are not as well-established (8). Each of these tests can be beneficial to diagnosing PPID; however, none are 100% accurate in detecting PPID. Ultimately, post-mortem evaluation of the pituitary gland of the horse is the only way to accurately determine PPID status, but in clinical practice this is not possible, and therefore, alternate diagnostic tests must
be performed. It is important to find a test that is highly correlated to the results of post-mortem evaluation and that can be easily performed in a clinical setting.

It is known that the adenoma on the pituitary gland of the horse can increase ACTH and α-MSH production. When the concentrations of these hormones are measured, levels outside of the normal range may indicate PPID status. Plasma ACTH and α-MSH concentrations are determined by taking a single blood sample collected in an EDTA tube. PPID diagnosis is based on an ACTH concentration greater than 40-50 pg/mL (35) and α-MSH concentration greater than 35 pmol/L (36). During the fall months, both of these tests can be greatly affected by seasonality, where a natural rise in ACTH and α-MSH occurs in the later part of August to mid-September and declines by October. α-MSH has the greatest seasonal effects as it increases over 3 times its normal range in the fall months from August to October (8). Beech et al. (37) determined that it might be necessary to have seasonal reference ranges of 90 pmol/L for the fall months in order to accurately diagnose PPID using α-MSH concentration. Additionally, α-MSH is not available as a commercial test, so this diagnostic method is not commonly used in veterinary practice, although it has proven useful in research settings. ACTH is not as seasonally affected as α-MSH (8); however, it can be elevated due to stress, exercise and other biological factors (38).

The dexamethasone suppression test was once considered the goal standard in PPID diagnosis. To perform this test, dexamethasone is administered intramuscularly at a dose of 0.04 mg/kg and then a blood sample is taken 19-20 hours post dexamethasone administration. Diagnosis for PPID is positive if dexamethasone does not suppress blood cortisol levels below 1 μg/dL, 19-20 hours later. In healthy aged horses, administering
Dexamethasone inhibits ACTH release from the pars distalis leading to the suppression of cortisol, whereas horses affected by PPID have a decreased ability to suppress cortisol since ACTH produced in the pars intermedia is not regulated by glucocorticoid feedback. A study by Dybdal et al. (39) found that DST has nearly 100% sensitivity and 100% specificity in diagnosing PPID at 19-20 hours post dexamethasone administration when evaluated post-mortem. In this case, sensitivity refers to the test’s ability to correctly identify a horse with PPID as having PPID and specificity refers to the test’s ability to correctly identify a non-affected horse as negative for PPID. However, later studies showed that this test might not be nearly as accurate as initially reported (40).

A later study compared the three diagnostic tests for specificity and sensitivity (40). These tests were evaluated based on post-mortem evaluation of the ratio of the pars intermedia to the total pituitary area. During this study, 105 clinically normal horses and 15 with the clinical signs of PPID were tested for PPID using all 3 diagnostic methods and then evaluated post-mortem. Using the ratio pars intermedia area to total pituitary area of greater than 0.3 as a positive diagnosis for PPID, sensitivity and specificity in non-fall samples for the ACTH test was 71% and 81%, for α-MSH was 63% and 90%, and for DST was 65% and 95%, respectively. While all three diagnostic tests were poor at correctly identifying non-end stage cases of PPID, the best results (sensitivity=80%, specificity=90%) were obtained when both DST and α-MSH were performed and a positive result from either test was used to indicate PPID status. The results indicate that it may be necessary to use more than one diagnostic test to more accurately diagnose PPID.
1.2.6 Treatments for PPID

Horses with PPID have additional considerations in both general geriatric management as well as pharmaceutical treatment of the disease. For horses with PPID, nutrition, parasite control and hoof care have even greater importance. Due to the possibility of increased incidence of insulin resistance and laminitis (41), feeds with highly soluble carbohydrates should be avoided. Currently, many companies have diets formulated specifically for horses with insulin resistance, such as Purina’s WellSolve L/S® and Intégri-T®. Proactive deworming strategies should be employed for horses with PPID due to the increased risk of strongyle infections (36). Additionally, due to their long hair coat, horses with PPID may need to be body clipped and provided with ample shade during the summer months.

There are two drugs commonly used to treat PPID: pergolide mesylate and cyproheptadine. Pergolide is currently the most commonly used drug due to its ability to down regulate POMC derived peptide production by acting as a dopamine D2 receptor agonist (8). The drug was originally used to treat Parkinson’s disease, but was voluntarily withdrawn from the market in March 2007 (42), due to the 33% increase in vascular heart disease seen in human Parkinson’s patients using pergolide, compared with those who had never used the drug (43). The withdrawal of human pergolide created a problem for veterinarians since there was not a commercially available product containing pergolide that was approved for use in horses. Since increased heart lesions have not been reported in horses, the Food and Drug Administration (FDA) issued a limited exemption, allowing pergolide to be compounded in bulk for veterinary use (42).
Reports have demonstrated pergolide’s ability to significantly lower plasma ACTH concentrations in horses with PPID (7, 44, 45). In 40-60% of the horses, ACTH was lowered to within normal range (9-35 pg/mL) (44, 45). Horses treated with pergolide had ACTH concentrations significantly lower than those horses treated with cyproheptadine (7, 44). Further Donaldson et al. (44) found that 85% (17/20) of owners with horses on pergolide reported an improvement in the clinical signs associated with PPID compared with only 28% (2/7) of owners of the horses treated with cyproheptadine.

In September 2011, PRASCEND® Tablets were approved for use in horses with PPID (NADA 141-331). According to Hagyard pharmacy, a major veterinary pharmacy in Lexington, KY, the approximate cost of PRASCEND® Tablets is $1.95/per tablet with most horses requiring 1 tablet (1 mg of pergolide mesylate per tablet) per day. Due to the high cost, it is essential that a horse be correctly diagnosed with PPID.

Cyproheptadine is a mixed-action drug, which acts as a serotonin antagonist and has antihistamine and antimuscarinic effects or the ability to block muscarinic receptors (8). Blocking muscarinic receptors helps to increase the level of dopaminergic activity. The use of cyproheptadine has limited success when compared to pergolide (7, 44); however, it may be beneficial for some equines. It has been suggested that may be useful in combination with pergolide when maximal doses of pergolide alone are unsuccessful in resolving the clinical signs (8). As well, cyproheptadine’s effectiveness may be underestimated because Perkins et al. (45) found that there was no difference in the amount that plasma ACTH decreased between horses treated with pergolide and horses treated with cyproheptadine. Additionally, the study found that 81% of horses (26/32) treated with cyproheptadine and 90% (9/10) treated with pergolide showed improvement
of at least one clinical sign. Currently, cyproheptadine is not approved by the FDA for use in horses, despite being used in accepted practice. According to Hagyard pharmacy, a major veterinary pharmacy in Lexington, KY, the cost of Cyproheptadine 4mg Tablets is $104.71 for 1,000 (4mg) tablets. With most horses requiring 0.5 mg/kg BW per day (45), a 500 kg horse will require 250 mg per day, at a cost of about $6.50 per day.

1.3 INSULIN SENSITIVITY IN THE HORSE

PPID is believed to be associated with decreased insulin sensitivity; however, as previously discussed, this relationship has not been well described in the scientific literature. Despite the lack of conclusive research, popular press articles and feed companies marketing low starch feeds continue to associate PPID with insulin resistance. The next section of this literature review will discuss the physiological roles of insulin, the concept of insulin resistance, methods used to measure insulin sensitivity in the horse, insulin resistance in aged horses and conditions other than PPID that may cause a decrease in insulin sensitivity in horses.

1.3.1 Physiological roles of insulin

Insulin is a dipeptide, containing A and B chains, that are linked by disulphide bridges and contains 51 amino acids. Insulin is synthesized as its precursor, proinsulin, by the β-cells of the pancreatic islets of Langerhans. Proinsulin is synthesized in the ribosomes of the rough endoplasmic reticulum (RER) from mRNA as pre-proinsulin. When the signal peptide is removed from pre-proinsulin, proinsulin is formed which is
transferred from the RER to the Golgi apparatus. Then, enzymes acting outside the Golgi apparatus convert proinsulin to insulin and C-peptide (46, 47).

Insulin secretion from the islet cells into the portal vein is pulsatile and increases post-feeding (48). In response to a glucose stimulus, insulin secretion is biphasic meaning an initial rapid phase of insulin secretion occurs followed by a less intense, but more sustained insulin release (49). Increased levels of glucose induce the first phase of glucose-mediated insulin secretion by the release of insulin from the secretory granules of the β-cells. The process occurs when glucose entering the β-cell is sensed by glucokinase, which phosphorylates glucose to glucose-6-phosphate, generating ATP. Then potassium-ATP dependent channels close, resulting in membrane depolarization and activation of calcium channels. When calcium channels open, intracellular calcium concentrations increase leading to a pulsatile secretion of insulin (50). Insulin secretion can also be stimulated by other factors such as stimulation of the vagus nerve when food is seen, smelled or acutely ingested (51). Peptide hormones such as glucagon-like peptide-1 (GLP-1) enhance insulin secretion (52) while somatostatin (53) and leptin (54) inhibit insulin secretion. Additionally, amino acids, particularly arginine (55), ornithine (56) and leucine (57), have been shown to stimulate insulin secretion.

Upon secretion, insulin interacts with its target cells by binding to specific sites on the plasma membrane called receptor sites (58). Insulin acts on cells in the skeletal muscle, liver and fat. Insulin binds to the α subunit of the receptor site causing a conformational change, which then allows ATP to bind the β subunit of the receptor site. The main insulin receptor in the cell involves tyrosine kinase. This interaction is rapid, reversible and triggers a series of internal events that results in the movement of glucose-
transporter proteins (GLUT4) to the cell surface for glucose uptake. GLUT4 appears the major transporter that mediates the insulin response (59). Ultimately, the role of insulin is to maintain normal blood glucose levels by facilitating cellular glucose uptake. The major sites of insulin mediated glucose uptake include the skeletal muscle and adipose tissues. Insulin secretion facilitates glucose uptake by the cells, promotes glycolysis, glycogenesis, protein synthesis and ion uptake and inhibits gluconeogenesis, glucogenolysis, lipolysis, ketogenesis and proteolysis (Figure 1.3) (60).

In the horse, insulin secretion has a distinct circadian rhythm with maximum secretion at 1200h. (61, 62). The pattern of this rhythm does not appear to be affected by PPID status or hay versus concentrate diet as seen in two independent studies (61, 62). Additionally, horses show a significant post-feeding rise in plasma insulin concentration when fed concentrate, but little increase in insulin concentration when hay is fed (63). This is due to increased levels of glucose post-feeding, inducing glucose-mediated insulin secretion (47) and dietary amino acids such as arginine and leucine which are known to increase insulin secretion (64). Insulin release is decreased during stress and exercise, due to the increase in circulating catecholamines, which inhibit insulin secretion (65). In the horse, this inhibition occurs at about 50% of VO$_{2\text{max}}$ which allows the horse to increase gluconeogenesis to maintain blood glucose during exercise (65).

1.3.2 Insulin Resistance

Insulin resistance (IR) is defined as a condition where normal concentrations of insulin produce a subnormal physiologic response (66). It occurs when tissues are not as responsive to the actions of insulin, usually due to defects in post receptor insulin
signaling. When the tissues no longer elicit the proper response to insulin, the body may compensate by increasing insulin secretion. This is known as compensatory hyperinsulinaemia and occurs when pancreatic β-cell secretion increases in order to maintain normal blood glucose. In humans, the state is maintained until pancreatic secretory defects occur and the inability to compensate for decreased insulin sensitivity results in increased blood glucose concentrations and type 2 diabetes. In humans, IR is a characteristic of type 2 diabetes and is different from the reduction in insulin production as seen in type 1 diabetes (67). However, diabetes mellitus in the horse is rare, but it may be underdiagnosed in aged horses (68). Horses commonly compensate for decreased insulin sensitivity in two ways. First, a horse may compensate by increasing pancreatic β-cell release and insulin secretion in order to maintain insulin-mediated glucose uptake. Alternatively, a horse may increase glucose-mediated glucose uptake (glucose uptake not related to insulin, which occurs primarily in the fasted state) in order to decrease the concentration of plasma and extracellular glucose (69).

In the horse, insulin resistance may occur due to a reduction in the density of insulin receptors on the cell surface, malfunction of insulin receptors, defects in internal signaling pathways or interference with the translocation or function of GLUT4 proteins (70). To distinguish between the different causes of IR, two terms were adopted. The first, insulin sensitivity, describes reduced insulin-mediated glucose transport into the cell. The second, insulin ineffectiveness, describes a failure of insulin-facilitated intracellular glucose utilization (21, 69). These two types of IR may exist separately or together depending on the specific case of IR.
1.3.3 Conditions associated with insulin resistance in the horse

As previously discussed, insulin resistance (IR) may be associated with PPID (18, 19); however, there are many other conditions that are also associated with IR. It has been suggested that IR is a factor causing many equine diseases, such as laminitis (71), hyperlipidemia (72) and osteochondritis dessicans (OCD) (73). This review will specifically discuss the role of IR in laminitis, as well as other factors that affect IR such as body condition, activity, diet and age.

1.3.3.1 Insulin resistance and laminitis

IR was first associated with laminitis in the 1980’s when two studies found that ponies with a history of laminitis were more likely to be insulin resistant than horses with no history of laminitis (71, 72). Additionally, more recent studies had similar findings using basal proxies, reciprocal of the square root of insulin (RISQI) and modified insulin to glucose ratio (MIRG) (74, 75). A further discussion of these proxies can be found in Section 1.3.4.2. Analysis of these proxies indicated that the ponies predisposed to laminitis had lower insulin sensitivity (RISQI) and higher insulin response (MIRG), indicating a compensatory exaggeration of pancreatic β-cell secretion (75). In healthy horses, a study demonstrated that laminitis can be induced due to prolonged hyperinsulinemia within 48 hours (76).

Although, it is well defined that decreased insulin sensitivity and laminitis are associated, the cause of this relationship is unknown. Studies have shown that laminitic episodes may be able to be predicted using insulin, leptin and measures of generalized and localized obesity (77). It was initially suggested that insulin-mediated
vasoconstriction may be responsible for causing laminitis (72). More recent hypotheses include compromised glucose transport into insulin-dependent cells (21, 78), since glucose deprivation has been shown to result in the separation of hoof connective tissue (79). While there are many hypotheses of the known relationship between insulin resistance and laminitis, none of them have been proven to be the cause of all laminitis cases; therefore, it is likely that this is complex relationship that will require further research in order to gain a better understanding.

1.3.3.2 Factors affecting insulin resistance in the horse

IR is affected by many different factors, but it may be particularly affected by age (80) body condition (81), activity level (82), and diet (83).

The relationship between insulin resistance and age is well known; however, the cause of this relationship is unclear (84). In human studies, insulin sensitivity was found to decrease with age, beginning at 30 years of age and continuing throughout the remainder of a person’s lifetime (85). However, a separate study found that when adjusted for increased body weight with age this relationship was no longer significant (86).

A study found that older horses (27±0.2 yrs) needed nearly 200% and 150% more insulin to respond to an oral glucose tolerance test than young (6.8±0.4 yrs) and middle-aged (15.2±0.4 yrs) mares, respectively (87). Additionally, tumor necrosis factor-α (TNF-α) has been suggested to lead the development of insulin resistance and a study of mice found the TNF-α increased with age. This relationship was investigated in the horse in a study by Vick et al. (88). The study used the EHC procedure to assess insulin
sensitivity in sixty mares and found that insulin sensitivity was negatively correlated with body condition score, age, and TNF-α. The results of this study support previous associations that high concentrations of circulating insulin and reduced insulin sensitivity occurs in mares with high BCS and these effects may be exacerbated by aging (88).

The study was hardly the first to associate IR and obesity in horses. Several studies have reported decreased insulin sensitivity in obese horses when compared to their lean counter parts (81, 83). The decrease in insulin sensitivity seen in obese horses seems to improve with weight loss due to both calorie restriction and exercise (81). Additionally, fat distribution, such as neck crest adiposity, has been shown to be negatively correlated with insulin sensitivity in horses and ponies (89). The relationship between insulin resistance and obesity may be due to increased inflammation that occurs with obesity. Administration of lipopolysaccharide (LPS) induces an inflammatory response with increased concentration of inflammatory cytokines, particularly TNFα. Administration of LPS was found to induce insulin resistance within 24 hours in horses (90).

In human studies, exercise has been shown to improve IR even when weight loss does not occur (91). Similarly, a study of both lean and obese mares found that exercise improved insulin sensitivity without a change in body weight after 7 days of round-pen exercise (82). Stewart-Hunt et al. (92) suggested that these findings are due to the effect of training on insulin action. They found that GLUT-4 content and glycogen synthase activity increased with short-term training and these enhancements were still evident after 5 days of inactivity. Alternatively, another study found that exercise training alone
without dietary restriction did not improve insulin sensitivity in overweight or obese horses (93).

It is also important to understand the relationship between diet and IR. A study by Hoffman et al. (83) showed that healthy horses adapted to sweet feeds that are high in non-structural carbohydrates, such as starch and sugars, had decreased insulin sensitivity. Additionally, the study results suggested that the effect of non-structural carbohydrates may be magnified in horses with preexisting IR. A second study found that weanlings adapted to a sugar and starch diet had decreased insulin sensitivity when compared to weanlings on a fat and fiber diet; however, this decrease in insulin sensitivity appeared to be compensated for with increased β-cell secretion of insulin (94).

Overall, there are many factors that can affect insulin sensitivity in the horse and many of these factors are interrelated. It is important to understand these risk factors in order to assess a horse’s risk for insulin resistance.

