Endocrine responses in mares undergoing abrupt changes in nutritional management

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ABSTRACT: Leptin, a protein hormone secreted by adipocytes, plays an important role in energy homeostasis and regulation of body composition. We previously observed that acute feed restriction resulted in a rapid decline in concentrations of leptin in obese pony mares. This acute response prompted us to characterize the temporal changes in concentrations of leptin, GH, and insulin in obese pony mares during the transition between fed and feed-restricted conditions. Nine obese pony mares of mixed breed, previously maintained on fescue pasture, were randomly allotted to 2 groups. Treatments consisted of a 48-h feed restriction, a 48-h refeeding, and a 24-h feed restriction (RFR; n = 4), or 48 h of alfalfa hay ad libitum, a 48-h feed restriction, and a 24-h refeeding (FRF; n = 5). Blood samples were taken every 15 min during restriction and feeding transitions (0600 to 1400 on d 2 and 4), and every 30 min thereafter until 0830 of the following days (d 3 and 5).

Key words: equine, feed deprivation, growth hormone, leptin

INTRODUCTION

The prevention or subsequent management of obesity in horses is of paramount concern among horse owners, as obese horses are often predisposed to maladies such as insulin resistance (Jeffcott et al., 1986; Hoffman et al., 2003), metabolic syndrome (Johnson, 2002), and potentially laminitis (Johnson et al., 2004). Among the population of obese horses exist those designated as metabolically efficient, and commonly known as “easy-keepers,” which have lower nutritional requirements than “normal” horses (NRC, 1989). Managing metabolically efficient horses can be difficult, in that: a) limiting their feed intake does not resolve feed-seeking behavior, and b) limiting access of metabolically efficient horses to poor quality roughage, as a management scheme, can result in nutritional deficiencies.

Of the factors that influence nutritional status, the adipocyte-derived hormone leptin has been reported to play a key role in the regulation of appetite in other animal models and has been positively associated with energy balance (Schwartz et al., 2000). Positive associations exist between peripheral concentrations of insulin and adiposity in horses and ponies, as assessed by ultrasonography (Fitzgerald and McManus, 2000) and BCS (Buff et al., 2002; Gentry et al., 2002). Several groups have reported low circulating concentrations of leptin after feed deprivation in meal-fed horses (McManus and Fitzgerald, 2000; Piccione et al., 2004; Cartmill et al., 2005) and obese pony mares fed ad libitum (Buff et al., 2005). The acute response to feed restriction in obese pony mares fed ad libitum led us to the current objective of characterizing temporal concentrations of leptin, GH, and insulin in obese pony mares during the transition between fed and feed-restricted conditions. We hypoth-
Animals and Procedures

of ad libitum alfalfa hay (hay followed by 48 h of feed restriction and then 24 h restriction (48 h of ad libitum alfalfa hay and then 24 h of feed restriction and then 24 h of ad libitum alfalfa hay (group FRF; n = 5). A pretreatment blood sample was taken at time 0 (indicated by the arrow). Blood samples were taken every 15 min beginning 2.5 h before the onset of the feeding transition and thereafter for 5.5 h, followed by collection every 30 min for 18.5 h. FED = ad libitum alfalfa hay; RES = feed restriction.

esized that in obese pony mares, peripheral concentrations of leptin and insulin would rapidly decrease; and GH would rapidly increase in response to feed deprivation and abruptly adjust to basal patterns after their return to feed.

MATERIALS AND METHODS

Animals and Procedures

All procedures with live animals were approved by the University of Missouri Animal Care and Use Committee.

Test subjects consisted of 9 obese crossbred [Quarter Horse × (Shetland or Welsh type)] pony mares ranging in age from 5 to 15 yr, with BW of 242 ± 16.8 kg. All mares were managed as a herd on a fescue pasture with no supplementation before experimentation, and maintained a BCS of ≥6 and ranging from 6 to 8 (Henneke et al., 1983). Mares were removed from pasture and offered ad libitum access to alfalfa hay for 1 wk before experimentation, during which their peripheral concentrations of leptin, insulin, and GH were measured and determined not to differ between animals or from the mean values previously reported for this group in experiments conducted 12 and 16 mo earlier (Buff et al., 2005).

