Modulation of Leptin, Insulin, and Growth Hormone in Obese Pony Mares under Chronic Nutritional Restriction and Supplementation with Ractopamine Hydrochloride*

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Horses that are fed beyond their nutritional requirement and are physically inactive will develop obesity, which is often accompanied by insulin resistance and a heightened risk of laminitis. The use of pharmacologic agents in combination with nutritional restriction may promote weight loss in obese horses that are unable to exercise because of laminitic pain. This study showed that reducing feed intake of brome grass hay to 75% of ad libitum intake in obese pony mares reduced body weight without induced exercise. Additional supplementation of ractopamine hydrochloride for 6 weeks resulted in a tendency for increased weight loss. Subsequent modulation of the obesity-associated hormones leptin and insulin was observed as a result of caloric restriction.

INTRODUCTION

Obesity is a problem in modern horses in large part because of the tendency of owners and trainers to feed and exercise horses in a manner that is not congruous with the way the species evolved.1 Obese horses have excessive depots of adipose, which can generally be determined by a body condition scoring system. Many horses have been identified as metabolically efficient or “easy keepers” and are suscep-
tible to developing obesity when fed based on the nutrient requirements of normal horses. Obese horses are often predisposed to insulin resistance and are potentially susceptible to metabolic syndrome and laminitis.

In January 2000, the US FDA approved the use of ractopamine hydrochloride (Paylean, Elanco) (RAC) in swine finishing rations to augment weight gain, feed efficiency, and carcass leanness in finishing pigs. To date, all in vivo experimentation with RAC has used a growing-animal model. Recently, RAC was shown to be absorbed and metabolized when fed to horses. RAC and other β-adrenergic agonists share similar structural characteristics with the naturally occurring catecholamines dopamine, norepinephrine, and epinephrine. These catecholamines regulate a wide range of physiologic responses in many different tissues. We hypothesized that RAC would alter weight loss and peripheral concentrations of leptin, insulin, and growth hormone in mature obese pony mares in a negative energy balance. Our objective was to determine the effects of a negative energy balance, achieved by feeding obese pony mares at 75% ad libitum feed intake, and the effects of treating with RAC during a period of 6 weeks.

MATERIALS AND METHODS
Animals and Procedures
All procedures with live animals were approved by the University of Missouri Animal Care and Use Committee. Fifteen obese pony mares with body condition scores greater than 7 were used in this experiment. Each mare was assigned to one of five statistical blocks based on initial body weight to ensure an even distribution of body size (249.9 ± 0.01 kg) in each treatment group. The three heaviest mares were assigned to block 1, and the allocation continued until the three lightest weight mares were assigned to block 5. Each block was represented equally in each of the three treatments, which consisted of a control group and two treatment groups that received either 0.6 or 1.0 mg/kg/day of RAC. Dosage was determined by a dose–response study to not increase body temperature, respiration, or heart rate.

Mares were housed individually in 16-m² box stalls and fed free-choice chopped brome hay for 5 days to determine daily ad libitum feed intake (days –4 to 0). Fresh hay was offered twice daily 12 hours apart, with each mare receiving 4,500 g/feeding. Refusals from each previous feeding were weighed and discarded before the next feeding. Once feed consumption had been determined (4,345.8 ± 304.8 g/day), each mare was then fed at 75% of individual ad libitum intake by weight (starting on day 1). The proportional amount fed was 90% chopped brome hay (2,933.4 ± 205.8 g/day) and 10% oats coated with molasses (325.9 ± 22.9 g/day) (used as a carrier for the RAC). This design ensured that all animals were nutritionally restricted at the same rate. The mares received this ration in two meals fed 12 hours apart (0700 and 1900) for the duration of the study.