1.3.4 Methods used to measure insulin sensitivity

There are many methods used to measure insulin sensitivity, each with its own advantages and disadvantages. Depending on the specific situation, it may be necessary to use one or a combination of these methods in order to quantify insulin sensitivity in the horse. While there are many methods that can be employed, this review will focus on five methods including, basal hyperglycemia and hyperinsulinemia, proxies and surrogate testing, glucose tolerance test, minimal model of glucose-insulin dynamics and the euglycemic-hyperinsulinemic clamp (EHC) technique.
1.3.4.1 Basal hyperglycemia and hyperinsulinemia

Measurement of fasting plasma glucose and insulin concentrations is a simple screening test that involves a single blood sample. The technique may be useful in obtaining some information about insulin sensitivity with minimal effort on the part of the person testing. Measuring plasma glucose is not extremely effective due to its inability to differentiate between inadequate secretion of insulin by pancreatic β-cells or a decrease in insulin sensitivity of tissues (95). Conversely, basal hyperinsulinemia was once considered a certain sign of IR; however, upon further research new methods were developed which gave a better indication of the insulin sensitivity of the horse. Basal hyperinsulinemia only occurs when insulin insensitivity of the tissues is overcompensated for by increased insulin secretion; however, decreased insulin sensitivity caused by decreased secretion of insulin will not be detected (95). Therefore, basal hyperinsulinemia may be useful to detect some cases of IR, but not others. When measuring plasma glucose and insulin concentrations, the cutoff value for hyperglycemia and hyperinsulinemia has not been well defined. The cutoff values of 5.5 mmol/L and 36 µIU/mL have been suggested for hyperglycaemia and hyperinsulinaemia in adult horses, respectively (20).

1.3.4.2 Proxies and surrogate tests

Several simple tests have been developed to determine IR in humans. These screening tests are based on equations that use fasting plasma glucose and insulin concentration. In human medicine, there are many screening tests used, including
HOMA-IS, QUICKI, RISQI and MIRG. Table 1.4 shows the full names and equations used to calculate these proxies.

While commonly used in humans, the use of these proxies has only been recently investigated in the horse. A study by Treiber et al. (96) compared the use of different proxies to the minimal model of glucose and insulin dynamics determined using a frequently sampled intravenous glucose tolerance test, a much more intensive procedure known to accurately measure insulin sensitivity. The study found that RISQI ($r=0.774$) was the best predictor of insulin sensitivity and MIRG ($r=0.754$) was the most accurate predictor of $\beta$-cell responsiveness. The combined use of these proxies allows for the assessment of insulin response and compensatory insulin secretion. The authors proposed that the use of these may be useful in research on large populations or in clinical situations requiring convenient and cost-effective evaluations of insulin resistance where a dynamic test cannot be performed and nonspecific indicators, such as basal hyperinsulinemia, do not characterize cause of insulin resistance (96).

1.3.4.3 Glucose tolerance testing

The glucose tolerance test measures the plasma glucose response to an oral or IV administration of a specified dose of glucose using the time integral or area under the curve. This test was originally developed to classify carbohydrate tolerance in humans. However, because plasma glucose and insulin responses during this test may reflect the ability of pancreatic $\beta$-cells to secrete insulin and tissues sensitivity to insulin, the test has also been used to evaluate IR (97).
Recently, the oral sugar test was developed to test horses in the field. The test works by orally administering corn syrup using a syringe at a dose of 15 mL per 100 kg BW. Then, a single blood sample is taken 60-90 minutes later. An insulin concentration greater than 60 µU/mL at this time is indicative of insulin resistance in the horse (98). The glucose tolerance test may be useful in horses since it does not require as much labor and time as some of the other diagnostic methods for IR. The major limitation of the test is that glucose intolerance does not necessarily coexist with IR. The test cannot distinguish between factors, such as insulin secretion or insulin sensitivity, which may cause failure of glucose homeostasis (99). Despite the limitations of this method, it has been considered evidence of IR in several studies of horses including horses with PPID (18), fasted ponies (100), mature obese ponies (101) and laminitic ponies (72).

1.3.4.4 Minimal model analysis of the frequently sampled intravenous glucose tolerance test

The minimal model of glucose-insulin dynamics is a dynamic test of insulin resistance, which divides glucose disposal into two parts, glucose and insulin-mediated disposal. The model describes the glucose time curve as 2 differential equations. The first represents glucose-mediated glucose disposal by use of as single rate constant and the second represents insulin mediated glucose disposal by use of a rate constant and insulin sensitivity (95).

An application of the minimal model is in the modified frequent sampling i.v. glucose tolerance test (FSIGT). To perform the technique, a bolus of glucose is administered intravenously. Then 20 minutes after the glucose bolus, an insulin bolus is administered at a dose, which would accelerate glucose disposal, but not promote
hypoglycemia. The technique lasts 3-4 hours and blood samples are taken frequently (1-3 minutes between samples) particularly in the first 30 minutes and then the time between samples increases to approximately 30 minutes in the last 1-2 hours of the procedure (83).

Plasma insulin and glucose concentrations during sampling are determined and used to calculate four output measures. Glucose effectiveness (Sg), insulin sensitivity (Si), acute insulin response to glucose (AIRg) and the disposition index (DI) must be calculated using computer software. A diagram of the minimal model used to interpret the modified FSIGT is shown in Figure 1.4. To define these terms, glucose effectiveness (Sg) refers to the capacity of glucose to mediate its own disposal independent of a change in plasma insulin and insulin sensitivity (Si) refers to the capacity of insulin to promote its own disposal. Acute insulin response to glucose (AIRg) quantifies the endogenous insulin secretion in response to the glucose dose. Disposition index (DI) describes β-cell responsiveness and accounts for the influence of endogenous insulin secretion (AIRg) and Si (83).

The frequent sampling makes this a labor-intensive procedure requiring many trained personnel to properly perform the technique. Additionally, concerns have been raised about the calculation of glucose-mediated disposal using a single-rate constant which may not always adequately describe the glucose component of the model (102).

1.3.4.5 Euglycemic hyperinsulinemic clamp technique

The euglycemic hyperinsulinemic clamp (EHC) technique was introduced in human medicine by DeFronzo et al. (103) and gained initial acceptance as the best
method to quantify IR. Later, this technique was applied for use in the horse (82). To perform the technique, the horse must be outfitted with two catheters, one to infuse glucose and insulin and the second to obtain blood samples. The method replicates a state of hyperinsulinemia by infusing insulin at a constant rate that is based on the horse’s body weight. During the insulin infusion, frequent blood samples are taken and analyzed for glucose concentration using an automated analyzer. Then the rate of glucose infusion is subsequently adjusted in order to maintain euglycemia. When the rate of glucose infusion reaches plateau the rate of glucose infusion is assumed to equal the rate of glucose disposal (104).

The EHC technique was found to be more repeatable than the minimal model analysis of the FSIGT for determining insulin sensitivity in the horse (105). This may make it more desirable for studies where the same horse is studied multiple times. Additionally, the EHC technique is advantageous for situations where the horse needs to be in an insulin-stimulated state at the end of the procedure as in the case of a muscle biopsy. While the EHC is a well-accepted technique, the method does not come without some disadvantages. It is labor intensive, as many samples need to be collected in order to maintain stable glucose concentrations. Additionally, the technique determines the sensitivity of tissues to insulin; however, it does not account for the sensitivity of β-cells to glucose. It has also been argued that the conditions of the EHC are non-physiologic, since the measured glucose disposal rate corresponds to an abnormally high insulin level (102). However, this can be avoided by using lower rates of insulin infusion that create a more physiologic state as seen in several studies (82, 88).
1.4 PROTEIN METABOLISM IN THE HORSE

Protein metabolism refers to the sum of all biochemical processes required for protein synthesis and protein breakdown. This review will cover protein synthesis as it is regulated through the mTOR pathway, suppression of the mTOR pathway by myostatin and protein breakdown as it is regulated through the ubiquitin-proteasome pathways and factors that interact with this pathways of protein breakdown including the FoxO family and NF-κB. This will be followed by a review of isotope infusion methodology to study protein metabolism and the effect of aging on whole body protein metabolism.

1.4.1 Protein synthesis and the mTOR pathway

The process of protein synthesis occurs when the nucleotides in mRNA are decoded into amino acids that are linked via peptide bonds in order to form a new protein. The process of protein synthesis can be divided into three stages: initiation, elongation, and termination. A number of the components involved in initiation and elongation stages of translation are controlled by the mechanistic, formally known as mammalian, target of rapamycin (mTOR) pathway (106). The mTOR signaling pathway was initially discovered in 1991 in yeast cells (107) and has been extensively studied in the skeletal muscle of humans (108, 109), rodents (110, 111), piglets (112-114) and to a lesser extent in the horse (115, 116). The mTOR pathway has been the subject of many recent reviews (106, 117, 118). Briefly, mTOR is phosphorylated and activated by a number of upstream pathways, which are activated by factors such as insulin, amino acids and exercise. Upon activation, mTOR affects a series of downstream regulators leading to an increase in protein synthesis. A simplified schematic of this pathway can be
seen in Figure 1.5. The effects of mTOR on protein synthesis will be further discussed in this review, including upstream pathway activators and downstream effectors. The ability of myostatin to suppress protein synthesis will be reviewed and this will be followed by a summary of what is currently known about mTOR signaling in the skeletal muscle of horses.

1.4.1.1 Activation of mTOR

Insulin is a major activator of the mTOR pathway through Akt, which is also known as protein kinase B (PKB). In this interaction, a subunit activated by insulin phosphorylates Akt at the Ser\textsuperscript{473} and Thr\textsuperscript{308} phosphorylation sites, activating Akt (119). Activation of Akt leads to activation of mTOR by inhibiting negative regulators of mTOR known as 40 kDa protein rich Akt substrate (PRAS40) and tuberous sclerosis 2 (TSC2) (120, 121). Akt is also responsible for phosphorylation of transcription factors, forkheadbox protein O1 (FoxO1) and forkheadbox protein O3 (FoxO3) (111, 122). These factors are involved in regulation of protein breakdown and activate the expression of genes regulating apoptosis. A further discussion of this regulation can be found in Section 1.3.2. Additionally, activation of Akt results in the movement of glucose transporter 4 (GLUT4) to the cell membrane resulting in the transport of glucose in the cell (123).

Movement of glucose into the cells does not directly influence mTOR signaling (124); however, cellular energy is needed to drive mTOR signaling. ATP is produced by glucose as a product of glycolysis. When levels of ATP in the cell are high, AMP activated protein kinase (AMPK) activity is inhibited (125). Similarly, when levels of ATP are low and levels of AMP are high, AMPK becomes active. Ultimately, through a
series of phosphorylations AMPK inhibits mTOR by phosphorylating regulatory-associated protein of mTOR (RAPTOR) (110, 126). Therefore, any physiological state, which effects glucose metabolism, will in turn affect mTOR (127).

Intracellular amino acids stimulate mTOR signaling through a system of amino acid transporters that move the amino acids from the blood to the muscle. Particularly, arginine and leucine have been identified as key amino acids that stimulate mTOR (128, 129). Studies have demonstrated the ability of leucine enriched mixture to decrease phosphorylation of eEF2, which promotes elongation and stimulated muscle protein fractional synthesis rates (130). It is unclear how amino acids stimulate mTOR activation; however, mitogen-activated protein 4 kinase kinase kinase (MAP4K3), PI3K catalytic subunit type 3 (VPS34) and Rag GTPases have been identified as potential mediators. When MAP4K3 is stimulated by amino acids it is believed to activate S6K1 independent of mTOR (131). VPS34 may be the primary modulator of mTOR signaling in response to amino acids and it has been suggested that VPS34 is required for leucine stimulation of S6K1 (132). The Rag GTPases become activated in the presence of amino acids and interact with RAPTOR resulting in mTOR relocation onto the surface of endosomes and lysosomes. This movement may enable allow mTOR to interact with Ras homolog enriched in brain (Rheb) leading to mTOR activation (133). Ultimately, amino acids stimulate protein synthesis specifically at the points of translation and elongation.

Exercise has been shown to increase protein turnover leading to an increase in muscle fiber size; however, during aerobic exercise mTOR signaling is suppressed. Several mechanisms have been proposed for the decrease in mTOR signaling during aerobic exercise. One way that exercise suppresses mTOR signaling is by increasing the
expression of regulated in development and DNA damage response 1 (REDD1), ahypoxia induced gene. REDD1 activates TSC1/2 complex, leading to a reduction inmTOR signaling (134). Other proposed mechanisms include suppression of mTOR byAMPK (135) and suppression of mTOR due to a decrease in eEF2 phosphorylation (136).Some exercise increases skeletal muscle contractile activity increasing the mechanicalstimulation of muscle fibers. Mechanical stimulation increases the activity of mTOR,thus increasing protein synthesis and leading to an enlargement in muscle fibers (137).Following short-term resistance exercise in rodents, an immediate increase in mTORsignaling factors was seen (138), particularly in the downstream effectors leading toprotein synthesis. mTOR signaling is suppressed during exercise and then increased toduring the recovery period leading to muscle hypertrophy (138).

1.4.1.2 Downstream regulators of mTOR

Following the activation of mTOR, a series of cell signaling events lead to theinitiation of mRNA translation into protein. When mTOR is activated, 70kDa S6 kinase 1(S6K1) and eukaryotic initiation factor (eIF) 4E-binding protein 1 (4EBP1) becomephosphorylated through RAPTOR (139).

Once activated, S6K1 phosphorylates several downstream proteins required forRNA processing and mRNA translation initiation, including ribosomal protein S6 (rps6)and eIF4B. rps6 is an important factor known to aid in the determination of cell growthby controlling cell size and cell division (140). rps6 knockout mouse embryo fibroblastshave been shown to have an accelerated cell division due to a shortened G1 phase (141).S6K1 and rps6 activation was initially thought to be important to up regulate translational
capacity by enhancing the translation of components required for protein synthesis; however this theory has been disproven (142, 143). When phosphorylated, eIF4B is required for ribosomal recruitment to mRNA through a ribosomal subunit pre-initiation complex (144). Ultimately, S6K1 plays an important role in the regulation of cell growth, cell cycle progression, and cell proliferation (145).

mTOR activation leads to the phosphorylation of 4EBP1, a regulator of the eIF4F complex that is required in the initiation of cap-dependent mRNA translation (146). When 4EBP1 is phosphorylated, it separates from eIF4E. This allows eIF4E to associate with eIF4G, which leads to the formation of the initiation complex, eIF4F. Once this complex is formed, initiation is followed by elongation and thus protein synthesis occurs (147).

1.4.1.3 mTOR signaling in the skeletal muscle of horses.

The molecular signaling pathway that regulates protein synthesis has been extensively studied in cell culture (107), rodents (110, 111), neonatal piglets (112-114) and humans (108, 109); however, only few recent studies have looked at mTOR signaling in the horse (115, 116). A study of mTOR in the horse demonstrated that the anabolic stimulus of meal consumption increased mTOR signaling, which agrees with the results of previous studies in other species (115). When the post-feeding response of yearlings, 2 year olds and mature horses were compared, the greatest increase in mTOR signaling factors occurred in the yearling horses (116). Another study investigated the effect of repeated muscle biopsies over a 5 days period on mTOR signaling. It was determined that mTOR signaling, specifically phosphorylation of S6K1, rpS6 and
4EBP1, is increased with repeated muscle biopsies. However, phosphorylation of these mTOR signaling factors was not altered by repeated biopsies when an oral anti-inflammatory was administered (148). Additionally, mTOR signaling does not appear to be affected by muscle biopsy depth in horses (149).

1.4.1.4 Myostatin

Myostatin, also known as growth and differentiation factor 8, is a member of the transforming growth factor-β superfamily and a negative regulator of skeletal muscle mass. Disrupted myostatin gene expression is associated with increased skeletal muscle mass resulting from muscle fiber hyperplasia and hypertrophy (150). Studies have demonstrated that recombinant myostatin protein inhibits muscle cell proliferation and protein synthesis in vitro (151). Additionally, dexamethasone administration, which has been shown to decrease skeletal muscle mass (152), leads to increased myostatin expression in rats (153). Further, when dexamethasone was administered to mice with the myostatin gene deleted, skeletal muscle atrophy did not occur (154).

Myostatin leads to muscle atrophy through phosphorylation of Smad2 and Smad3, which block muscle differentiation. Smad2 and Smad3 activation are both required in order for myostatin to inhibit protein synthesis (155). It has been demonstrated that myostatin inhibits the activation of Akt, TORC1 and p70 leading to decreased protein synthesis and muscle loss (155). Interestingly, although inactivation of Akt usually leads to upregulation of MuRF1 and atrogin-1, the study found MuRF1 and atrogin-1 expression were downregulated by myostatin (155). Therefore, the authors hypothesized that myostatin can block the induction of muscle’s differentiation program (155).
1.4.2 Protein breakdown

The degradation of protein is important for the control of growth and in normal metabolism. Protein breakdown allows for protein turnover and provides quality control by selectively eliminating abnormally folded proteins. However, when the rate of protein breakdown exceeds the rate of protein synthesis, muscle atrophy occurs (156). Muscle atrophy is defined as a decrease in muscle mass including a decrease in size and number of skeletal muscle fibers. The pathways regulating protein synthesis and breakdown are highly interrelated. When protein synthesis occurs, it inhibits factors associated with muscle atrophy and when atrophy occurs protein synthesis pathways are inhibited (157). A simplified schematic of the proposed relationship between protein synthesis and breakdown can be seen in Figure 1.6. Muscle protein breakdown is regulated predominantly through the ubiquitin-proteasome pathway, as well as through the lysosomal and calpain pathways (158). These pathways associated with protein breakdown may be altered by various physiologic states such as aging (111, 159), insulin resistance (157), and during postprandial and post-absorptive states (160). This review will specifically cover the ubiquitin-proteasome pathways and factors that interact with this pathways of protein breakdown, including the FoxO family and NF-κB.

1.4.2.1 Ubiquitin-proteasome pathway

The ubiquitin proteasome pathway is responsible for the degradation of most of the skeletal muscle proteins (158). Ubiquitin is a small highly conserved regulatory protein that, when added to a protein substrate, regulates signaling processes associated with muscle atrophy. Three unique enzymatic components are required in this signaling
process, including E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and E3 ubiquitin-ligating enzyme (161). Currently, several hundred distinct E3s have been identified and it is likely that each of these ubiquitin-ligating enzymes regulates ubiquitination of a specific set of substrates (162). Therefore, the regulation of ubiquitination can be part of a coordinated signaling pathway, analogous to phosphorylation. Rates of protein degradation increase through the ubiquitin proteasome pathways protein synthesis is blocked (163) and the amount of polyubiquitin conjugation compared to total protein increase (164). Additionally, mRNA levels of genes that encode ubiquitin-conjugating enzymes that are part of the ubiquitin pathway were found to increase during atrophy (165).