The mares were subsequently allotted randomly to 1 of 2 treatments that were applied concurrently, and which consisted of 48 h of feed restriction followed by 48 h of ad libitum alfalfa hay and then 24 h of feed restriction (RFR; n = 4), or 48 h of ad libitum alfalfa hay followed by 48 h of feed restriction and then 24 h of ad libitum alfalfa hay (FRF; n = 5; Figure 1). The feeding paradigms were designed, based on our previous study of the effects of feed restriction on endocrine variables (Buff et al., 2005), to determine transitional changes in hormone concentrations at feed introduction and removal.

Additionally, this experiment was conducted when the daily amount of visible ambient light ranged from 15 h 52 min to 15 h 49 min (sunrise and sunset occurred from 0545 to 0550 and 2037 to 2036, respectively). During the study, mares were maintained in their respective treatment groups in identical 53.5-m² corrals with 26.7 m² covered by a 3-sided shed with the open side facing west. Furthermore, during the period of ad libitum feeding, we provided each animal with an individual hay bunk. We concede the possibility that the treatment-group penning arrangement may have introduced a pen bias, but maintain that our objective was to determine individual animal responses over time. Water was available to mares at all times.

One day before treatment, each mare was fitted with a jugular cannula to aid in the collection of blood samples, and the mares were loosely tethered (permitting recumbency) within the shed during the nutritional transition periods. The mares were maintained untethered in the corrals, in their respective treatment groups, during nonsampling periods. During the nonsampling periods, mares that were not being nutritionally restricted were fed alfalfa hay ad libitum provided in hay bunks that equaled the number of mares being fed to minimize the effect of group feeding.

Blood samples were collected every 15 min beginning 2.5 h before the onset of the feeding transition and thereafter for 5.5 h, followed by collection every 30 min for 18.5 h. Mares were then allowed to remain in their fed or restricted condition without being sampled for 21.5 h, after which the sampling was repeated as before, beginning at 2.5 h before the onset of the subsequent nutritional transition (Figure 1). To minimize light exposure, nighttime samples were collected with the aid of flashlights fitted with red lenses. Samples collected between 0700 and 1100 were designated as AM samples, and those collected from 2200 to 0200 were designated as PM samples. The specific sampling intervals were selected to include 4-h periods of light and 4-h periods of darkness after the events of sunrise and sunset, as adapted from a previous study conducted to examine the variation in concentration of leptin during a 24-h period (Buff et al., 2005). Blood was collected (3 mL) into tubes containing 100 μL of 0.05 M EDTA. Blood samples were stored at 4°C for no more than 12 h and were then centrifuged at 3,000 × g for 25 min at 4°C. Plasma samples were stored at −20°C until assayed.

Hormonal Analysis

Plasma samples were analyzed for leptin, in triplicate 200-μL aliquots, with the double-antibody RIA procedures previously validated for equine plasma (Buff et
Table 1. Criteria for defining a pulse in secretion of growth hormone

<table>
<thead>
<tr>
<th>Item</th>
<th>Constant CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Evaluation of individual point SD</td>
<td>5%</td>
</tr>
<tr>
<td>Assay precision (CV)</td>
<td></td>
</tr>
<tr>
<td>Assay replicate df</td>
<td>1</td>
</tr>
<tr>
<td>Time between data points</td>
<td>15 and 30 min</td>
</tr>
<tr>
<td>Number of points nadir</td>
<td>1</td>
</tr>
<tr>
<td>Number of points peak</td>
<td>1</td>
</tr>
<tr>
<td>t-Statistic for upstroke</td>
<td>2</td>
</tr>
<tr>
<td>t-Statistic for downstroke</td>
<td>2</td>
</tr>
<tr>
<td>Minimum data value pulse</td>
<td>2 ng</td>
</tr>
</tbody>
</table>

1Pulse analysis was conducted using Cluster Analysis computer software (Veldhuis and Johnson, 1986) and parameters that have been optimized using a multiple-parameter deconvolution model (Veldhuis and Johnson, 1988).

al., 2002). The intra- and interassay CV were <10%, and the sensitivity was 0.04 ng/mL. Plasma concentrations of equine GH were determined as described by Thomas et al. (1998) in duplicate 200-μL aliquots. The intra- and interassay CV were <10%, and the sensitivity was 0.2 ng/mL. Plasma concentrations of insulin were measured in duplicate 200-μL aliquots with an RIA kit (Diagnostic Products Corporation, Los Angeles, CA) that was validated for use with equine plasma by Free- stone et al. (1991). The intra- and interassay CV were <10%, and the sensitivity was 4.6 μIU/mL.