Twice-daily administration of RAC began on day 3 and continued for 6 weeks. RAC was premeasured for each animal and then mixed thoroughly with the oats before each feeding to ensure consumption. The RAC–oats mixture or oats (control) were fed to the mares first, followed by hay fed no more than 30 minutes after consumption of oats. Mares had access to water and salt throughout the study and received turnout (by treatment group) in a dry lot for 1 hour once every third feeding. Feces was removed from stalls and dry lot twice daily to prevent coprophagy. Blood samples were collected daily for the first 10 days and then weekly for the remaining 5 weeks of the study; samples were collected before the evening feeding was provided. Three blood samples were collected from each mare over a 1-hour period.
to account for variations caused by hormone pulse or variability. Blood was collected via jugular venipuncture using Vacutainer tubes containing EDTA (Becton, Dickinson and Company, Franklin Lakes, NJ). Blood samples were immediately centrifuged at 3,000 \( \times g \) for 25 minutes at 4°C, and plasma samples were stored at –20°C until assayed. Body weights were measured weekly throughout the study using a digital scale.

**Hormonal Analysis**

Plasma samples were analyzed for leptin via double-antibody radioimmunoassay (RIA) procedures previously validated for equine plasma using an in-house rabbit anti–ovine leptin primary antiserum (number 7105).\(^{11}\) Serial dilutions of a pool of equine serum were parallel to the ovine leptin standard curve. The intra- and interassay coefficients of variation were less than 10%. Plasma insulin concentrations were measured using a single RIA kit validated for equine plasma (Diagnostic Products, Los Angeles, CA).\(^{12}\) Serial dilutions of equine plasma were parallel to the human insulin standard curve. The interassay coefficient of variation was less than 10%. Procedures of RIA were used to determine equine growth hormone (GH)\(^ {13}\) using monkey anti–porcine-GH antisera (AFP1038545, obtained from A. F. Parlow, PhD, Harbor-UCLA Medical Center, Torrance, CA). Serial dilutions of a pool of equine serum were parallel to the equine GH standard curve. The intra- and interassay coefficients of variation were less than 10%.

**Statistical Analysis**

A repeated measures design was used to determine the effect of RAC treatment on body weight and concentrations of plasma leptin, insulin, and GH. An analysis of variance (ANOVA) using the mixed procedures of SAS V8 (SAS Institute, Cary, NC) was used to determine statistical differences between treatments. Effects within the model included individual, block, treatment, time, and the interactions of treatment and time. Initial values of hormones and body weights were used as covariates. The repeated statement was time and the error term was individual within treatment by block. Akaike information criterion and Schwarz Bayesian criterion were used to determine the compound symmetry heterogenous model as best fitting for analyses of plasma concentrations of leptin and body weight, compound symmetry model as best fitting for analysis of plasma concentrations of insulin, and antidependent model as the best fit for GH.\(^ {14}\) Least squares means and SE were generated in the body weight model for time.
and treatment by time. Least squares means, SE, and least squares differences were generated in the leptin, insulin, and GH models for time, treatment, and treatment by time.

RESULTS

No significant differences existed in any variable measured before the onset of treatment ($P > .10$). Body weights significantly changed throughout the duration of the study following feed restriction ($P < .01$; Figure 1). This steady decline in body weights was observed in all mares regardless of treatment, as expected. Although the finding was not significant, mares treated with 1.0 mg/kg RAC exhibited a tendency to have a greater decrease in body weight compared with the mares in the control and 0.6-mg/kg RAC groups in the first 2 weeks of the study ($P = .091$; Figure 1). After the immediate changes in weight in the 1.0-mg/kg RAC group, body weight appeared to decrease at a similar rate in all of the groups.

Plasma concentrations of leptin changed over time in all mares regardless of treatment ($P < .01$; Figure 2). These concentrations decreased by 16% immediately following the restriction of feed. After the initial decrease, the concentrations of leptin increased over the next 2 days followed by a progressive decrease for the next 4 days. Observations throughout the remaining weeks indicated that concentrations of leptin increased to prerestriction values within 3 weeks and then decreased for the remaining 3 weeks. No significant differences were observed in concentrations of leptin between treatments ($P = .33$; Figure 3).

![Figure 2](image_url). Changes in leptin concentration over time (least squares means ± SEM) in pony mares regardless of treatment. Feed restriction began on day 1. A significant change in leptin concentration occurred over time ($P < .01$).