1.4.2.2 MuRF1 and atrogin-1

The genes for two E3 ubiquitin ligases have been shown to increase in expression in multiple models of skeletal muscle atrophy. These genes are known as muscle-RING (really interesting new gene)-finger protein 1 (MuRF1) and muscle atrophy F-box (MAFbx; also known as atrogin-1) (166). Consistent increases in the gene expression of these two factors have been observed in models of skeletal muscle atrophy including diabetes, cancer, and adrenal failure (167). MuRF1 encodes a protein that contains three domains required for ubiquitin-ligase activity (168) and formation of heterodimers between MuRF1 and MuRF2 (169). MuRF1 has been demonstrated to be in the nucleus and may have a role in both regulation of myofibril assembly and muscle gene expression (170). Atrogin-1 contains three domains including an F-box domain. An experiment in mice revealed that when MuRF1 and atrogin-1 knockout mice were compared to their littermates, all mice appeared phenotypically normal. However, when subject to
physiologic conditions that would usually cause muscle atrophy, significantly less muscle mass was lost in the knockout animals (166). The requirement of MuRF1 and atrogin-1 for skeletal muscle atrophy makes them potential targets for drug discoveries since they are required for muscle atrophy, are expressed specifically in muscle cells, and do not seem to be required for normal muscle growth and function (171).

1.4.2.3 FoxO family

The FoxO family is an important factor in the ubiquitin proteasome pathway of protein breakdown that when activated leads to protein breakdown through increasing the expression of atrogin-1 (172). Members of the forkhead box containing protein, O sub-family or FoxO family of transcription factors have been shown to play a variety of roles in the cellular processes including longevity, metabolism and reproduction and regulation of gene transcription (173). There are 3 primary members of the FoxO family: FoxO1, FoxO3a and FoxO4. FoxO1 has been suggested to have a crucial role in vascular formation (174). Both FoxO3a and FoxO4 null mice were viable and did not appear phenotypically different from their littermates. However, female FoxO3a null mice were infertile and had abnormal ovarian follicular development (174). Interesting, the FoxO3a and FoxO1a genotype has been found to be strongly associated with longevity in humans (175, 176). It is believed that the expression of the FoxO genes may help explain the increased longevity seen in females (176).

Akt regulates the FoxO family through phosphoinositide-3-kinase (PI3K) signaling. Thus, when Akt/PI3K is phosphorylated, FoxO is subsequently phosphorylated and inactivated leading to decreased expression of atrogin-1 and
ultimately a decrease in muscle atrophy (172). However, a study showed that FoxO3 could also be regulated in the absence of phosphorylated Akt through interaction with IKKβ, a major catalytic subunit of IkB kinase complex (IKK). When this interaction occurs, IKK phosphorylates FoxO3a, inhibiting it, and causes proteolysis of FoxO3a via the ubiquitination-dependent proteasome pathway. Downregulation of FoxO3a by IKK can induce cell proliferation and tumorigenesis (177). Ultimately, the FoxO family of transcription factors play an important role in upregulating factors associated with muscle atrophy.

1.4.2.4 NF-κB

NF-κB is stimulated by inflammatory cytokines, particularly TNF-α, and leads to increased expression of MuRF-1 (178, 179). In cells of the immune and inflammatory systems, NF-κB was demonstrated to be an integration site for pro-inflammatory signals and a regulator of inflammatory target genes (180). NF-κB activation increases the production of cytokines and begins a positive feedback loop (178). Activation and phosphorylation of NF-κB is controlled by phosphorylation of IKK (181). When the NF-κB is activated, significant atrophy is induced due to upregulation of MuRF1, but not atrogin-1 (179). However, without the presence of MuRF-1, activation of NF-κB did not induce significant atrophy. Thus, it appears transcriptional activation of MuRF1 by NF-κB is a necessary step in NF-κB induced atrophy and a linear signaling pathway had been proposed between IKK, NF-κB, and MuRF1 (Figure 1.6). Alternatively, atrogin-1 is stimulated by p38, a mitogen-activated protein kinase (MAPK), not NF-κB. A study determined that TNFα stimulates atrogin-1 expression and atrogin-1 could not be upregulated when p38 was blocked (182). p38 MAPK has been identified as a potential
regulator of muscle catabolism and activity of p38 is increased in aging (183) and type II diabetes (184). A study of endotoxin induced laminitis in horses found that phosphorylation and thus activation of p38 peaked at 12 h of LPS infusion (185).

1.4.3 Introduction to whole body protein metabolism

The regulation of protein synthesis and breakdown is important to overall metabolic and physiological homeostasis. The overall sum of protein synthesis and breakdown can be measured and expressed as whole-body protein metabolism. Whole-body protein metabolism is affected by factors such as diet (186), age and exercise (187). These changes can be measured using isotope infusion techniques to measure rates of whole-body protein synthesis and breakdown and muscle protein synthesis. This section of the literature review will focus specifically on the isotope infusion methodology and the effect of aging on whole body protein metabolism.

1.4.3.1 Isotope methodology

Whole body protein metabolism can be studied by infusing a stable amino acid isotope, such as labeled leucine, glycine and phenylalanine. In studies of the horse, [1-\(^{13}\)C]phenylalanine has been used for isotope infusion (188, 189). An estimation of whole-body protein synthesis is based on the principle that phenylalanine flux is equal to the rate phenylalanine enters and exits the free amino acid pool and is calculated from the dilution of labeled to unlabeled phenylalanine in the plasma. By measuring phenylalanine flux and rates of phenylalanine oxidation to carbon dioxide (CO\(_2\)) during isotope infusion, rates of whole body synthesis and breakdown can be measured using a stochastic approach (Figure 1.7) (190)
The measurement of whole body protein synthesis is based on the ability to calculate the amount of labeled phenylalanine isotope to unlabeled phenylalanine in the plasma free amino acid pool. Urschel et al. (188) used the formulas established in human studies (191) and applied these equations for use in the horse. Entering the pool, dietary intake (I) refers to the estimated amount of phenylalanine coming from the diet. For the horse, I was estimated as the dietary phenylalanine multiplied by 0.5 in order to account for prececal phenylalanine digestibility and the dietary phenylalanine that is extracted during the first-pass splanchnic metabolism (188). Also entering the pool, the release of phenylalanine from protein breakdown (B) is measured by finding the difference between phenylalanine flux (Q) and I.

Leaving the pool, phenylalanine oxidation to CO\textsubscript{2} (E), known as oxidative disposal, is measured by collecting breath samples. Also leaving the pool, phenylalanine used in protein synthesis (Z) is measured by subtracting phenylalanine oxidation (E) from flux (Q). Conversion to other metabolites (M) is assumed to be negligible as long as tyrosine intake is equal between all treatment groups and in excess of estimated requirements. The sum of these two parts (Z+M) is known as non-oxidative disposal. Therefore, phenylalanine flux (Q) is assumed to equal the rate phenylalanine enters (I+B) and exits (E+Z+M) the free amino acid pool.

1.4.3.2 Effect of aging on whole body protein synthesis

Studies of the effect of age on protein metabolism in humans have provided largely mixed results. Some studies have shown that whole body protein metabolism decreases with age (192), while others have shown no difference (187). Discrepancies
between human studies may be due to differences in body composition and activity that usually occur with increased age.

Short et al. (187) found that the rates of whole body muscle protein synthesis declined with age. Although there was a reduction in the amount of fat-free mass that occurred with age, when the rates of whole body protein synthesis were adjusted for fat free mass, a negative correlation was still found between age and whole body protein turnover. Additionally, the study suggested that some of the negative effects of aging on whole body protein synthesis could be reversed with the addition of an exercise regimen. Conversely, a study by Chevalier et al. (192), found that aging did not alter whole-body insulin sensitivity and protein anabolic responses. The authors hypothesized that this was due to similar muscle mass index and level of physical activity between the elderly and young group. The study suggests that increased muscle atrophy that occurs with age may be the result of increased adiposity and inactivity rather than from chronological age. Ultimately, the relationship between aging and whole body protein metabolism is complex and may be influenced by a number of factors including diet, body composition and activity level.

1.4.3.3 Effect of aging on protein digestibility

Studies in humans have found that protein availability may decrease with age (193). In human studies of young men, a slowly digested dietary protein (casein) lead to greater protein gain than a quickly digested protein (whey); however, a subsequent study showed that elderly males may benefit from a quickly digested protein compared with a slowly digested protein in order to limit protein loss (193, 194). In horses, there is some
evidence to support that protein digestion may decrease with advanced age. In an early study, apparent crude protein digestion was reduced in aged horses (~25 yrs) when compared with younger horses (~2 yrs) (195); however, in later studies of aged horses, protein digestion was within the normal range of apparent protein digestion reported for younger horses (196). Despite some evidence that protein requirements are different in aged horses when compared to younger horses, the current edition of the NRC does not make separate requirements due to inadequate support in the literature.
### 1.5 TABLES:

#### Table 1.1: Reported prevalence of clinical signs of PPID in horses and ponies.

<table>
<thead>
<tr>
<th>Clinical Sign</th>
<th>McFarlane et al. (11)</th>
<th>McGowan et al. (61)</th>
<th>Donaldson et al. (44)</th>
<th>Schott et al. (7)</th>
<th>Couëtil et al. (35)</th>
<th>Hillyer et al. (27)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hirsutism</td>
<td>58% (7/12)</td>
<td>100% (20/20)</td>
<td>59% (16/27)</td>
<td>83% (64/77)</td>
<td>95% (21/22)</td>
<td>94% (16/17)</td>
<td>82% (144/175)</td>
</tr>
<tr>
<td>Laminitis</td>
<td>-</td>
<td>80% (16/20)</td>
<td>74% (20/27)</td>
<td>52% (40/77)</td>
<td>59% (13/22)</td>
<td>82% (14/17)</td>
<td>63% (103/163)</td>
</tr>
<tr>
<td>Hyperhidrosis</td>
<td>-</td>
<td>30% (6/20)</td>
<td>26% (7/27)</td>
<td>33% (25/77)</td>
<td>14% (3/22)</td>
<td>59% (10/17)</td>
<td>31% (51/163)</td>
</tr>
<tr>
<td>Weight Loss/Muscle wasting</td>
<td>42% (5/12)</td>
<td>65% (13/20)</td>
<td>15% (4/27)</td>
<td>47% (36/77)</td>
<td>50% (11/22)</td>
<td>88% (15/17)</td>
<td>48% (84/175)</td>
</tr>
<tr>
<td>Abnormal fat distribution</td>
<td>17% (2/12) a</td>
<td>50% (10/20) a</td>
<td>33% (9/27)</td>
<td>29% (22/77)</td>
<td>9% (2/22) a</td>
<td>12% (2/17) a</td>
<td>27% (47/175)</td>
</tr>
<tr>
<td>Behavioral changes</td>
<td>8% (1/12)</td>
<td>95% (19/20)</td>
<td>19% (5/27)</td>
<td>-</td>
<td>41% (9/22)</td>
<td>82% (14/17)</td>
<td>49% (48/98)</td>
</tr>
<tr>
<td>PU/PD</td>
<td>0% (0/12)</td>
<td>55% (11/20)</td>
<td>7% (2/27)</td>
<td>34% (26/77)</td>
<td>32% (7/22)</td>
<td>76% (13/17)</td>
<td>34% (59/175)</td>
</tr>
<tr>
<td>Secondary infections</td>
<td>-</td>
<td>35% (7/20)</td>
<td>30% (8/27)</td>
<td>-</td>
<td>36% (8/22)</td>
<td>48% (8/17)</td>
<td>36% (31/86)</td>
</tr>
</tbody>
</table>

*a* = reports describing only suborbital fat distribution.

It is important to note that the prevalence of many of these clinical signs may be overestimated due to more advanced cases of PPID selected due to the available diagnostic methods.
Table 1.2: Signs and symptoms of human Cushing’s syndrome\textsuperscript{1}.

<table>
<thead>
<tr>
<th>Sign/symptom</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Truncal obesity</td>
<td>96</td>
</tr>
<tr>
<td>Facial fullness</td>
<td>82</td>
</tr>
<tr>
<td>Diabetes or glucose tolerance</td>
<td>80</td>
</tr>
<tr>
<td>Gonadal dysfunction</td>
<td>74</td>
</tr>
<tr>
<td>Hirsutism, acne</td>
<td>72</td>
</tr>
<tr>
<td>Hypertension</td>
<td>68</td>
</tr>
<tr>
<td>Muscle weakness</td>
<td>64</td>
</tr>
<tr>
<td>Skin atrophy and bruising</td>
<td>62</td>
</tr>
<tr>
<td>Mood disorders</td>
<td>58</td>
</tr>
<tr>
<td>Osteoporosis</td>
<td>38</td>
</tr>
<tr>
<td>Oedema</td>
<td>18</td>
</tr>
<tr>
<td>Polydipsia, polyuria</td>
<td>10</td>
</tr>
<tr>
<td>Fungal infections</td>
<td>6</td>
</tr>
</tbody>
</table>

The group consisted of 302 patients, 239 females and 63 males, with an average age of 38.4 years (SD: 13.5; range 8-75)

\textsuperscript{1} Adapted from Boscaro et al. (30)
Table 1.3: Characteristics of human, canine and equine Cushing’s disease \(^1\).

<table>
<thead>
<tr>
<th>Clinical Sign</th>
<th>Human</th>
<th>Canine</th>
<th>Equine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatigue</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>Weight Gain</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Truncal obesity</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Muscle Atrophy</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Osteoporosis</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hypertension</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Polyuria/polydipsia</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Thinning of skin</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Easy bruising</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hirsutism</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Alopecia</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Hypercortisolism</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^1\) Adapted from de Bruin et al. (31)
Table 1.4: Proxies adapted for basal glucose (mg/dL) and insulin (mU/L) concentrations.

<table>
<thead>
<tr>
<th>Proxy</th>
<th>Full name</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOMA-IS</td>
<td>Homeostasis model assessment- insulin sensitivity</td>
<td>$\frac{22.5}{(G \times I)}$</td>
</tr>
<tr>
<td>QUICKI</td>
<td>Quantitative insulin sensitivity check index</td>
<td>$(\log(G \times I))^{-1}$</td>
</tr>
<tr>
<td>RISQI</td>
<td>Reciprocal of the square root of insulin</td>
<td>$I^{-0.5}$</td>
</tr>
<tr>
<td>MIRG</td>
<td>Modified insulin-to-glucose ratio</td>
<td>$\frac{(800 - 0.30 \times (I-50)^2)}{(G-30)}$</td>
</tr>
</tbody>
</table>

$G =$ glucose (mg/dL); $I =$ insulin (mU/L)

\(^1\)Adapted from Treiber et al. (96)
Figure 1.1: Schematic drawing showing the four lobes of the pituitary gland. The pars intermedia is the region affected when a horse has PPID.
Figure 1.2: Schematic drawing illustrating the production of hormones in the pituitary gland of the horse. Both the corticotropes of the pars distalis and the melanotropes of the pars intermedia produce POMC. In the pars distalis, PC1 converts POMC to ACTH and β-lipotropin. In the pars intermedia, PC1 functions similarly, but PC2 is also present to further cleave ACTH to α-MSH and CLIP and cleave β-LPH to β-END. In a horse affected by PPID, PC1 activity increases 4-5 fold and PC2 activity increases 2-3 fold. These changes cause ACTH to be produced at a faster rate than it can be cleaved and thus there is a net production of ACTH in the pars intermedia. (Adapted from Dybdal et al. (39))
Figure 1.3: Schematic drawing of the effects of insulin secretion on the body. Insulin secretion increases glucose uptake, glycolysis, glycogen synthesis, protein synthesis and ion uptake. When insulin is secreted, gluconeogenesis, glucogenolysis, lipolysis, ketogenesis and proteolysis decrease.
Figure 1.4: Schematic represents the output measures of the minimal model used to interpret the FSGTT. $S_g$ represents glucose effectiveness (glucose-mediated glucose disposal) and $X$ represents insulin action (insulin-mediated glucose disposal). $\text{AIR}_g$ represents the acute insulin response to glucose in the first 10 minutes after glucose infusion. Insulin sensitivity ($S_i$) is calculated as $p_3/p_2$ where $p_3$ is the contribution of plasma insulin to the remote compartment and $p_2$ is the fractional rate of insulin clearance from the remote compartment (Adapted from (99, 197))
Figure 1.5: Schematic drawing of the mTOR pathway. Briefly, insulin stimulates the phosphorylation and activation of Akt, which leads to activation of mTOR by inhibiting negative regulators of mTOR known as PRAS40 and TSC2. mTOR activates S6Ks and inhibits 4E-BPs leading to protein synthesis.
Figure 1.6: Schematic drawing of the relationship between protein synthesis and breakdown. The mTOR signaling pathway is responsible for some of the signaling associated with muscle atrophy. Akt inhibits the transcriptional upregulation of Atrogin-1 and MURF1 via inhibition of the FoxO family of transcriptional factors. Multiple factors can induce muscle atrophy including TNF-α and IL-1. Briefly, TNF-α activates NF-κB, which leads to the activation of MuRF1. A second ligase atrogin-1 is upregulated by MAPK p38 (Adapted from Glass (162)).
Figure 1.7: Schematic drawing of whole body phenylalanine kinetics. Entering the pool dietary intake (I) is measured phenylalanine in the feed and release of phenylalanine from protein degradation (B) is measured by difference of phenylalanine flux and I. Leaving the pool phenylalanine oxidation to CO$_2$ (E) is measured by collecting breath samples and phenylalanine use in protein synthesis (Z) is measured by subtracting phenylalanine oxidation from flux. Conversion to other metabolites (M) is assumed to be negligible assuming Tyr intake is equal in all treatment groups. Therefore, phenylalanine flux equals the rate phenylalanine enters (I+B) and exits (E+Z+M) the free amino acid pool. Flux is calculated from the dilution of the isotope by unlabeled phenylalanine in the plasma.
Chapter II
Rationale and Objectives

2.1 SCOPE OF THESIS

The overall objectives of the presented research are to examine the effects of PPID on protein metabolism and insulin sensitivity. The horses used in these studies were aged horses older than 20 years of age and consisted of horses with PPID and age-matched, non-PPID controls. Stable isotope infusion was used to measure whole-body protein metabolism and euglycemic hyperinsulinemic clamp (EHC) procedures were used to measure insulin sensitivity. Additionally, muscle biopsies were collected in both studies to examine the effects of PPID on the mTOR signaling pathway of protein synthesis and markers in the protein degradation pathways.