Statistical Analysis

To determine whether changes in concentrations of leptin, GH, or insulin occurred over time in response to nutritional status, the data were analyzed using a repeated measures design with the MIXED procedure of SAS (SAS Inst., Inc., Cary, NC). Effects used in the model were treatment (FRF vs. RFR), sample (time), and the interaction of these variables. The variable “sample” was used in the repeated statement and the error term used was “animal by treatment”. Covariance structure compound symmetry was determined by the Akaike information criterion and the Schwarz Bayesian criterion as the best fitting for analyses of concentrations of all hormones (Littell et al., 1998).

To determine the effect of diurnal changes and treatment on concentrations of leptin, samples from each treatment were grouped as AM or PM. The AM samples were collected from 0700 to 1100, whereas the PM samples were collected from 2200 to 0200. These data were analyzed using the GLM procedure of SAS. Effects within the model included treatment (FRF vs. RFR), diurnal rhythm (AM vs. PM), nutritional status (restriction vs. ad libitum feeding), and all interactions of these variables. Least squares means and differences were generated for treatment and treatment × diurnal rhythm × nutritional status.

Mean concentration, area under the curve (AUC), pulse frequency, and pulse amplitude were determined for GH for each animal within each 24-h sampling period using the CLUSTER pulse analysis program (Veldhuis and Johnson, 1986). The criteria for determining pulsatile secretion of GH, previously described by Veld- huis and Johnson (1988), are outlined in Table 1. The GLM procedure of SAS was used to determine if frequency or amplitude differed between feed restriction (RES) and ad libitum feeding (FED) treatments. The

![Figure 2](image_url)

Figure 2. Changes in concentrations of leptin over time in obese pony mares receiving alfalfa hay ad libitum for 48 h, then feed restriction for 48 h, then alfalfa hay ad libitum for 24 h (FRF), or feed restriction for 48 h, then alfalfa hay ad libitum for 48 h, then feed restriction for 48 h (RFR). The solid vertical line indicates a break in sampling for 21.5 h. Blood samples were collected every 15 min beginning 2.5 h before the onset of the feeding transition and thereafter for 5.5 h, followed by collection every 30 min for 18.5 h. Arrows indicate changes between fed vs. restricted feeding. A significant sample by treatment interaction was observed (P < 0.01).
model tested the effects of animal and treatment on pulse frequency and pulse amplitude, using the residual error as the error term.

RESULTS

Peripheral concentrations of leptin at time 0 were 21.3 ± 1.8 ng/mL. When mares were maintained on pasture, concentrations of leptin were 28.2 ± 1.7 ng/mL for PM samples and 22.1 ± 1.7 ng/mL for AM samples (Buff et al., 2005). Mean leptin concentrations from all samples collected within this study were 13.0 ± 0.4 ng/mL for RFR and 18.0 ± 0.3 ng/mL for FRF ponies (P < 0.001). Plasma concentrations of leptin declined precipitously 6 h after the initial removal of feed (approximately 1430; sample by treatment interaction; P < 0.01; FRF; Figure 2) and remained low during refeeding. We observed a rapid decline in the concentrations of leptin in 2 mares in FRF group at the same time point, which emphasized the decline in Figure 2. Similarly, in the RFR ponies, plasma concentrations of leptin were initially low and did not respond to feeding during the second (FED) sampling period or during the final interval of feed restriction (Figure 2). An interaction between treatment × photoperiod × nutritional status was observed (P < 0.001, Figure 3). In the FRF group, AM concentrations of leptin were greater than PM concentrations when mares were feed-restricted and PM concentrations were greater when provided ad libitum alfalfa hay. In the RFR group, PM concentrations were greater when feed was restricted and no differences were observed when they were fed ad libitum alfalfa hay.