![Figure 3](image_url). Changes in leptin concentration over time (least squares means ± SEM) in nutritionally restricted pony mares (control animals and those receiving daily treatment of 0.6 or 1.0 mg/kg RAC). Treatment began on day 3. No differences were observed between any treatment group ($P = .33$).
We observed a significant decline in concentrations of insulin in all mares following the feed restriction \((P < .01; \text{Figure 4})\). Concentrations of insulin declined overall following the onset of restriction. During the first 10 days, insulin increased on days 4 to 5 and 8 followed by subsequent decreases (Figure 4). No significant differences in concentrations of insulin were observed between treatments \((P = .10; \text{Figure 5})\).

GH concentrations changed over time in all mares, regardless of treatment \((P < .01; \text{Figure 6})\). Concentrations of GH appeared to decrease during the first week following the initiation of feed restriction and increase during the remaining weeks of the study. No significant differences were observed in concentrations of GH between treatments \((P = .10; \text{Figure 7})\).

**DISCUSSION**

Many researchers believe that equine metabolism evolved for survival based on the seasonally variable availability of forage.\(^1\) The temporary development of additional body fat at times when forage is plentiful provides a survival mechanism for times when conditions are harsh and forage is scarce. One theory is the natural selection of a genotype for metabolic efficiency, similar to the thrifty genotype proposed by Neel in humans.\(^15\) Through centuries of breeding for traits inconsistent with metabolic efficiency, humans have produced horses that are considered to have “normal” metabolism. We theorize that some horses referred to as metabolically efficient or “easy keepers” may have retained

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**Figure 4.** Changes in insulin concentration over time (least squares means ± SEM) in pony mares regardless of treatment. Feed restriction began on day 1. A significant change in insulin concentration occurred over time \((P < .01)\).

**Figure 5.** Changes in insulin concentration over time (least squares means ± SEM) in nutritionally restricted pony mares (control animals and those receiving daily treatment of 0.6 or 1.0 mg/kg RAC). Treatment began on day 3. No differences were observed between any treatment group \((P = .10)\).
these “thrifty” genes and thus have lower nutrient requirements than “normal” horses. Circadian and seasonal variations of leptin have recently been reported in horses, which supports this theory of body fat accumulation for survival. During periods in which forage is relatively unavailable, the body fat stores, which were never intended to become excessive, are depleted to provide energy for survival. Under many modern horse-management systems, the combination of feeding starch-rich rations over many years and protracted periods of stall confinement can lead to the acquisition and maintenance of substantial adiposity in domesticated horses.

The β-adrenergic agonists are reported to be metabolic modifiers and are orally active; the main effects on the carcass of the animal are increasing skeletal muscle and reducing adipose tissue mass. When growing animals are treated orally with RAC, they respond by increasing muscle mass and decreasing adipose stores; this has been seen in swine, rats, and steers. Similar results are observed when genetically obese and genetically lean pigs are treated with RAC. Skeletal muscle deposition is dramatically enhanced by the β-adrenergic agonists clenbuterol and cimaterol, both of which are specific for the β₂ receptor subtype. Conversely, RAC is primarily a β₁ agonist and has been shown to have a high affinity for middle subcutaneous adipose tissue. Clenbuterol has been investigated as a possible therapeutic treatment for obesity in horses and has resulted in a reduction of adipose, but it has been reported to negatively affect cardiac function. Recently, RAC

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**Figure 6.** Changes in GH concentration over time (mean ± SEM) in nutritionally restricted pony mares regardless of treatment. A significant change occurred over time regardless of treatment (P < .01). Treatment began on day 3. No effect of treatment was observed (P = .10).

a, b Differences of preplanned comparisons between different points in time are indicated by different letters (P < .05).