2.2 RATIONALE

Estimates of the prevalence of PPID vary greatly, with high estimates suggesting 15 to 30% of aged horses are affected by PPID (8). While PPID may affect a large number of horses, few studies have been conducted in order to understand the clinical signs associated with this multi-faceted condition.

The effect of PPID on whole body muscle protein synthesis has not been looked at in the current literature. Studies of horses with PPID have found that horses with PPID have increased proteolysis, atrophied type 2 muscle fibers, sarcoplasmic lipid accumulation and increased variation in myofiber size compared to age matched controls (16, 17). It is possible that the muscle atrophy associated with these horses may be a
result of decreased whole body protein synthesis due to decreased protein synthesis and increased protein breakdown. Therefore, it is also important to look at some of these factors associated with protein synthesis and breakdown in order to better characterize muscle changes associated with PPID.

Further, the relationship between PPID and insulin sensitivity has not been well characterized. Two studies found horses with PPID have decreased insulin sensitivity compared to younger cohorts (18, 19); however, the difference in insulin sensitivity between horses with PPID and age matched controls has not been studied. Understanding the relationship between insulin sensitivity and PPID will be essential to better management of horses with PPID.

Beyond just looking at insulin sensitivity in the horse, it is well known that insulin affects a variety of metabolic functions in the body. One example of this is insulin stimulating muscle protein synthesis through the mTOR pathway. Since horses with PPID are believed to have muscle atrophy, specifically in the form of decreased type 2 fibers (17); it was important to understand the relationship between insulin response and muscle protein synthesis. For this reason, muscle biopsies were taken both before and after the euglycemic hyperinsulinemic clamp procedure and analyzed for factors associated protein synthesis and degradation in order to better characterize the effects of insulin on a muscle level.
2.3 SPECIFIC HYPOTHESES AND OBJECTIVES

2.3.1 Hypothesis 1.

**Hypothesis 1a:** Horses with PPID will have lower rates of whole body protein synthesis when compared to aged, non-PPID horses. **Hypothesis 1b:** Horses with PPID will have decreased gluteal muscle mTOR signaling, following feeding, compared to aged, non-PPID horses. **Hypothesis 1c:** Horses with PPID will have an increase in protein degradation factors, following feeding, when compared with aged, non-PPID horses.

The objective of the first study entitled, "Pituitary pars intermedia dysfunction does not alter whole-body and muscle protein metabolism in aged horses", was to determine the effect of PPID on whole body protein synthesis and the pathways of muscle protein synthesis and degradation when compared to aged, non-PPID controls. In order to achieve this objective, whole body protein synthesis was assessed using isotope infusion techniques (Hypothesis 1a). Additionally, a gluteal muscle biopsy was performed 90 minutes post feeding on the day prior to the isotope infusion procedures. The muscle samples were prepared and analyzed for factors associated with mTOR signaling (Hypothesis 1b) and protein degradation (Hypothesis 1c) using Western blotting.

2.3.2 Hypothesis 2.

**Hypothesis 2a:** Horses with PPID will have decreased insulin sensitivity when compared to aged, non-PPID horses. **Hypothesis 2b:** Horses with PPID will have decreased mTOR signaling, in response to insulin administration, when compared to aged, non-PPID horses. **Hypothesis 2c:** Horses with PPID will have increased protein
degradation factors, in response to insulin administration, when compared to aged, non-PPID horses.

The objective of the second study entitled, “Pituitary pars intermedia dysfunction does not affect insulin sensitivity in aged horses”, was to determine if horses with PPID had reduced insulin sensitivity compared to age-matched, non-PPID controls. In order to achieve this objective, insulin sensitivity was assessed using a euglycemic-hyperinsulinemic clamp procedure and calculation of two proxies, RISQI and MIRG (Hypothesis 2a). Additionally, gluteal muscle biopsies were performed on the day prior and immediately after the EHC procedure. The muscle samples were prepared and analyzed for factors associated with mTOR signaling (Hypothesis 2b) and protein degradation (Hypothesis 2c) using Western blotting.
Chapter III
*Pituitary pars intermedia dysfunction does not alter whole-body and muscle protein metabolism in old horses.*

3.1 INTRODUCTION

Pituitary pars intermedia dysfunction (PPID; also known as equine Cushing’s disease) is believed to affect 15-30% of aged horses (1, 2). The disease results from the loss of dopaminergic inhibition of the pituitary pars intermedia leading to increased synthesis of proopiomelanocortin-derived peptides, α-melanocyte-stimulating hormone, β-endorphin-related peptides, corticotropin-like intermediate lobe peptide and adrenocorticotropic hormone (ACTH) (9). Clinical signs of PPID include hypertrichosis (198), laminitis (41), increased secondary infections (36), decreased insulin sensitivity (19), and muscle atrophy (17).

In human studies, muscle atrophy has been associated with decreased whole-body protein synthesis (199). In human patients with Cushing’s disease, whole-body protein metabolism was altered due to increased protein breakdown, presumably due to changes in body composition and glucocorticoid excess associated with Cushing’s disease (200). In horses with PPID, basal cortisol levels are generally within the normal references range (14, 15); however, it has been hypothesized that the cortisol circadian rhythm in horses with PPID may be abolished leading to increased cumulative cortisol over a 24-hour period (39). Therefore, it is unclear if similar alterations in whole-body protein metabolism will be seen in horses with PPID compared to control horses. Isotope infusion techniques to measure whole-body protein metabolism have been used extensively in humans studies (187) and they have recently been validated for use in the
horse (188). Studies in the horse have looked at the effects of old age on whole-body protein synthesis; however, no differences were seen in whole-body protein metabolism between the healthy aged (~ 25 yrs) and mature (~11 yrs) horses (201). Even though age had no effect on whole-body protein metabolism, it may be possible that differences in protein metabolism exist within an aged population, such as horses with PPID, where a loss of muscle mass has been previously described (17).

Muscle atrophy occurs when the rate of protein degradation exceeds the rate of protein synthesis. PPID is associated with muscle loss due to atrophy of type 2A and 2B muscle fibers and loss of type 2B myofibers (17). The overall increase in glucocorticoids proposed in horses with PPID (39) may lead to decreased protein synthesis and increased protein breakdown at the muscle level. Horses with PPID do have increased expression of a factor, m-calpain, which is part of the non-lysosomal calcium protease-dependent system associated with protein breakdown (16). The results from the previous study suggest that calpains may be associated with muscle atrophy that occurs in horses with PPID; however, whether the pathways of protein synthesis or other pathways of protein breakdown are involved still requires elucidation.

Protein synthesis is regulated through the mechanistic (formally mammalian) target of rapamycin (mTOR) signaling pathway (106) and previous studies of the horse have looked specifically at the factors, protein kinase B (Akt), eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), and riboprotein S6 (rpS6), which when phosphorylated lead to an increase in translation initiation and subsequently protein synthesis (202). Akt activation also plays a role in protein degradation as it has been shown to phosphorylate and inactivate nuclear transcription factors Forkhead box O (FoxO) (203). When FoxO is
activated, it increases the expression of key genes expressed during proteosomal protein degradation, atrogin-1 and muscle RING finger 1 (MuRF1) (203). Muscle atrophy in the horse may also be influenced by decreased protein synthesis due to upregulation of negative regulators of protein synthesis, AMP-activated protein kinase (AMPK) (204) and myostatin (155).

The objective of this study was to compare whole-body protein metabolism in horses with PPID compared to age-matched, non-PPID controls. Further, the study aimed to characterize differences between PPID and age-matched, non-PPID horses in the activation of signaling pathways associated with muscle protein synthesis and breakdown in response to feeding.

3.2 MATERIALS AND METHODS

3.2.1 Animals, housing, diets.

All procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee (2009-0562). Twelve horses consisting of six horses with PPID and six age-matched, non-PPID controls (CON) were obtained from the Department of Veterinary Science’s Maine Chance Farm at University of Kentucky. Horses in the two groups were of comparable age (PPID: 25.0±2.5 yrs; mean±SD; CON: 25.7±2.0 yrs) and weight (PPID: 542±35 kg; CON: 500±50 kg). Additionally, both groups were comprised of 4 mares and 2 geldings and all horses were of moderate body condition score (PPID: 5.5±0.5 CON: 5.0±0.7; [scale 1-9] (205)).
Since the horses used in this study could not be evaluated post-mortem to assess the presence of pituitary lesions, two diagnostic methods were used to determine PPID status with the greatest possible accuracy (40). Horses were confirmed as PPID based on resting ACTH greater than 50 pg/mL and serum cortisol greater than 1 μg/dL 19-20 hours after dexamethasone administration. Horses classified as PPID based on these methods had many of the clinical signs consistent with PPID, such as hirsutism and hair coat abnormalities, hyperhydrosis, muscle atrophy, and a history of laminitis. In April 2012, blood samples were collected in EDTA tubes (Vacutainer; Becton-Dickinson, Franklin Lakes NJ) and analyzed for ACTH concentration (PPID: 103.6±73.7 pg/mL; CON: 23.3±8.2 pg/mL) using a hormone assay performed by an external laboratory (Animal Health Diagnostic Center; Cornell University, Ithaca, NY). In May 2012, dexamethasone suppression tests were performed according to previously described procedures (39) where blood samples were collected into glass evacuated tubes containing no additive (Vacutainer; Becton-Dickinson, Franklin Lakes NJ) before and 19-20 hours post intramuscular administration of dexamethasone (DexaJect; Butler Schein Animal Health, Dublin OH ) at 0.04 mg/kg. Serum cortisol was then analyzed using a hormone assay by an external laboratory (Animal Health Diagnostic Center; Cornell University, Ithaca, NY).

Horses were adapted to diets and housing for at least 2 weeks prior to the isotope infusion procedures. All horses were housed in a single paddock with free access to mixed grass hay (mean ± SD, as fed; 10.1± 0.1% crude protein, 33.0± 0.6% acid detergent fiber, 50.9± 0.6% neutral detergent fiber, 2.7± 0.4% fat, and 6.5± 0.4% ash), water, and salt blocks. Twice daily at 0700 and 1500, horses were fed a commercial
concentrate designed for senior horses (mean ± SD, as fed; 14.1± 0.1% crude protein, 22.5± 0.7% acid detergent fiber, 37.2± 1.3% neutral detergent fiber, 6.2± 0.2% fat, and 7.4± 0.2% ash) (Purina® Equine Senior® Horse Feed, Purina Mills LLC, Gray Summit, MO) individually in stalls (12g/kg BW/day, split between two meals), with the exception of study days where the feeding procedure was modified as described below. Analysis of the commercial concentrate and hay were performed by an external laboratory (Dairy One Forage Laboratory; Ithaca NY).

3.2.2 Study procedures.

On the morning prior to the isotope infusion procedures, horses were weighed and blood was collected immediately before (t=0) and 90 minutes after their 0700 meal to measure postprandial changes in plasma glucose, insulin and amino acid concentrations. According to previous research, horses reach peak amino acid concentration 90 to 180 minutes following a meal (206).

The following morning, a catheter (14 gauge × 14.0 cm, Abbocath; Abbott Laboratories, North Chicago IL) was inserted into each jugular vein: one for isotope infusion and a second for blood collection. In order to determine total CO\textsubscript{2} production, each horse received a 2 h primed (7.1 μmol/kg BW), constant (6.0 μmol/kg BW/h) infusion of a [\textsuperscript{13}C]sodium bicarbonate solution using a cordless IV pump attached to a surcingle (J-1097 VetPro Infusion Pump, Jorgensen Laboratories Inc., Loveland, CO) attachd to a surcingle worn by the horse. The sodium bicarbonate solution was prepared by dissolving [\textsuperscript{13}C]sodium bicarbonate (Isotec, Miamisburg, OH) in 0.9% sterile saline, which was then transferred into a sterile ethylene vinyl acetate bag (Baxter Healthcare,
Breath samples were taken at -30, -60, 0, 30, 60, 75, 90, 105 and 120 minutes of sodium bicarbonate infusion. Breath samples were obtained using a modified Equine Aeromask® (Trudell Medical International, London ON, Canada) with a reversed one-way valve to allow air to be trapped in gas impermeable bags (Wagner Analysen Technik Vertriebs GmbH, Bremen, Germany). To collect breath samples, horses wore the mask for one minute prior to sample collection to allow the air to equilibrate inside the mask. Then the bag was attached to the one-way valve and was removed and capped once it became full. During sodium bicarbonate infusion, baseline blood samples for phenylalanine enrichment were taken at 90 and 120 minutes into the infusion.

At the end of the two hours, sodium bicarbonate infusion was stopped and a second isotope infusion began immediately. Each horse received a 4-hour primed (10.2 μmol/kgBW), constant (7.2 μmol/kgBW/h) infusion of L-[1-13C] phenylalanine (Isotec, Miamisburg, OH) in order to measure whole-body phenylalanine kinetics (207). The 1.5 prime to constant ratio was based on the findings of a previous study in horses (188). The preparation and administration of the infusion solution and the breath sampling protocols were as described above for the [13C]sodium bicarbonate infusion. Blood and breath samples were taken and feed was given every 30 minutes after the start of isotope infusion.

On the day of the isotope infusion procedures, horses received 1/48 of their daily concentrate allocation every 30 minutes, beginning at least 90 minutes prior to the start of the [13C]sodium bicarbonate infusion. To minimize changes in plasma phenylalanine concentration as a result of the isotope infusion, the meals given prior to the start of [1-13C]phenylalanine isotope infusion were top dressed with 0.6 mg/kg (3.6
μmol/kg BW) of L-phenylalanine powder (L-phenylalanine, Sigma-Aldrich, St. Louis MO).

3.2.3 Muscle biopsy.

Immediately prior to the biopsy, horses were lightly sedated with xylazine hydrochloride (0.5 mg/kg; AnaSed; Lloyd, Shenandoah IA) administered IV. The muscle biopsy was performed in accordance to previously described procedures (115) where a Bergstrom needle was used to aseptically collect muscle (~500 mg) from the gluteus medius muscle. Following the biopsy, horses were given 2 g of the non-steroidal anti-inflammatory phenylbutazone paste (ButaPaste; Butler Animal Health Supply, Dublin OH), with additional doses given 24 and 48-hours later. After biopsy, horses were returned to their stalls to be monitored. There were no incidences of biopsy site infections, with the exception of minor swelling, in any horses in this study.

3.2.4 Plasma analysis.

The blood samples were transferred into evacuated glass tubes (Vacutainer; Becton-Dickinson, Franklin Lakes NJ) coated with sodium heparin and refrigerated (4°C) immediately until centrifuged at 1,000 x g at 4°C and plasma was aliquoted and stored at -20°C. The two plasma samples taken before and after feeding were analyzed using an automated analyzer (YSI 2300 STAT Plus™ Glucose and Lactate Analyzer, YSI Inc., Life Sciences, Yellow Springs, OH) to measure plasma glucose concentrations enzymatically and a commercially available kit (Coat-A-Count RIA® kit Siemens, Healthcare Diagnostics Inc., Deerfield, IL) to measure plasma insulin concentration. The kit used has been previously validated for use in horses (208, 209). Additionally, plasma
free amino acid concentrations were measured for these samples by HPLC analysis of phenyisothiocyanate derivatives, as described previously (115).

Plasma samples collected during the isotope infusion procedures were analyzed by an external laboratory (Metabolic Solutions, Nashua, NH) for the isotopic enrichment of a t-butyldimethylsilyl derivative of phenylalanine (m+1) using GC-MS as previously described (201, 210).

3.2.5 Breath sample analysis.

On the day of isotope infusion, the ratio \(^{13}\text{CO}_2 : ^{12}\text{CO}_2\) in exhaled breath samples was determined using an isotope selective non-dispersive infrared absorption (NDIR) analyzer (IRIS-2; Wagner Analysen Technik Vertriebs, Bremen, Germany).