At time 0 (i.e., after a 1-wk acclimation period to ad libitum access to alfalfa hay), plasma concentrations of insulin were 97.2 ± 18.7 μIU/mL. Plasma concentrations of insulin were 50.5 ± 4.5 μIU/mL when mares had ad libitum access to alfalfa hay (Buff et al., 2005). During the experimental phase of the study, plasma concentrations of insulin were low after feed restriction and elevated when animals were returned to feed after a restriction in both RFR and FRF paradigms (sample by treatment interaction; P < 0.001; Figure 4). In the FRF paradigm, insulin declined after feed restriction (P < 0.001) and increased after the return of feed (P < 0.001) but reached a plateau at concentrations less than the prerestriction concentrations (P = 0.003). In the RFR paradigm, insulin also increased after feeding (P < 0.05), yet the magnitude of this increase was less than that after reintroduction of feed in the FRF paradigm (P = 0.001).

At time zero (i.e., after a 1-wk acclimation period to ad libitum alfalfa hay), mean plasma concentrations of GH were 0.5 ± 0.1 ng/mL. Plasma concentrations of GH were 1.1 ± 0.3 ng/mL for mean concentration, 1.5 ± 0.4 pulses/24 h for pulse frequency, and pulse amplitude of 7.2 ± 1.5 ng/mL when mares had ad libitum access to alfalfa hay in our previous study (Buff et al., 2005). During the experimental phase of the study, plasma GH was greater in RES than FED mares (P = 0.03; Figure 5A). Pulse amplitude of GH was greater in RES than FED mares (P < 0.05; Figure 5B), as was AUC (P < 0.05; Figure 5C). Pulse frequency of GH was greater in RES than FED mares (P < 0.05; Figure 5D).

DISCUSSION

Horses evolved as animals capable of surviving on seasonally available forage (Epstein, 1971). The sea-
Figure 4. Changes in concentrations of insulin over time in obese pony mares receiving alfalfa hay ad libitum for 48 h, then feed restriction for 48 h, then alfalfa hay ad libitum for 24 h (FRF), or feed restriction for 48 h, then alfalfa hay ad libitum for 48 h, then feed restriction for 48 h (RFR). Solid vertical line indicates a break in sampling for 26.5 h. Arrows indicate changes between fed vs. restricted feeding. A significant sample by treatment interaction was observed ($P < 0.001$).

Seasonal variation in feed availability may have entrained horses to increase feed intake and accrue greater fat mass when forage was readily available as an anticipatory survival mechanism that could be drawn upon during intervals of limited feed availability (Berger, 1986). When ambient temperatures decrease, horses attempt to increase consumption of feed to increase and maintain energy stores (Brody, 1945). Neel (1962) put forth the concept (in terms of human evolution) of a thrifty genotype as an explanation of why some humans develop obesity and type II diabetes and others do not. Neel (1962) contends in his hypothesis that Paleolithic humans evolved to be thrifty to survive. Today, although that survival mechanism is no longer needed, it persists and unfortunately contributes to the occurrence of obesity and type II diabetes. In contrast, the human population that evolved without the thrifty genes do not suffer from metabolic disorders. A similar scenario may exist among metabolically efficient horses, which have a propensity to develop obesity; tending to gain BW when fed a diet that, for most horses, only satisfies maintenance requirements (NRC, 1989). Arguably, selective breeding of horses, which has been practiced for centuries, for traits that are counter to the thrifty mode of survival may have favored horses (i.e., today’s “normal horses”) that are distinctly different from the metabolically efficient horses. Cunningham et al. (2001) have reported a low genetic diversity among Thoroughbreds and that lineages of this closed breeding population are linked to 1 founding stallion. The group of obese pony mares in the present experiment consisted of crossbred animals that developed and remained obese while fed a maintenance diet. This group of mares was used as a model for metabolically efficient horses to investigate the physiology of energy balance and endocrine variables associated with these processes.

The hormone leptin, which is primarily produced by adipose tissue, is a key regulator of appetite and energy homeostasis (Schwartz et al., 2000). Concentrations of leptin increase as BCS increases in horses and ponies (Buff et al., 2002; Gentry et al., 2002). Although BCS may be an indicator of degree of adiposity, it only assesses subcutaneous depots. Recent findings in horses indicate that some horses have greater or lesser concentrations of leptin within similar BCS (Cartmill et al., 2003). Leptin works in concert with insulin in some species to affect the neuroendocrine mediators of appetite control as signals of adiposity (Schwartz et al., 2000). The mechanisms by which GH interacts with leptin in the regulation of energy balance and body composition are not well understood. However, infusion of leptin has been reported to increase GH secretions in sheep (Nagatani et al., 2000; Henry et al., 2001; Morrison et al., 2001) and pigs (Barb et al., 1998).