**Figure 7.** Changes in GH concentrations over time (least squares means ± SEM) in nutritionally restricted pony mares (control animals and those receiving daily treatment of 0.6 or 1.0 mg/kg RAC). Treatment began on day 3. No differences were observed between any treatment group (P = .10).
was tested in horses and was determined to be metabolized by the detection of metabolites in urine. That study showed that when RAC is fed to horses in the same manner as in the present study, it is absorbed via the gastrointestinal tract. The metabolites recovered indicated that RAC is highly metabolized via similar methods in other species. In the present study, RAC was delivered in the same method and consistent dosage as the previous study. In that study, however, horses were dosed once, whereas in the present study, animals were dosed twice daily for 6 weeks. Based on the previous data, we believe that RAC was absorbed and metabolized by the animals in the present study. However, no tests were performed in the present study to determine if RAC was absorbed or metabolized.

In the current study, we observed a tendency for the group receiving RAC at 1.0 mg/kg to have a greater decrease in body weight compared with the control and 0.6-mg/kg groups. The differences seen in this group appear to have occurred very soon after treatment began. It can be speculated that the lack of a continued response is a result of treatment with RAC twice daily. This dose frequency may not allow the \( \beta_1 \) receptors sufficient time to recover; thus, a greater refractory period may be needed to elicit a greater response. It is possible that feeding RAC twice daily may not allow time for the molecular actions following receptor binding to recover and, therefore, may not allow a full response to each treatment. Reducing treatment to once daily or less frequently could potentially affect receptor activity. Recently reported data support our contention that the dose and route of administration that we used were appropriate in terms of bioavailability for horses. Because of housing constraints, the maximum number of animals per group was five, which limited statistical power.

Changes in all hormones measured were attributed to restriction of feed and subsequent weight loss. We did not observe changes in hormone concentrations attributable to treatment with RAC. However, the changes in hormone profiles as a result of feed restriction do offer insight regarding metabolism in obese horses. Most notable was the change in the leptin concentration, which decreased during the first day following the feed reduction and then gradually increased over the next 2 days. This increase was followed by a daily decrease for the next 4 days. The remaining weekly samples revealed another rise and fall in the concentrations of leptin. The observed changes in concentrations of leptin may be explained by Kennedy’s lipostatic theory. Briefly, Kennedy suggested that animals regulate appetite and energy expenditure according to the amount of energy storage (body fat) and food available. Leptin is believed to be a key regulator in this mechanism, acting to signal adiposity and regulating energy balance. We conclude that the changes observed in concentrations of leptin were attributable to regulation (or resetting) of the “lipostat” as time passed and feed was continually restricted. While the horses were maintained in a negative energy balance, their body weight declined and, presumably, metabolism and energy expenditure were adjusted. When feed is restricted at a constant rate, body weight decreases until equilibrium is reached. We attribute the rise and fall of leptin to adjustments in metabolism as the body is reaching equilibrium. The concentrations of leptin observed in the current study agree with previous observations in which concentrations of leptin decreased following an acute feed restriction.

Concentrations of insulin rapidly declined as a result of feed restriction and continued to be suppressed for the remainder of the experiment. Within the first 10 days of declining insulin concentrations, we observed increases on days 4 to 5 and day 8 followed by subsequent decreases. These transient rises in concentrations of insulin
could be an adaptation response to a negative energy balance. Because insulin has lipogenic and antilipolytic properties, this suppression of insulin is indicative of a feed restriction. Suppression of this hormone therefore allowed the body to maintain homeostasis by using adipose stores.

A modest decrease followed by a modest increase in GH was observed after feed restriction. In a previous experiment, we observed a twofold increase in concentrations of GH when horses were feed deprived for 48 hours, indicating that a modulation of GH likely depends on nutrient availability. Thus, we conclude that reducing feed intake to 75% of ad libitum intake is below the threshold required to increase GH to a maximum concentration.

**CONCLUSION**

One interpretation of these data—that a tendency existed for RAC to increase weight loss at the 1.0-mg/kg dose—offers insight for the use of β1-adrenergic agonists for weight loss in obese horses. The frequency or quantity of treatment with RAC may have limited our success to moderate weight loss. However, a reduction in feed consumption to 75% of ad libitum intake with minimal exercise resulted in significant weight loss without any apparent adverse effects. Assessing the ad libitum forage intake for an individual obese horse and restricting the intake based on this recommendation may represent the best approach for obesity reversal in horses at this time.

**REFERENCES**

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