3.2.6 Western immunoblot analysis.

Muscle homogenates were prepared according to previously described procedures (116, 202). Briefly, following the muscle biopsy collection, a portion of muscle (~100 mg) was homogenized in a lysis buffer. Homogenates were centrifuged at 10,000 x g for 10 min at 4°C and the supernatant was removed and frozen at -80°C until the time of analysis. Protein content of the supernatant was determined using a Bradford assay (Bradford Reagent, Sigma-Aldrich, St. Louis MO) and all samples were diluted in a Laemmli to a 2 μg/μL concentration to be loaded into polyacrylamide gels. The proteins were separated via electrophoresis, transferred to polyvinylidene difluoride membranes and incubated for 1 hr in a 5% fat free milk solution with the exception of myostatin, which was incubated in a 5% BSA solution. Next, membranes were incubated with the
following primary antibodies: Akt (total and phosphorylated Ser\textsuperscript{473}; 1:2000 dilutions for each; Cell Signaling Technology, Beverly MA), rpS6 (total and phosphorylated Ser\textsuperscript{235/236} and Ser\textsuperscript{240/244}; 1:10000 and 1:2000 of each antibody dilutions, respectively; Cell Signaling Technology, Beverly MA), 4E-BP1 (total and phosphorylated; 1:1000 dilutions of each; Cell Signaling Technology, Beverly MA), AMPK\(\alpha\) (total and phosphorylated Thr\textsuperscript{172}; 1:1000 dilutions of each; Cell Signaling Technology, Beverly MA), FoxO1 (total and phosphorylated Ser\textsuperscript{256}; 1:4000 and 1:5000 dilutions, respectively; Santa Cruz Biotechnology, Dallas TX), NF-\(\kappa\)B p65 (total and phosphorylated Ser\textsuperscript{536}; 1:4000 and 1:1000 dilutions, respectively; Cell Signaling Technology, Beverly MA), MuRF1 (total; 1:5000 dilution; ECM Biosciences, Versailles KY), atrogin-1 (total; 1:1000 dilution; ECM Biosciences Versailles, KY), myostatin (total; 1:10,000 dilution; abcam, Cambridge MA) and \(\alpha\)-tubulin (total; 1:1000; Cell Signaling Technology, Beverly MA). Membranes were then washed and incubated with a goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (BioRad, Hercules CA) for 1 hr at 20\(^{\circ}\)C. Following washing, membranes were developed with a chemiluminescent kit (Abersham ECL Plus Western Blotting Detection System; GE Healthcare, Piscataway NJ) and developed using a film processor (Kodak X-OMAT film processor, Kodak Health Imaging Division, Rochester, NY). After developing, membranes were stripped and re-probed with a second antibody in order to account for the total amount of the protein of interest. In the case of phosphorylated proteins, the antibody recognized the total form of the protein. \(\alpha\)-tubulin, which has previously been used to standardize protein abundance in equine skeletal muscle (211), was used to standardize the abundance of MuRF1, atrogin-1 and myostatin, because only the total abundance of protein was measured. Band densities were measured
using a densitometric computer software (ImageJ, National Institutes of Mental Health, Bethesda MD). The abundance of phosphorylated forms of Akt, S6K1, rps6, 4E-BP1, AMPK, NF-κB and FoxO1 was corrected for the density of the total protein band and the value for the control treatment was set at 1.0 arbitrary units. The abundance of MURF1, atrogin-1 and myostatin were determined and compared to abundance of α-tubulin in order to correct for total protein. All gels were run in duplicate.

3.2.7 Calculations.

3.2.7.1 Plasma phenylalanine enrichment

Plasma enrichment of [1-13C] phenylalanine, in molecules percent excess, was calculated using a previously described formula (212).

3.2.7.2 Breath CO₂ enrichment

For the breath samples collected during the [13C] sodium bicarbonate infusion, total CO₂ production was calculated based on breath CO₂ enrichment using a previously published formula (213). For all breath samples, the δ enrichment values obtained from the nondispersive infrared absorption analyzer was converted to atoms percent excess using a previously described formula (213).

3.2.7.3 Primed, constant infusion, whole-body phenylalanine kinetics

During phenylalanine enrichment, a plateau, defined as at least 4 values having a slope not significantly different from 0 (P > 0.05) was determined using linear regression analysis (GraphPad Prism 4 Software). The average plasma enrichment at isotopic steady
state (plateau) was used to calculate whole-body phenylalanine kinetics. The plateau enrichment values were then used to calculate the whole-body phenylalanine flux using previously described formulas in the horse (188). Briefly, flux is equal to the amount of phenylalanine entering the plasma pool through dietary intake (I), de novo synthesis (N) and protein breakdown (B) or leaving the pool through protein synthesis (Z), oxidation (E) or conversion to other metabolites (M):

\[ Q = I + N + B = Z + E + M. \]

Intake was determined by multiplying dietary phenylalanine intake by 0.5 to account for prececal phenylalanine digestibility (214) and the dietary phenylalanine that is extracted during first-pass splanchnic metabolism in other monogastric species (215, 216). In the horse, phenylalanine is an indispensible amino acid, so de novo synthesis does not occur; therefore protein breakdown can be estimated by:

\[ B = Q - I. \]

Phenylalanine oxidation was calculated using the equations previously described (191). Non-oxidative phenylalanine metabolism can be calculated by taking the difference between phenylalanine flux and oxidation and is used as an indication of whole-body protein synthesis. The major non-CO₂ product of phenylalanine metabolism is tyrosine; however, in the current study, the only source of dietary tyrosine was the pelleted complete feed, which all of the horses received at the same intake level corrected for body weight. Therefore, any reduction in whole-body phenylalanine oxidation would suggest an increase in phenylalanine use for whole-body protein synthesis.
3.2.8 Statistical analysis.

All data were analyzed using the mixed procedure of SAS (version 9.3; SAS Institute; Cary NC) and data were considered significant at P < 0.05. Statistical trends were considered at 0.05 < P <0.10.

Analyses of pre-feeding and post-feeding plasma metabolite concentrations were assessed using a repeated-measures analysis, with group, time, and the interaction between group and time as the fixed effects and horse nested in group as the random effects. The variance-covariance matrix was chosen for each repeated-measures analysis based on the lowest value for Schwarz’s Bayesian Criterion. Phenylalanine kinetics parameters and western immunoblot data were analyzed using a 1-way ANOVA with PPID status (group) as the fixed effect and horse nested in group as the random effect. All data are presented as least square means and pooled standard errors.

3.3 RESULTS

3.3.1 Plasma insulin, glucose, and amino acids.

Plasma insulin, glucose, and amino acid concentrations were measured prior to feeding and 90 minutes post-feeding. Plasma insulin (Table 3.1) and glucose (Table 3.1) concentrations increased post-feeding (P< 0.01). Plasma glucose concentration was higher post-feeding in the PPID horses compared to the CON horses (P= 0.02); however, plasma insulin concentrations were not different between PPID and CON horses. There was no effect of the interaction between time and PPID status on either plasma glucose or
insulin concentrations (P> 0.05). Plasma amino acid concentrations were not affected by
time, PPID status or the interaction between time and PPID status (P > 0.05) (Table 3.1).

3.3.2 Western immunoblot data.

There was no effect of PPID status on the post-feeding activation of Akt at Ser\textsuperscript{473}
(P= 0.93; Figure 3.1), rpS6 at Ser\textsuperscript{235/236 \& 240/244} (P= 0.20; Figure 3.1), 4E-BP1 at Thr\textsuperscript{37/46}
(P= 0.36; Figure 3.1), NFkB p65 at Ser\textsuperscript{536} (P= 0.48; Figure 3.2), AMPK\alpha at Thr\textsuperscript{172} (P=
0.54; Figure 3.3), myostatin (P= 0.27; Figure 3.3), FoxO1 (P= 0.85; Figure 3.4), MuRF1
(P= 0.67; Figure 3.4) or atrogin-1 (P= 0.53; Figure 3.4) (P > 0.05).

3.3.3 Whole-body phenylalanine kinetics.

There was no effect (P > 0.05) of PPID status on carbon dioxide production,
phenylalanine flux, phenylalanine intake, phenylalanine oxidation, phenylalanine release
from protein breakdown and non-oxidative phenylalanine disposal (Table 3.2).

3.4 DISCUSSION

Although a previous study examined some of the pathways associated with
muscle protein breakdown in horses with PPID compared to age-matched controls (16),
to the best of the authors’ knowledge, this was the first study to compare whole-body
protein synthesis and mTOR signaling between horses with PPID and age-matched, non-
PPID horses. The current study showed that despite an increase in plasma insulin and
glucose concentrations post-feeding, there was no concurrent increase in the plasma
concentration of any of the indispensable amino acids 90 minutes following a meal.
There were no differences in whole-body protein kinetics between the PPID and CON horses, nor were differences seen in the abundance of any of the signaling factors associated with protein synthesis and degradation that were studied. These findings suggest that although PPID status does not affect whole-body protein metabolism or any of the signaling pathways studied, advanced age may lead to a decreased ability to release amino acids into the bloodstream post-feeding.

The only significant difference that was measured between the PPID and CON groups in this study was that the PPID horses had a higher plasma glucose concentration in response to feeding when compared to the CON horses, although pre-feeding values were not different between the groups. Despite the differences in glucose concentration, there were no differences in basal insulin concentrations or insulin concentrations post-feeding between the two groups of aged horses. This suggests that the PPID horses may some dysregulation in insulin-mediated glucose uptake, since similar concentrations of insulin were not able to stimulate glucose uptake as effectively in the PPID horses. Previous studies have suggested increased insulin resistance in horses with PPID (18, 19) and increased incidence of hyperinsulinemia (1); however, in all of these previous studies the control group was much younger than the PPID horses due to the nature of PPID affecting primarily aged horses. Differences in insulin sensitivity may help to explain some of the associations between muscle atrophy and PPID. Understanding differences in insulin sensitivity in horses with PPID compared to age-matched, non-PPID controls is an area where additional research is warranted.

In the current study, plasma glucose and insulin concentrations at 90 minutes post-feeding were increased compared to pre-feeding levels; however, feeding did not
result in an increase in the plasma concentrations of any of the indispensable amino acids. Previous research in horses has shown that plasma amino acid concentrations reach peak concentrations around 90 minutes post-feeding (206); therefore the lack of postprandial increase in the plasma amino acids at the t= 90 min sample in the current study was unexpected. We have previously shown that feeding of a high protein meal (1.32g protein/kg BW, divided between 2 meals which were separated by 30 minutes) resulted in large increases in plasma glucose, insulin and amino acids concentrations and also resulted in an increase in the activation of mTOR signaling factors in the skeletal muscle of both mature (115, 116) and growing horses (116). The current study provided 0.86 g/kg BW protein in the meal, which is lower than the previous studies of mTOR signaling in horses (115, 116); however, it is greater than in another previous study (0.55 g protein/kg BW) (188) in mature horses where increases in plasma amino acid concentrations were measured at 120 min post-feeding. Therefore, based on the literature, it appears unlikely that the timing of the postprandial sample was not appropriate or that the amount of protein fed was inadequate to measure the expected postprandial increase in plasma amino acids concentrations.

It is possible that the lack of a postprandial increase in plasma amino acid concentrations is a function of advanced age of the horses studied (~25 years) and therefore affected both groups of horses in the current study. This hypothesis is consistent of studies in old rats, which revealed that age alters splanchnic extraction of amino acids. In aged rats, arterial plasma concentrations of AA were lower (20-30% less on average) when compared to adult rats infused with amino acids (217). In horses, there is some evidence to support that protein digestion may decrease with advanced age. In an early
study, apparent crude protein digestion was reduced in aged horses (~25 yrs) when compared with younger horses (~2 yrs) (195); however, in later studies of aged horses, protein digestion was within the normal range of apparent protein digestion reported for younger horses (196). Therefore, it is difficult to speculate whether the lack of postprandial increase in plasma amino acid concentrations in the current study was due to age-related alterations in dietary protein digestibility. The lack of increase in plasma amino acid concentrations in these old horses is of interest because it could affect the amount of anabolic stimuli that is present to stimulate the postprandial increase in muscle protein accretion. The underlying cause of the lack of postprandial increase in plasma amino acid concentrations in the aged horses in the current study warrants additional research.

The muscle atrophy that has been described in horses with PPID may be due to increased circulating inflammatory cytokines, because these have been shown to stimulate protein breakdown (178). In the horse, inflammation has been shown to increase with advancing age as part of a process known as inflamm-aging (218). Interestingly, horses with PPID have been shown to have decreased (219) or similar (16) expression of several inflammatory cytokines when compared to healthy aged-matched controls. In the present study, inflammation was not measured directly, but no differences were found in the relative abundance of phosphorylated NF-κB between the PPID and CON. The phosphorylation and activation of NF-κB is stimulated by inflammatory cytokines (179), thus it is unlikely that there was a difference in inflammatory signaling in the skeletal muscle between the two groups of aged horses in the current study.
In studies of aging in humans, some studies have found that the rates of whole body muscle protein synthesis declined with age (187), whereas other studies have found that aging does not alter whole-body muscle protein synthesis (192). The authors hypothesized that the lack of difference in protein anabolic responses may have been due to similar muscle mass index and level of physical activity between the elderly and young group (192). It has been demonstrated that up to 87% of the variance seen in whole-body protein kinetics is due to differences in fat free mass (187). In the current study, fat free mass was not measured directly, but both groups were of moderate body condition score suggesting differences in fat-free mass between the two groups was minimal. This is consistent with another study where no differences in whole-body protein metabolism were measured between aged and mature horses of similar body condition scores (201). In the current study, no significant differences were seen in any of the measures of whole-body protein kinetics between the PPID and CON groups. These results differ from what would be expected based on previous studies that have shown increased muscle atrophy and increased expression of a factor associated with protein breakdown pathway non-lysosomal calcium protease-dependent systems. The results from the current study also differ from human studies where protein breakdown was increased in patients with Cushing’s disease (200), further highlighting the differences between human Cushing’s disease and PPID.

It is possible that no differences in whole-body protein kinetics were seen due to the horses that were included in this study. All of the aged horses, regardless of PPID status, included in the present study were relatively healthy, capable of being managed in a group setting and did not exhibit visible signs of the extreme losses in fat free mass that
can occur with age. It is possible that if more advanced cases of PPID had been included in the current study, differences in whole-body protein metabolism may have been measured between the PPID and CON groups. Whole-body protein kinetics only give information about overall rates of protein synthesis and breakdown occurring in the body, but do not provide specific information about the contribution of the individual tissues. Therefore, although no differences in whole-body protein synthesis or breakdown were seen in the present study, it is still possible that changes may exist on the muscle level and thus the activation of the signaling pathways were studied in the skeletal muscle.

Protein synthesis is regulated through the mTOR pathway. Briefly, Akt is activated by insulin and subsequently activates downstream factors of mTOR signaling, rps6 and 4E-BP1, leading to protein synthesis. Glucocorticoids, which have been hypothesized to be elevated over a 24-hr period in horses with PPID, have been shown to decrease Akt phosphorylation in the horse (211). In the current study, no differences were seen between the PPID and CON groups in the phosphorylation of any of the mTOR signaling factors studied, suggesting that PPID status does not alter muscle protein synthesis in response to feeding in the horse. Protein synthesis is stimulated by insulin and negatively affected by glucocorticoid excess and this suggests that insulin sensitivity and cumulative daily glucocorticoids may not be different between the two groups in the current study, although this was not measured.

Myostatin acts as a negative regulator of skeletal muscle mass by inhibiting activation of factors associated with mTOR signaling (155). Dexamethasone administration, which has been shown to decrease skeletal muscle mass (152), leads to increased myostatin expression in rats (153). In the current study, no differences were
seen in muscle abundance of myostatin, which is consistent with previous studies of PPID horses compared to age-matched controls (16). Another negative regulator of protein synthesis is AMPK, a sensor of cellular energy, that is activated by rising AMP levels as a result of energy starvation and results in mTOR inhibition (220). Due to the differences in plasma glucose post feeding in the current study, it would be possible that differences in AMPK phosphorylation could exist between the two groups of horses. However, no differences were seen in phosphorylation of AMPK between the PPID and CON groups, indicating no differences in cellular energy status exist between the two groups of aged horses.

In addition to activating protein synthesis, Akt phosphorylation also has the ability to affect protein breakdown through phosphorylation and subsequent inactivation of FoxO (203), leading to an increase in protein breakdown through the ubiquitin-proteosomal pathway. FoxO phosphorylation has been show to increase the expression of MuRF1 and atrogin-1, two genes highly associated with muscle atrophy (166). Due to the muscle atrophy previously associated with PPID (16), it would be expected to see an increase in factors associated with ubiquitin-proteosomal protein breakdown pathway in the PPID versus CON horses; however, this was not observed in the present study. There were no differences seen between PPID and CON groups in the phosphorylation of FoxO1, which is consistent with the lack of differences in the expression of MuRF1 and atrogin-1. In a previous study of protein breakdown in horses with PPID versus age matched controls, no significant differences were detected the expression of the major proteolytic systems between the two groups with the exception of m-calpain, which had greater expression in horses with PPID (16). Therefore, the results from the current study
generally support the results from the previous study (16) that it is not the ubiquitin-proteosomal pathway of protein breakdown that is affected by PPID status in aged horses.

Alternatively, it is possible that the muscle biopsy at 90 minutes post-feeding did not effectively capture differences in the protein synthesis and breakdown pathways between the two groups of aged horses. Further, the study only looked at the abundance or phosphorylation of these factors post-feeding, so it is possible that response to other anabolic stimuli, such as exercise, may in fact be different between the two groups. In the current study, muscle biopsies were taken at 90 minutes post-feeding based on previous studies of younger horses (115, 116). However, in the current study, no changes were seen in plasma amino acid concentrations post-feeding, indicating 90 minutes may not have been an appropriate time to take the muscle biopsy in aged horses. Since it is known amino acids stimulated mTOR signaling (221), differences in the signaling pathways between the two groups may have been missed. Further, the current study did not take a pre-feeding muscle biopsy, so it is impossible to speculate if the meal was able to stimulate a post-prandial increase in mTOR signaling, as seen in previous studies (115, 116). In human studies, aging has been shown to decrease the responsiveness of mTOR signaling in response to anabolic stimuli, but differences are not always seen in a basal state (222). The inability to stimulate mTOR or suppress protein breakdown in response to feeding could be a potential mechanism to explain muscle loss as seen in aged horses and in particular horses with PPID (17), although additional research would be needed to specifically examine this hypothesis.
3.5 CONCLUSION

The study highlights the importance of understanding amino acid requirements and digestibility in all aged horses, because none of the horses in this study showed an increase in plasma amino acid concentration in response to feeding. Although muscle atrophy has been shown to be associated with PPID, no differences were seen in whole-body protein kinetics between the two groups of aged horses in the current study. Additionally, although there is some evidence that insulin sensitivity may be lower in horses with PPID, it does not seem to affect abundance or phosphorylation of the factors associated with protein synthesis and breakdown that were studied in the muscle. None of the factors associated with protein synthesis and breakdown in the muscle were different between the two groups; supporting the idea that whole-body protein metabolism is not affected by PPID status. The mechanisms underlying the loss of muscle mass in horses with PPID requires additional investigation.
### Table 3.1. Glucose, insulin and amino acid concentrations in PPID and CON horses before (0 min) and 90 min following a meal

<table>
<thead>
<tr>
<th></th>
<th>CON 0 min</th>
<th>CON 90 min</th>
<th>PPID 0 min</th>
<th>PPID 90 min</th>
<th>Pooled SE</th>
<th>Group</th>
<th>Time</th>
<th>Group *Time</th>
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<td>Glucose</td>
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<td>5.35</td>
<td>6.28*</td>
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<td>20.2</td>
<td>61.4*</td>
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</tbody>
</table>

\(^1\)Values are lease square means. \(^*\)Values that differed significantly (P < 0.05) from baseline. \(^a,b\) Values that were significantly different between the CON and PPID group at the same time point.

\(^2\)Plasma glucose concentrations are reported as least squares means in mmol/L

\(^3\)Plasma insulin concentrations are reported as least squares means in µIU/mL.

\(^4\)Plasma amino acid concentrations are reported as least squares means in µmol/L.
Table 3.2: Whole-body phenylalanine kinetics in PPID and CON horses

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>PPID</th>
<th>Pooled SE</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylalanine flux, ( \mu \text{mol-kg}^{-1}\cdot\text{h}^{-1} )</td>
<td>38</td>
<td>39</td>
<td>1</td>
<td>NS</td>
</tr>
<tr>
<td>Carbon dioxide production, ( \mu \text{mol-kg}^{-1}\cdot\text{h}^{-1} )</td>
<td>14</td>
<td>15</td>
<td>1</td>
<td>NS</td>
</tr>
<tr>
<td>Phenylalanine entering the free phenylalanine pool</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylalanine from dietary intake(^3), ( \mu \text{mol-kg}^{-1}\cdot\text{h}^{-1} )</td>
<td>7</td>
<td>7</td>
<td>0</td>
<td>NS</td>
</tr>
<tr>
<td>Phenylalanine from protein breakdown(^2) ( \mu \text{mol-kg}^{-1}\cdot\text{h}^{-1} )</td>
<td>30</td>
<td>31</td>
<td>1</td>
<td>NS</td>
</tr>
<tr>
<td>Phenylalanine leaving the free phenylalanine pool</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylalanine oxidation(^3), ( \mu \text{mol-kg}^{-1}\cdot\text{h}^{-1} )</td>
<td>9</td>
<td>8</td>
<td>1</td>
<td>NS</td>
</tr>
<tr>
<td>Non-oxidative phenylalanine disposal, ( \mu \text{mol-kg}^{-1}\cdot\text{h}^{-1} )</td>
<td>29</td>
<td>31</td>
<td>1</td>
<td>NS</td>
</tr>
</tbody>
</table>

\(^1\) Values are least square means. Labeled means in a row without a common letter differ, \( P < 0.05 \).

\(^*\) Differs from the rate of non-oxidative phenylalanine disposal for that treatment, \( P < 0.05 \).

\(^2\) The following stochastic model of phenylalanine kinetics was used: \( \text{flux} = \text{rate of phenylalanine entry} = \text{rate of phenylalanine leaving} \); \( \text{rate of phenylalanine entry} = \text{phenylalanine intake} + \text{phenylalanine release from protein breakdown} \); \( \text{rate of phenylalanine leaving} = \text{phenylalanine oxidation} + \text{non-oxidative phenylalanine disposal} \).

\(^3\) NS, not significant, \( P > 0.05 \).
Figure 3.1. Postprandial gluteal muscle phosphorylation of factors involved in the upregulation of protein synthesis, Akt at Ser$^{473}$, rpS6 at Ser$^{235/236}$ & Ser$^{240/244}$, and 4E-BP1 at Thr$^{37/46}$ in CON (white bars) and PPID (black bars) horses. The phosphorylated forms of the translation initiation factors were corrected by the respective total form abundance, with the value for CON horses set to 1.0 AU. Values are least square means ± pooled SE, n=6 per group. Representative images of the immunoblots are shown above.
Figure 3.2. Postprandial gluteal muscle phosphorylation of NFκB p65 at Ser$^{536}$, an indicator of inflammatory status, in CON (white bars) and PPID (black bars) horses. The phosphorylated form of NFκB was corrected by the respective total form abundance, with the value for CON horses set to 1.0 AU. Values are least square means ± pooled SE, n=6 per group. Representative images of the immunoblots are shown above.
Figure 3.3. Postprandial gluteal muscle phosphorylation of AMPKα at Thr$^{172}$ and relative abundance of myostatin, negative regulators of protein synthesis, in CON (white bars) and PPID (black bars) horses. The phosphorylated form of αAMPK was corrected by the respective total form abundance, and myostatin abundances were normalized to α-tubulin, with the value for CON horses set to 1.0 AU. Values are least square means ± pooled SE, n=6 per group. Representative images of the immunoblots are shown above.
Figure 3.4. Postprandial gluteal muscle phosphorylation of FoxO1 and relative abundances of atrogin-1 and MuRF1 in CON (white bars) and PPID (black bars) horses. The phosphorylated form of FoxO1 was corrected by the respective total form abundance and atrogin-1 and MuRF1 abundances were normalized to total α-tubulin, with the value for CON horses set to 1.0 AU. Values are least square means ± pooled SE, n=6 per group. Representative images of the immunoblots are shown above.
Chapter IV

*Pituitary pars intermedia dysfunction does not affect insulin sensitivity in aged horses.*

4.1 INTRODUCTION

Pituitary pars intermedia dysfunction (PPID), also known as equine Cushing’s disease, is believed to affect 15-30% of aged horses (1, 2). The disease is caused by a hypertrophy, hyperplasia, or adenoma formation on the pars intermedia of the pituitary gland and typically occurs in horses older than 15 years of age (5). Clinical signs of PPID include hypertrichosis (198), muscle atrophy (17), laminitis (41), increased secondary infections (36) and decreased insulin sensitivity (19).

Insulin resistance is defined as a condition in which normal concentrations of insulin produce a subnormal physiologic response (66). Currently, two studies have investigated the effects of PPID on insulin sensitivity in the horse; however, in both studies it is likely that the PPID horses were much older than the control horses, due to the nature of PPID affecting primarily aged horses (5). Garcia and Beech (18) determined that horses with PPID (> 12 yrs old) did not respond to intravenous glucose administration with an increase in insulin similarly to control horses (3 to 13 years old) on the same diet. Another study used a euglycemic hyperinsulinemic clamp (EHC) and found that horses with PPID (~21 yrs) had decreased insulin sensitivity compared with healthy controls (~10 yrs) (19). Insulin sensitivity has been shown to decrease with age (88); therefore, it is likely age was a confounding factor in both of these studies and it is still unknown whether insulin sensitivity is different between PPID and non-PPID aged horses.
Additionally, PPID has been associated with muscle atrophy (17). Muscle atrophy occurs when the rate of protein degradation exceeds the rate of protein synthesis. The signaling pathways associated with both protein accretion and breakdown are affected by insulin (171, 223); therefore, insulin resistance could be a potential mechanism to explain the muscle atrophy that occurs in the horse. It has been demonstrated that insulin increases the phosphorylation of factors in the mechanistic target of rapamycin (mTOR) signaling pathway, which is responsible for muscle protein synthesis (106), specifically factors protein kinase B (Akt), eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), and riboprotein S6 (rpS6) are phosphorylated, ultimately leading to an increase in protein synthesis. A previous study determined that there was an increase in the activation of mTOR signaling factors of the skeletal muscle of mature (~14 year old) horses in response to hyperinsulinemia (Urschel et al., unpublished); however, there has only been limited research regarding muscle protein degradation in the horse (16).

Increased circulating inflammatory cytokines have been shown to induce loss of skeletal muscle proteins through the activation of NFκB (179); however, inflammatory cytokines in horses with PPID have been shown to have similar (16) or decreased (219) expression when compared to age-matched controls. Akt activation also plays a role in protein degradation as it has been shown to phosphorylate and inactivate nuclear transcription factors Forkhead box O (FoxO) (203). When FoxO is activated, it increases the expression of E3 ubiquitin ligases, atrogin-1 and muscle RING finger 1 (MuRF1), key genes expressed during protein degradation (203). Muscle atrophy in the horse may also be influenced by decreased protein synthesis due to AMP-activated protein kinase (AMPK) (204) and myostatin (155), both negative regulators of protein synthesis.
The objective of this study was to compare insulin sensitivity in horses with PPID compared to age-matched, non-PPID controls using a dynamic test and proxies to measure insulin sensitivity. Further, the study aimed to characterize differences in the activation of signaling pathways associated with muscle protein synthesis and breakdown in response to insulin administration between the PPID and age-matched, non-PPID control horses.

4.2 MATERIALS AND METHODS

The University of Kentucky Institutional Animal Care and Use Committee approved all procedures in this study (2009-0562). Horses were obtained from an already established herd of aged horses housed at the Department of Veterinary Science’s Maine Chance Farm at the University of Kentucky.

4.2.1 Animals, housing and diets.

Twelve horses, older than 20 years of age, were classified as either PPID (n=6; 25.0±2.5 yrs; mean±SD) or age-matched, non-PPID control (CON) (n=6; 25.7±2.0 yrs). The selected horses consisted of 10 Thoroughbreds (PPID: n=5; CON: n=5), one mixed breed horse (CON: n=1) and one Quarter horse (PPID: n=1). Both the PPID and CON group were comprised of 4 mares and 2 geldings of moderate body condition scores (PPID: 5.5±0.5; CON: 5.0±0.7; [scale 1-9] (205)). Horses classified as PPID exhibited a variety of the classic physical signs associated with the disease including hirsutism, hyperhydrosis, muscle atrophy, a history of laminitis, and hair coat abnormalities (8). PPID classification was confirmed based on a resting ACTH greater than 50 pg/mL and
serum cortisol greater than 1 μg/dL 19-20 hours following dexamethasone administration.

Blood for the plasma ACTH hormone assay was collected in April using EDTA tubes (Vacutainer; Becton-Dickinson, Franklin Lakes, NJ) and sent to an external laboratory for analysis (Animal Health Diagnostic Center; Cornell University, Ithaca, NY) that used a chemiluminescent immunoassay to measure plasma ACTH. Plasma ACTH concentrations were well above the 50 pg/mL cutoff for PPID horses (98.4±73.7 pg/mL) and much lower in the CON horses (23.3±8.2 pg/mL). Dexamethasone suppression tests were performed in May according the previously described procedures (39). Blood samples were collected into glass evacuated tubes containing no additive (Vacutainer; Becton-Dickinson, Franklin Lakes, NJ) before and 19-20 hours following an intramuscular injection of dexamethasone at a dose of 0.04 mg/kg (DexaJect; Butler Schein Animal Health, Dublin OH). Serum cortisol concentrations were determined by an external laboratory (Animal Health Diagnostic Center; Cornell University, Ithaca, NY), which used a chemiluminescent immunoassay to measure cortisol concentrations. Cortisol was suppressed in the CON horses (0.46±0.32 μg/dL), but it did not suppress in the PPID horses (2.27±0.92 μg/dL) consistent with a diagnosis of PPID. Horses were returned to the Department of Veterinary Science’s herd of horses at the end of the study and therefore it was not possible to perform a post-mortem examination for a more definitive diagnosis of pituitary pars intermedia abnormalities.

All horses were housed in one paddock with free access to mixed grass hay (mean ± SD, as fed; 10.1± 0.1% crude protein, 33.0± 0.6% acid detergent fiber, 50.9± 0.6% neutral detergent fiber, 2.7± 0.4% fat, and 6.5± 0.4% ash), water, and salt blocks. Horses were fed a commercial concentrate (mean ± SD, as fed; 14.1± 0.1% crude protein, 22.5±
0.7% acid detergent fiber, 37.2± 1.3% neutral detergent fiber, 6.2± 0.2% fat, and 7.4±
0.2% ash) (Purina® Equine Senior® Horse Feed, Purina Mills LLC, Gray Summit, MO)
individually in stalls twice daily (12g/kg BW/day), divided equally between meals at
0700 and 1500. Feed and hay samples were collected throughout the study by random
sampling and analyzed by Dairy One Forage Laboratory (Ithaca, NY). Horses were
adapted to the diets and housing for a minimum of 2 weeks prior to the euglycemic
hyperinsulinemic clamp (EHC) procedures

4.2.2 Experimental design and procedures.

4.2.2.1 Determination of basal values.

Prior to the day of the EHC procedures, horses were kept in stalls with free access to hay
and water. Twenty-four hours prior to the end of the EHC procedure, a 10 mL blood
sample was collected, via jugular vein venipuncture, to measure basal values for the
plasma concentrations of glucose, insulin and amino acids. Immediately after the blood
sample, horses were lightly sedated with xylazine hydrochloride (0.5 mg/kg BW)
administered IV (AnaSed; Lloyd, Shenandoah, IA). Then, a muscle biopsy was
performed in accordance with previously described procedures (202) where a muscle
tissue sample (~500 mg) was collected from the gluteus medial muscle using a Bergstrom
needle. Following the biopsy, horses were given 2 g of a non-steroidal anti-inflammatory
phenylbutazone (ButaPaste; Butler Animal Health Supply, Dublin, OH) and a meal was
fed. Horses were kept in stalls overnight (~18 hours) with free access to hay and water.
4.2.2.2 The EHC procedure

The following day, catheters (14 gauge X 14.0 cm, Abbocath; Abbott Laboratories, North Chicago, IL) were inserted into each jugular vein after the overlying skin had been desensitized with 2% lidocaine (Lidocaine Injectable; Sparhawk Laboratories, Inc., Lenexa, KS). One catheter was used for infusion of a 50% dextrose solution (Bimeda Inc., Le Sueur MN) and insulin (Humulin N; Eli Lilly and Company, Indianapolis, IN) while the second catheter was used for obtaining blood samples.

Immediately (-25 to -5 min) prior to the EHC procedures, four blood samples were taken at 5-minute increments and analyzed for glucose concentration using a hand held glucometer (Accu-Chek Aviva Plus; Roche Diagnostics, Indianapolis, IN). These values were then averaged in order to determine resting glucose concentration. Horses were outfitted with a surcingle and two infusion pumps. The insulin solution was prepared by diluting recombinant human insulin into a 0.9% sterile saline solution for a final concentration of 360 mU/mL. A priming dose of 0.4mU/kg BW insulin was given at t=0 and then insulin infusion began at a rate of 1.2 mU/kg BW/minute as recommended by previous research (224, 225), using a cordless intravenous pump (J-1097 VetPro Infusion Pump, Jorgensen Laboratories Inc., Loveland, CO). Insulin infusion at this rate has also been shown to activate factors in the mTOR pathway in the gluteus medius muscle of mature horses (Urschel et al., unpublished). The EHC procedure was performed as previously described (104), where insulin was infused at a constant rate and the glucose rate was adjusted to maintain euglycemia. Once the procedure began, small blood samples were taken every 5 minutes and glucose concentrations were measured.
with the hand held glucometer. When the blood glucose concentration fell below 10% of the average baseline values, the infusion of the 50% dextrose solution was initiated using a second peristaltic pump. Dextrose infusion was then adjusted throughout the procedure in order to keep glucose values within 10% of baseline. In addition to the small blood samples, 10 mL blood samples were taken every 15 minutes over the 120-minute procedure for the determination of plasma glucose, insulin and amino acid concentration. The blood samples were transferred into evacuated glass tubes (Vacutainer; Becton-Dickinson, Franklin Lakes NJ) coated with sodium heprin and refrigerated (4°C) immediately until centrifuged at 1,500 x g at 4°C. Plasma was aliquoted and stored at -20°C until the time of analysis.

Following the EHC procedure, a second muscle biopsy was taken from the opposing gluteus medius muscle using the same techniques as basal muscle biopsy. For this biopsy, insulin and glucose infusion continued until the biopsy sample was collected and thus this biopsy will be referred to as the insulin stimulated state biopsy (INS). Similarly to the basal biopsy, horses were given 2 g of the non-steroidal anti-inflammatory phenylbutazone (ButaPaste; Butler Animal Health Supply, Dublin, OH) immediately following the muscle biopsy and then 24 and 48-hours following the procedure. After the INS biopsy, insulin and dextrose infusion was stopped and horses were returned to their stalls to be monitored. There were no evidence of any incidences of hypoglycemia following the second biopsy or any signs of biopsy site infections, with the exception of minor swelling, in any horses in this study.
4.2.3 Plasma analysis.

An automated analyzer (YSI 2300 STAT Plus™ Glucose and Lactate Analyzer, YSI Inc., Life Sciences, Yellow Springs, OH) was used to measure plasma glucose concentrations enzymatically. A commercially available kit (Coat-A-Count RIA® kit Siemens, Healthcare Diagnostics Inc., Deerfield, IL) was used to analyze plasma insulin concentrations. The kit used has been previously validated for use in horses (208, 209). Plasma free amino acid concentrations were measured by use of HPLC analysis of phenylisothiocyanate derivatives, as described elsewhere (202).

4.2.4 Western immunoblot analysis.

Muscle homogenates were prepared as previously described (202). Briefly, following the muscle biopsy collection, a portion of muscle (~100 mg) was homogenized in a lysis buffer and centrifuged at 10,000 x g for 10 min at 4°C. Then the supernatant was removed and frozen at -80°C until the time of analysis. Supernatant protein content was determined using a Bradford assay (Bradford Reagent, Sigma-Aldrich, St. Louis MO). All samples were diluted in a Laemmli to a 2 μg/μL concentration prior to being loaded into polyacrylamide gels. The proteins were separated via electrophoresis and then transferred to polyvinylidene difluoride membranes, as previously described (148). All proteins were incubated for 1 hr in a 5% fat free milk solution with the exception of myostatin which was incubated in a 5% BSA solution. Then, membranes were incubated with the following primary antibodies: Akt (total and phosphorylated Ser<sup>473</sup>; 1:2000 dilutions for each; Cell Signaling Technology, Beverly MA), rpS6 (total and phosphorylated Ser<sup>235/236</sup> and Ser<sup>240/244</sup>; 1:10000 and 1:2000 of each antibody dilutions,
respectively; Cell Signaling Technology, Beverly MA), 4E-BP1 (total and phosphorylated; 1:1000 dilutions of each; Cell Signaling Technology, Beverly MA), AMPKα (total and phosphorylated Thr\(^{172}\); 1:1000 dilutions of each; Cell Signaling Technology, Beverly MA), FoxO1(FKHR) (total and phosphorylated Ser\(^{256}\); 1:4000 and 1:5000 dilutions, respectively; Santa Cruz Biotechnology, Dallas TX), NF-κB p65 (total and phosphorylated Ser\(^{536}\); 1:4000 and 1:1000 dilutions, respectively; Cell Signaling Technology, Beverly MA), MuRF1 (total; 1:5000 dilution; ECM Biosciences, Versailles KY), atrogin-1 (total; 1:1000 dilution; ECM Biosciences Versailles, KY), myostatin (total; 1:10,000 dilution; abcam, Cambridge MA) and α-tubulin (total; 1:1000; Cell Signaling Technology, Beverly MA). Membranes were then washed and incubated with a goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (1:10000 dilution; BioRad, Hercules CA) for 1 hr at 20°C. Following a final wash, membranes were developed with a chemiluminescent kit (Abersham ECL Plus Western Blotting Detection System; GE Healthcare, Piscataway NJ) and viewed using a film processor (Kodak X-OMAT film processor, Kodak Health Imaging Division, Rochester, NY). After developing, the membranes were striped and re-probed in order to measure the total amount of the protein of interest or α-tubulin in the cases of MuRF1, atrogin-1 and myostatin, where only total abundance of protein was measured. α-tubulin has been previously used in other studies of equine skeletal muscle to standardize protein abundance in Western blot analysis (211). Band densities were determined using a densitometric computer software (ImageJ, National Institutes of Mental Health, Bethesda MD). The abundance of phosphorylated forms of Akt, S6K1, rps6, 4E-BP1, AMPK, NF-κB and FoxO1 was corrected for the density of the total protein band and the value for
the control treatment was set at 1.0 arbitrary units. The abundance of MURF1, atrogin-1 and myostatin were determined and compared to abundance of α-tubulin in order to correct for total protein. All gels were run in duplicate.

4.2.5 Calculations.

Glucose infusion rate, in μmol/kg/min, was plotted against time so area under the curve (AUC) could be calculated using the trapezoidal rule (GraphPad Prism 4; GraphPad, La Jolla CA). Tissue sensitivity to exogenous insulin (M/I₃₀) was calculated by dividing the rate of glucose infusion during the last 30 minutes (M) by the mean plasma insulin (I) during the last 30 minutes of the EHC procedure. Additionally, insulin sensitivity was assessed by the calculation of two proxies of insulin sensitivity that used baseline glucose and insulin concentrations: the reciprocal of the square root of insulin (RISQI) and the modified insulin-to-glucose ratio (MIRG), as previously described (96).

4.2.6 Statistical analysis.

Data was analyzed using a mixed procedure of SAS (version 9.3; SAS Institute Inc, Cary NC) with significance reported at P <0.05. Statistical trends were considered at 0.05 < P <0.10.

Measures of glucose metabolism and insulin sensitivity were analyzed using a one-way ANOVA with PPID status (group) as the fixed effect and horse nested in group as the random effect. Plasma glucose, insulin and amino acid concentrations were analyzed using repeated measures analysis with group, time and the interaction between group and time as the fixed effects and horse nested in group as the random effects. A
single baseline was determined by taking the average of the two baseline values for each variable. For each repeated measures analysis, the appropriate variance-covariance matrix was determined based on the lowest value for Schwarz Bayesian criterion. Basal and INS plasma glucose, insulin and amino acid concentrations at the time of biopsy and western blotting data were analyzed using a 2x2 factorial with group, insulin state (basal and INS) and the interaction between group and time as the fixed effects and horse nested in group as the random effects. All data are presented as least square means and pooled standard errors.

4.3 RESULTS

4.3.1 Plasma metabolite concentrations at the time of biopsy.

Plasma glucose, insulin and indispensable amino acid concentrations were not affected by PPID status (P > 0.05) or the interaction between PPID status and insulin state (P > 0.05) (Table 4.1). Glucose concentrations were also not affected by insulin state (P > 0.05) (Table 4.1). Plasma insulin concentrations were significantly greater in the INS compared to the basal state (P < 0.05) (Table 4.1). Plasma isoleucine, leucine, methionine, threonine and valine concentrations were lower in the INS than in the basal state (P < 0.05), whereas the plasma concentrations of the other indispensable amino acids were not different between the basal and INS state (P > 0.05) (Table 4.1).

4.3.2 Plasma metabolite concentrations during the EHC procedures.

During the EHC procedures, there was an effect of time (P< 0.001) and an interaction between PPID status and time (P=0.04), but no effect of PPID status (P= 0.22)
on plasma insulin concentrations (Figure 4.1). Plasma insulin concentrations were increased ~9 fold at the time of the biopsy (t=120) when compared to baseline values. Despite the increase in plasma insulin concentration over the course of the procedure, plasma glucose concentrations were not different from baseline with the exception of a few transient changes, particularly immediately after the initiation of insulin infusion (Figure 4.1), consistent with the goal of the EHC procedure to maintain euglycemia. The PPID horses had a trend to have higher plasma glucose concentrations (P=0.07) during the EHC procedures compared to the CON group (Figure 4.1). There was a significant effect of time (P< 0.001), but no interaction between PPID status and time (P= 0.89) for plasma glucose concentrations during the EHC procedure (Figure 4.1).

For all of the plasma amino acids studied, no interaction between PPID status and time (P> 0.05) was seen (Table 4.2). Plasma histidine and phenylalanine concentrations were not affected by PPID status or time (P> 0.05). Plasma isoleucine (P=0.002), leucine (P= 0.001), methionine (P= 0.01) and valine (P=0.04) concentrations decreased during the EHC procedures, but there was no effect of PPID status (P> 0.05). Plasma threonine (P= 0.01), lysine (P= 0.03) and tryptophan (P= 0.05) were all affected by PPID status, with higher concentrations seen in the PPID horses. Plasma threonine concentration was also affected by time (P= 0.05), but plasma lysine and tryptophan were not influenced by time of insulin infusion (P> 0.05).

4.3.3 Measures of glucose infusion rate and insulin sensitivity.

During the last 30 minutes of the EHC procedure, glucose infusion reached a plateau. At this time, the mean glucose infusion rate required to maintain euglycemia in
horses with PPID was similar to that of the CON horses (P=0.67) (Table 4.3) indicating no differences in insulin-dependent glucose disposal between the two groups. Further, there was no effect of PPID status on AUC for total glucose infusion (P=0.96), the M/I_{30} ratio (P= 0.26), RISQI (P= 0.16) or MIRG (P= 0.61) indices (Table 4.3).

4.3.4 Abundance of factors associated with protein synthesis and breakdown.

Representative blots for the proteins of interest are shown (Figure 4.2-4.5). No differences were found between the PPID and CON groups for any of the factors studied, nor were there any significant interactions between PPID status and insulin status (P> 0.05). Insulin infusion significantly affected the phosphorylation of Akt (P< 0.001), rps6 (P= 0.003) and 4E-BP1 (P= 0.003) in the gluteus medius muscle (Figure 4.2). The phosphorylation of NF-κB (Figure 4.3), AMPK (Figure 4.4), FoxO1 (Figure 4.5), and the abundance of myostatin (Figure 4.4), atrogin-1 (Figure 4.5), and MuRF1 (Figure 4.5) were not affected by insulin infusion (P> 0.05).

4.4 DISCUSSION

Although insulin sensitivity in horses with PPID has been compared to younger controls (18, 19), to the authors’ knowledge, this is the first time that horses with PPID have been compared to age-matched, non-PPID controls using a dynamic test. The results of this study demonstrated that insulin sensitivity was not different between horses with PPID and CON horses using the EHC procedure and proxies, RISQI and MIRG, to measure insulin sensitivity (226). Further, this study marks for the first time that the abundance of many of these protein breakdown factors has been studied in equine
skeletal muscle. Increases in factors associated with protein synthesis were seen in response to insulin administration; however, no differences were seen between the PPID and CON groups for any of the protein synthesis and breakdown factors studied. Together these findings indicated that PPID status does not affect insulin sensitivity on a whole-body level or the ability of insulin to affect insulin-mediated signaling pathways at the muscle level.

The human form of Cushing’s disease is highly associated with insulin resistance, primarily due to high levels of circulating cortisol (226). Although horses with PPID generally have basal cortisol levels within the reference range (14, 15), it has been hypothesized that the cortisol circadian rhythm in horses with PPID may be abolished leading to increased cumulative cortisol over a 24-hour period (39), and ultimately insulin resistance in PPID horses. With the exception of slightly elevated plasma glucose concentrations during the EHC procedure, our study shows little evidence to support the hypothesis that the horses with PPID were more insulin resistant than CON horses. Initially, it was reported that measuring basal insulin had a high sensitivity in diagnosing PPID in horses (15); however, it does not appear to be specific indicator of PPID (227) as many non-PPID horses may also be hyperinsulinemic. A large epidemiological study compared horses with PPID to aged controls and found that basal hyperinsulinemia occurred in 32% and 3% of horses, respectively. However, aged horses with PPID (~24 years) were significantly older than the group of aged horses without PPID (~19 years) (1). Age is a factor that continually presents a problem when investigating the relationship between PPID and insulin sensitivity due to the nature of PPID affecting horses of advanced age. The results of this study suggest that age rather than PPID status
may be responsible for the decrease in insulin sensitivity that has previously been reported.

It is likely that a decrease in insulin sensitivity occurs with advancing age. A study found that older horses (~27 yrs) needed nearly 200% and 150% more insulin to respond to an oral glucose tolerance test than young (~7 yrs) and middle-aged (~15 yrs) mares, respectively (87). Further, a relationship has been found between age, inflammatory cytokines, and insulin sensitivity where older horses have increased inflammatory cytokine production and decreased insulin sensitivity (88). A weakness of the present study was that a control group of younger horses was not included in order to compare the insulin sensitivity of older horses with younger adult horses using the EHC. However, when the results of the present study are compared to another study done by our lab group using the same EHC procedure in younger adult horses (~8 yrs), the aged horses, both PPID and CON, showed ~75% decrease in rate of glucose infusion compared with the younger adult horses in the previous study (228). The RISQI and MIRG values for the PPID and CON horses can also be compared to the established references ranges defined by Treiber et al. (229) using a group of healthy, mature horses (~9 – 18 years). Using the established quintiles (229), the CON horses fell into the 3rd quintile and PPID horses fell into the 1st quintile, with both groups in the bottom 50% of the established range for RISQI, a proxy measure for insulin sensitivity. Both groups fell into the 5th quintile for the MIRG (229), indicating that while these horses were somewhat insulin resistant, they were able to compensate for this resistance with the increased β-cell release of insulin.
Alternatively, it is possible that insulin resistance may only affect horses with advanced PPID at a greater rate than aged-matched, non-PPID controls. Advanced PPID has been defined as those with hirsutism plus 3 or more clinical signs (1). Using this definition, only one PPID horse in the current group had clinical signs consistent with advanced PPID. This horse also had a basal insulin value of 34.1 μIU/ml, the highest of any horse included in this study and consistent with a diagnosis of hyperinsulinemia (230). All of the PPID horses included in the present study were relatively healthy and capable of being managed in a group setting due to the nature of maintaining an aged research herd. It is possible that if a greater number of horses with advanced PPID horses were included in the present study, differences may have been detected between the PPID and CON groups. A study found that serum insulin concentrations were higher in PPID horses that did not survive the 1-2 year treatment period than in those horses that survived the entire study period (61). This suggests that hyperinsulinemia and, thus insulin resistance, may be associated with end stage cases of PPID.

Inflammation in the horse has been shown to increase with advancing age as part of a process known as inflamm-aging (218), leading to decreased insulin sensitivity (88) and increased protein breakdown (178). Interestingly, one study found that horses with PPID have decreased expression of several inflammatory cytokines when compared to healthy aged-matched controls that are similar to those of younger adult horses (219). In the present study, inflammation was not measured directly, but phosphorylation of NF-κB is indicative of inflammatory status (179), with greater phosphorylation of NF-κB being suggestive of increased inflammatory signaling. No differences were found in the relative abundance of phosphorylated NF-κB between the PPID and CON groups in the
basal or insulin-stimulated state, thus it is unlikely that there was a difference in inflammatory signaling in the skeletal muscle between the two groups of aged horses in the current study.

Insulin has the potential to affect protein synthesis through the activation of the mTOR signaling pathway, by phosphorylating Akt (119). In the present study, no differences were seen between PPID and CON groups in phosphorylation of factors leading to protein synthesis, in either the basal of INS state. There were no differences between the PPID and CON horses in the plasma concentrations of glucose, insulin, or concentration of any of the amino acids at the time of either biopsy, which likely explains why no differences were seen in the activation of any of the mTOR signaling factors studied between these two groups of horses. However, the activation of both upstream factors, Akt, and downstream factors, rps6 and 4E-BP1, in the mTOR signaling pathway increased with the infusion of insulin by ~156%, 40% and 31%, respectively. The results of this study were consistent with a previous study that found insulin infusion increased the phosphorylation of factors associated with protein synthesis (Urschel et al., unpublished).

At the time of biopsy, the plasma concentration of many of the indispensable amino acids were lower in the INS state than in the basal state, providing evidence that these amino acids were removed from the plasma and used in protein synthesis. A decrease in the concentration of the indispensable amino acids in the plasma is consistent with other studies using the EHC procedure in horses (Urschel et al., unpublished), humans (231) and pigs (232). Although it is likely that the horses used in this study had decreased insulin sensitivity due to their advanced age, it appears they still had the ability
to respond to insulin with increased phosphorylation of factors associated with protein synthesis. It is important to note that the signaling factors studied are simply indicators of protein synthesis; however, studies in other species have shown that activation of mTOR signaling factors also coincided with an increase in muscle protein fractional synthesis rates (233). It is difficult to speculate if the aged horses had a decreased mTOR signaling response to insulin compared to younger horses, since a younger control group was not included in this study. However, in another study no differences were found in the phosphorylation of Akt, rps6 and 4E-BP1 between mature and aged horses in a post-absorptive state (Wagner et al., in press), suggesting protein synthesis in the skeletal muscle in the horse may not be greatly affected by age specifically, although in the previous study differences between mature and aged horses were not determined in a stimulated (ie. post-feeding) state and therefore additional research is still needed.

Myostatin acts as a negative regulator of skeletal muscle mass by inhibiting activation of Akt, mTOR and p70 S6 kinase 1 (155). In human studies of insulin resistance, plasma myostatin has been shown to be increased (234); however, no differences were seen in muscle abundance of this protein in the current study, which is consistent with previous studies of PPID horses compared to age matched controls (16). These results are in agreement with our conclusion that there is not an apparent difference in insulin sensitivity between PPID and CON horses. Another negative regulator of protein synthesis is AMPK, which is a sensor of cellular energy that is activated by rising AMP levels as a result of energy starvation and results in mTOR inhibition (220). AMPK may also have an important function in muscle wasting as it has been shown to increase the expression of MuRF1 and atrogin-1 (235). In the present study, no differences were
seen in phosphorylation of AMPK between the PPID and CON groups, indicating no differences in cellular energy status.

Insulin mediates protein breakdown through the phosphorylation of Akt which has been demonstrated to phosphorylate and inactivate FoxO (203), leading to a decrease in protein breakdown. Muscle atrophy occurs when the rate of protein breakdown exceeds the rate of protein synthesis. Due to the muscle atrophy associated with PPID (16), it would be expected to see an increase in factors associated with protein breakdown in the PPID compared to age-matched, non-PPID control horses; however, this was not observed in the present study. It has been proposed that major causes of muscle atrophy include insulin resistance and inflammation (178); however, it appears that there was no difference in insulin resistance and inflammation between horses with PPID and CON horses in the present study.

In particular two genes, MuRF1 and atrogin-1 have been shown to have increased expression in multiple models of skeletal muscle atrophy, such as including diabetes, cancer, and adrenal failure (166). In the present study, no differences in MuRF1 and atrogin-1 expression or phosphorylation of FoxO were seen between the two groups of aged horses, indicating no differences in skeletal muscle protein breakdown. Moreover, insulin infusion did not reduce the abundance or activation of any of the studied factors associated with protein breakdown. In a study using the EHC procedure in humans, a biopsy at 30 minutes into the insulin infusion revealed marked increases in phosphorylated FoxO expression (236), indicating that the 2 hour time period of insulin infusion should have been long enough to cause an increase in FoxO phosphorylation in the current study. It is possible that because FoxO regulation of MuRF1 and atrogin-1
abundance occurs at the transcriptional level, that 2 hours may not have been sufficient to measure changes in the abundance of MuRF1 and atrogin-1. However, the lack of an effect of PPID or insulin status on MuRF1 and atrogin-1 is consistent with the lack of change in FoxO phosphorylation under these same conditions. It is known that insulin mediated signaling through the Akt pathway is non-linear (236), suggesting that even though Akt signaling had a normal response to insulin in the current study, it is possible that defects still exist insulin-dependent phosphorylation of factors downstream of Akt, such as FoxO. Therefore, the lack of insulin-mediated suppression of protein breakdown pathways provides some evidence that a portion of the muscle atrophy associated with old horses may be due to a decreased ability to suppress protein breakdown in response to anabolic stimulus. This is consistent with studies in rats and humans that have shown a decreased stimulatory effect of food intake on protein synthesis and its inhibitory effect on protein breakdown with old age (237, 238). The effect of aging on the balance between protein synthesis and breakdown pathways in the skeletal muscle of the horse is an area where additional research is warranted.

It is still possible that there were differences in whole-body protein metabolism between the PPID and CON groups that were not captured by measuring the abundance or activation of the selected factors in the skeletal muscle. The horses with PPID had increased plasma concentrations of lysine, threonine and tryptophan when compared to the CON horses during the EHC procedures, indicating that there may be some differences in whole-body amino acid utilization between these two populations of aged horses. Plasma indispensable amino acid concentrations represent a balance between the processes that add and remove amino acids from the blood. Both groups had similar
access to hay during the EHC procedure with minimal intake in both groups. Therefore, the other main mechanism where additional amino acids could have been added to the plasma would be through an increase in whole-body protein breakdown. Alternatively, elevated plasma amino acid concentrations could also indicate a reduction in amino acid use for protein synthesis in the PPID horses. A previous study in our laboratory utilized isotope infusion techniques in order to measure whole-body protein synthesis in horses with PPID compared to age-matched, non-PPID controls (Chapter III). The whole-body protein kinetics data provides no evidence that there were any differences in whole-body protein amino acid utilization between the PPID and age-matched, non-PPID control horses and therefore the cause of the differences in amino acid concentrations between the two groups of aged horses during the EHC procedures is an area that requires additional research.

4.5 CONCLUSION

Although reduced insulin sensitivity is commonly associated with PPID, it had never been evaluated using a dynamic test comparing horses with PPID and CON horses. The results of this study indicate that reduced insulin sensitivity may occur as a normal part of aging, but is not directly associated with PPID status. Further, PPID status does not seem to alter the ability of insulin to affect insulin-mediated signaling pathways at the muscle level. The decrease in insulin sensitivity as seen in aged horses compared to their younger cohorts appears to be a multi-faceted condition with many other causes other than just PPID. Since all aged horses, regardless of PPID status, have the potential to be affected by reduced insulin sensitivity, it is important that all aged horses be monitored for potential problems associated with insulin resistance.
4.6 TABLES

Table 4.1. Plasma glucose, insulin and indispensable amino acid concentrations at the time of biopsy collection in PPID and CON horses in the basal and insulin stimulated state

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>PPID</th>
<th>Pooled SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BASAL</td>
<td>INS</td>
<td>BASAL</td>
</tr>
<tr>
<td>Glucose²</td>
<td>4.37</td>
<td>4.64</td>
<td>4.90</td>
</tr>
<tr>
<td>Insulin³</td>
<td>10.4</td>
<td>109.0*</td>
<td>16.0</td>
</tr>
<tr>
<td>Histidine⁴</td>
<td>45</td>
<td>41</td>
<td>48</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>60</td>
<td>40*</td>
<td>63</td>
</tr>
<tr>
<td>Leucine</td>
<td>101</td>
<td>65*</td>
<td>119</td>
</tr>
<tr>
<td>Lysine</td>
<td>71</td>
<td>66</td>
<td>85</td>
</tr>
<tr>
<td>Methionine</td>
<td>33</td>
<td>22*</td>
<td>33</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>59</td>
<td>50</td>
<td>47</td>
</tr>
<tr>
<td>Threonine</td>
<td>94</td>
<td>74*</td>
<td>110</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>43</td>
<td>39</td>
<td>58</td>
</tr>
<tr>
<td>Valine</td>
<td>190</td>
<td>138</td>
<td>231</td>
</tr>
</tbody>
</table>

²Values are least square means, n=6/group, as determined using a 2x2 factorial with group, time (basal and EHC) and the interaction between group and time as the fixed effects and horse nested in group as the random effects. No differences were seen between group or an interaction between group and time. *Within a group, value differed significantly (P < 0.05) from baseline.
Plasma glucose concentrations are reported as least squares means in mmol/L.

Plasma insulin concentrations are reported as least squares means in µIU/mL.

Plasma amino acid concentrations are reported as least squares means in µmol/L.
Table 4.2: Plasma indispensable amino acid concentrations (mmol/L) in CON and PPID horses in the basal and insulin stimulated state

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>PPID</th>
<th>Pooled SE</th>
<th>group</th>
<th>time</th>
<th>group*time</th>
</tr>
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<tbody>
<tr>
<td><strong>Histidine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>42</td>
<td>56</td>
<td>5</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>30 min</td>
<td>41</td>
<td>46</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120 min</td>
<td>40</td>
<td>43</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Isoleucine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>53</td>
<td>65</td>
<td>4</td>
<td>NS</td>
<td>0.002</td>
<td>NS</td>
</tr>
<tr>
<td>30 min</td>
<td>52</td>
<td>59</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120 min</td>
<td>40*</td>
<td>46*</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Leucine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>86</td>
<td>122</td>
<td>11</td>
<td>NS</td>
<td>0.001</td>
<td>NS</td>
</tr>
<tr>
<td>30 min</td>
<td>88</td>
<td>104</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120 min</td>
<td>65</td>
<td>80*</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lysine</strong></td>
<td></td>
<td></td>
<td></td>
<td>0.03</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Baseline</td>
<td>71a</td>
<td>107b</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 min</td>
<td>74</td>
<td>94</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120 min</td>
<td>66</td>
<td>77</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Methionine</strong></td>
<td></td>
<td></td>
<td></td>
<td>NS</td>
<td>0.01</td>
<td>NS</td>
</tr>
<tr>
<td>Baseline</td>
<td>26</td>
<td>30</td>
<td>3</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>30 min</td>
<td>25</td>
<td>31</td>
<td>3</td>
<td></td>
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<td></td>
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</tbody>
</table>
Table 4.2 continued: Plasma indispensable amino acid concentrations (mmol/L) in CON and PPID horses in the basal and insulin stimulated state

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>PPID</th>
<th>CON</th>
<th>PPID</th>
<th>CON</th>
<th>PPID</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phenylalanine</strong></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>54</td>
<td>59</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 min</td>
<td>54</td>
<td>58</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120 min</td>
<td>50</td>
<td>45</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Threonine</strong></td>
<td>0.05</td>
<td>0.05</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>88(^a)</td>
<td>113(^b)</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 min</td>
<td>79</td>
<td>100</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120 min</td>
<td>74</td>
<td>91(^*)</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Tryptophan</strong></td>
<td>0.01</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>39(^a)</td>
<td>64(^b)</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 min</td>
<td>41(^a)</td>
<td>56(^b)</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120 min</td>
<td>39</td>
<td>52</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Valine</strong></td>
<td>NS</td>
<td>0.04</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>155</td>
<td>210</td>
<td>18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 min</td>
<td>158</td>
<td>190</td>
<td>18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120 min</td>
<td>138</td>
<td>166(^*)</td>
<td>18</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)Values are least square means (in µmol/L), n = 6/treatment, as determined using a repeated measures analysis with compound symmetry used as the variance-covariance matrix. \(a,b\) Values with different subscripts differ significantly at the same time point. *Within a group, value differed significantly from baseline, P < 0.05.

\(^2\)NS, not significant, P > 0.05
Table 4.3: Measures of glucose metabolism and insulin sensitivity for PPID (n=6) and CON groups (n=6) during the euglycemic hyperinsulinemic clamp procedures

<table>
<thead>
<tr>
<th>Group</th>
<th>CON</th>
<th>PPID</th>
<th>Pooled SE</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RISQI ($mIU/L$)</td>
<td>0.34</td>
<td>0.27</td>
<td>0.03</td>
<td>NS(^1)</td>
</tr>
<tr>
<td>MIRG ($mIU_{insulin}^2/[10\cdot L\cdot mg_{glucose}]$)</td>
<td>6.61</td>
<td>7.27</td>
<td>0.89</td>
<td>NS</td>
</tr>
<tr>
<td>Glucose AUC (mmol/kg)(^3)</td>
<td>1239</td>
<td>1230</td>
<td>147</td>
<td>NS</td>
</tr>
<tr>
<td>$M_{30}$ (mmol/kg/min)(^4)</td>
<td>13.0</td>
<td>11.7</td>
<td>2.04</td>
<td>NS</td>
</tr>
<tr>
<td>$M/I_{30}$ (mmol/kg/min per mIU/mL)(^5)</td>
<td>0.129</td>
<td>0.094</td>
<td>0.022</td>
<td>NS</td>
</tr>
</tbody>
</table>

\(^1\) All values are least square means, n=6/treatment. No differences were seen between the two groups (P > 0.05).

\(^2\) NS = not significant, P > 0.05

\(^3\) Total glucose infusion during the clamp procedures.

\(^4\) Mean glucose infusion rate during the final 30 minutes of the clamp.

\(^5\) Tissue sensitivity to exogenous insulin.
Figure 4.1. Plasma insulin concentrations (µIU/mL) in PPID (filled circle) and CON (open circle) and plasma glucose concentrations (mmol/L) in PPID (filled square) and CON (open square) during euglycemic hyperinsulinemic clamp procedures. Values are least square means ± pooled SE, n=6/group, determined by repeated measures analysis using the compound symmetry variance-covariance matrix for glucose and the autoregression heterogeneous variance-covariance matrix for insulin. Overall, insulin concentrations were effected by time (P< 0.001) and interaction between group and time (P= 0.04); however, there was no effect of PPID status (P=0.22) on plasma insulin concentrations during the EHC procedure. Plasma glucose concentration showed a trend...
(P=0.07) to be higher in the PPID horses than in the CON horses. There was an effect of time (P< 0.001), but no interaction between treatment and time (P= 0.89).

*Significantly different from baseline, P < 0.05.
Figure 4.2. Gluteal muscle phosphorylation of factors involved in the upregulation of protein synthesis, Akt at Ser^{473}, rpS6 at Ser^{235/236} & 240/244, and 4E-BP1 at Thr^{37/46} in CON and PPID horses during the basal (white bars) and insulin stimulated state (black bars). The phosphorylated forms of the translation initiation factors were corrected by the respective total form abundance, with the value for CON horses in the basal state set to 1.0 AU. Values are least square means ± pooled SE, n=6 per group. Representative images of the immunoblots are shown above. *Values with significantly (P<0.05) different from baseline.
Figure 4.3. Gluteal muscle phosphorylation of NF-κB p65 at Ser^{536} in CON and PPID horses during the basal (white bars) and insulin stimulated state (black bars).

The phosphorylated form of NF-κB was corrected by the respective total form abundance, with the value for CON horses in the basal state set to 1.0 AU. Values are least square means ± pooled SE, n=6 per group. Representative images of the immunoblots are shown above.
Figure 4.4. Gluteal muscle phosphorylation of negative regulators of protein synthesis, AMPKα at Thr$^{172}$ and relative abundance of myostatin, in CON and PPID horses during the basal and insulin stimulated state. The phosphorylation of αAMPK was not affected by PPID status and did not differ between the basal (white) and insulin stimulated state (black). There was no interaction between PPID and insulin status. The phosphorylated form of αAMPK was corrected by the respective total form abundance, with the value for CON horses in the basal state set to 1.0 AU. Values are least square
means ± pooled SE, n=6 per group. Representative images of the immunoblots are shown above.
Figure 4.5. Gluteal muscle phosphorylation of FoxO1 and relative abundances of atrogin-1 and MuRF1, factors associated with protein degradation, in CON and PPID horses during the basal (white bars) and insulin stimulated state (black bars). The phosphorylated form of FoxO1 was corrected by the respective total form abundance and atrogin-1 and MuRF1 abundances were normalized to total α-tubulin, with the value for CON horses in the basal state set to 1.0 AU. Values are least square means ± pooled SE, n=6 per group. Representative images of the immunoblots are shown above.
Chapter V

Summary, General Discussion and Future Directions

5.1 SUMMARY OF THE EFFECTS OF PPID ON THE AGED HORSE

The primary objective of this thesis was to determine the effects of PPID on protein metabolism (Chapter III) and insulin sensitivity (Chapter IV) in aged horses, with and without PPID. The first study in this thesis (Chapter III) examined the effect of PPID on protein metabolism at the whole-body and skeletal muscle levels, by using isotope infusion techniques and analyzing specific factors associated with muscle protein synthesis and breakdown. The second study included in this thesis (Chapter IV) determined the effect of PPID on insulin sensitivity using euglycemic-hyperinsulinemic clamp techniques to measure insulin sensitivity at a whole-body level and further characterized insulin-mediated signaling by looking at factors associated with protein synthesis and breakdown. The results of these studies suggest:

1. PPID does not significantly alter protein metabolism in the horse at the whole-body or skeletal muscle levels.
2. Aging may lead to a decrease in insulin sensitivity in all aged horses, not only those affected by PPID.
3. PPID status does not significantly alter insulin sensitivity; however, some deregulations in glucose metabolism may exist in aged horses with PPID.
4. Aging may produce an abnormal response to feeding where amino acids do not reach the bloodstream of the aged horse post-feeding to the same extent as in younger horses.

5. Overall, the studies suggest that age rather than PPID status may be responsible for the abnormalities in muscle mass and insulin sensitivity that have previously been listed as characteristics of PPID.

5.2 GENERAL DISCUSSIONS AND FUTURE DIRECTIONS CONCERNING RESEARCH OF PPID AND AGED HORSES.

Although it is estimated that PPID affects 15-30% of aged horses (1, 2), few studies have compared the clinical signs associated with PPID to age-matched controls. For most of the clinical signs associated with PPID the association is purely anecdotal based on observations from researchers and veterinarians; however, very few studies have actually compared these clinical signs to healthy aged horses, in order to understand the prevalence of these clinical signs in the normal aging horse population. Currently, the NRC does not make specific nutritional recommendations for aging horses, due to the lack of research in this field. However, it appears that physiologic differences, such as insulin resistance, may exist between the mature and aged horses making more research on aged horses needed. The research presented in this thesis did not look at the entire aging horse population, but instead chose to look at horses with PPID, a subset of the total population. Interestingly, the overall results from both studies showed very few differences between the two groups of aged horses.
When making comparisons to previous research in younger horses, it appears that some of the abnormalities seen in both studies may be a function of age rather than PPID status. Both studies presented in this thesis would have benefited from having a younger control group. The original objective of this research was to see how whole-body protein metabolism would differ between a population of horses that presumably had increased insulin resistance and increased muscle atrophy compared to the normal aging population. Initially, when making this comparison, it would seem that a younger control group was not necessary to complete the described objective. However, the differences between the two populations turned out to not be as distinct as previously suggested in the literature.

Part of the discrepancy between the results of the studies presented in this thesis and previous research is likely due to age and the progressive nature of PPID. As previously mentioned, prior studies of insulin sensitivity in aged horses compared horses with PPID to a younger population of horses (18, 19). This was likely due to the nature of PPID affecting horses of advanced age, and lack of understanding of the decrease in insulin sensitivity associated with age. Further, accurately diagnosing PPID, particularly in the early stages, is difficult due to the low sensitivity of diagnostic tests. It is likely that in previous studies, advanced cases of PPID were chosen, since those were the horses visibly affected, making them easy to characterize as PPID.

In the current research, the opposite problem may have occurred. This is to say that our study criteria likely selected for a healthier PPID horse. The horses included in this study were initially selected from a herd of 60 aged research horses. All of the horses in the herd were tested for plasma ACTH concentrations. Then, all horses with an ACTH
concentration greater than 50 pg/mL were given a dexamethasone suppression test to see if they would qualify for inclusion into the study. Using this method, some horses that did not stand out from the herd as being PPID based on appearance, since they did not present with substantial hirsutism, were included in the study. Further, PPID is a progressive degenerative disease, and studies have shown that an increase in clinical signs occurs with increasing age (1). PPID also affects horses differently and a variety of clinical signs can be seen. To be included as part of the research herd at University of Kentucky’s Department of Veterinary Sciences, aged horses must be healthy and capable of being managed in a group setting. Due to these criteria, the PPID horses selected may have been atypical. For example, it has been demonstrated that horses with PPID have an increased predisposition for laminitis (41); however, if a horse in the research herd had chronic bouts of laminitis, it would likely be culled from the herd. Laminitis has been shown to be prevalent in horses with hyperinsulinemia or insulin resistance (22, 76); therefore, our herd may have been unknowing selected toward the healthier PPID horses, with clinical signs that do not require additional maintenance, and away from the extremely insulin resistant horse.

Even if horses in the aged research herd were selected toward the healthier aged horse, it still doesn’t explain some of the unexpected findings that occurred in both groups of horses studied. Thus, additional research is needed in the aged population as a whole to explain some of the derailments in insulin-mediated glucose uptake and abnormal amino acid responses post-feeding. Both groups of aged horses had a glucose infusion during the last 30 minutes of the EHC that was about 75% lower than horses (~8 years old) in another study by our laboratory group (228). This observation suggests, that
age rather than PPID status may be responsible for the decreased insulin sensitivity that is seen in aged horses. Further, both the PPID and age-matched, non-PPID horses had an increase in glucose and insulin post-feeding as seen in previous studies. However, neither group had an increase in plasma amino acid concentrations post-feeding which suggests a potential decrease in amino acid digestibility or increased amino acid utilization by the splanchnic tissues. Previous studies have demonstrated that apparent crude protein digestion was reduced in aged horses (~25 yrs) when compared with younger horses (~2 yrs) (195); however, the low protein digestibility seen in the aged horses was not observed in subsequent studies (196). However, the later studies did not include a younger control group, so it is difficult to speculate if differences in protein digestibility may have existed between the aged horses and younger controls. The current study suggests that this research needs to be revisited, as neither of the groups of aged horses included in this study showed the normal response to feeding of an increase in plasma amino acid concentrations. Further investigation of protein digestibility and absorption in the horse is warranted and may have important implications for the feeding of the senior equine.

The studies included in this thesis leave many unanswered questions about PPID and the effects it may have on the aged horse. The problem with studies of PPID is the variety and severity of symptoms that may present in the horse. In future studies of PPID, it seems a larger population of PPID horses may be needed in order to better characterize a typical PPID horse. Further, it may be necessary to classify PPID horses as early stage or advance stage PPID, based on their clinical signs, in order to better characterize PPID horses. New research of PPID should focus on looking at improved diagnostic methods in
order to better diagnose early stage cases of PPID, which is currently limited by the available diagnostic methods.

Ultimately, the research presented in this thesis suggests that horses with PPID and non-PPID aged horses may not have as many differences as initially believed. No differences were seen between the two groups of horses in insulin sensitivity or whole-body protein metabolism. Further, no differences were seen between the PPID and age-matched, non-PPID control groups in signaling factors associated with protein synthesis and breakdown pathways, nor did PPID status affect the ability of insulin to stimulate these pathways.
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