Previous reports have provided evidence that concentrations of leptin are suppressed by feed deprivation in obese ad libitum fed pony mares (Buff et al., 2005) and nonobese meal-fed horses (McManus and Fitzgerald, 2000; Piccione et al., 2004; Cartmill et al., 2005). Recent findings in horses fed ad libitum hay combined with meal-fed concentrate have illustrated patterns of leptin in relation to time of feeding (Gordon and McKeever, 2005). In that study, it was found that leptin increased after the twice-daily concentrate meal. The diurnal variations of leptin observed in the current study were
Nutritional endocrinology in mares

Figure 5. Growth hormone responses in obese pony mares to feed restriction (RES) or alfalfa hay ad libitum (FED) measured in 24-h intervals. A) Mean concentration of GH was greater in RES vs. FED mares ($P = 0.04$); B) Pulse amplitude of GH was greater in RES vs. FED ($P < 0.05$); C) Area under the curve (AUC) was greater in RES vs. FED mares ($P < 0.05$); D) Pulse frequency of GH was greater in RES vs. FED mares ($P < 0.05$).

unexpected. Previously, we found a nocturnal increase in leptin concentrations in obese pony mares when fed alfalfa hay ad libitum, which was suppressed under feed deprivation (Buff et al., 2005). Piccione et al. (2004) discovered the same rhythm in horses fed meals thrice daily and observed no rhythm when feed deprivation was imposed. Cartmill et al. (2005) also reported diurnal changes that were related to time of feeding in meal-fed horses. Gordon and McKeever (2005) did not find diurnal variation in horses fed ad libitum hay with twice-daily concentrate. The findings from the present and other recent studies suggest that patterns of feeding may override the natural diurnal rhythm of leptin.

In our previous report, pulse frequency, pulse amplitude, and mean concentrations of GH were elevated and insulin was suppressed in feed-deprived obese ponies (Buff et al., 2005). Christensen et al. (1997) reported an increase in GH in mature geldings after feed deprivation with no evidence of pulsatile secretion. The lack of discovery of pulsatile secretion is most likely due to the infrequency of sampling conducted in that study. In contrast to these findings, no effect of feed removal on GH was reported in horses conditioned to meal feeding (DePew et al., 1994; Sticker et al., 1995). In comparing these studies, it seems that GH is more responsive to feed deprivation in horses and ponies that are fed ad libitum than in horses entrained to meal feeding.

The findings in our initial studies (Buff et al., 2005) led to the current objective of characterizing temporal changes in plasma concentrations of leptin, GH, and insulin as obese pony mares conditioned to ad libitum feeding made the transition between fed and feed-restricted conditions. In the current study, we observed concentrations of leptin abruptly decreasing 6 h after removal of feed in the FRF group, yet when feed was replaced, leptin did not return to the prerestriction concentrations. A rapid decline was observed in 2 mares at the same time point, which emphasized the overall decline in the FRF group. In RFR mares, concentrations of leptin were suppressed by the feed restriction when the sampling period began and did not increase when feed was made available. Similar findings have been reported for weanling pigs, in which concentrations of leptin decreased after the initiation of feed deprivation (Salzen et al., 2003). Interestingly, in that study, leptin increased within 12 h of refeeding, but did not return to prerestriction concentrations within 24 h of refeeding. In an earlier study with lean and obese (ob/ob) mice, quantitative expression of leptin mRNA from adipose resulted in a decrease in leptin mRNA during feed restriction with no difference in refed lean animals and no effect of feed on obese mice (Trayhurn et al., 1995). The current observations agree with the previously reported data collected from commercial crossbred pigs.
in which the secretion of leptin was highly responsive to a period of abrupt feed deprivation and less responsive to a period of ad libitum feeding. The failure of peripheral concentrations of leptin to return to prerestriction concentrations in these species may be due to a downregulation of feedback mechanisms during a period of feed deprivation. This may be a function of compensatory mechanisms that function to shuttle metabolic fuels to tissues that have a greater demand for the metabolic fuel (e.g., brain, muscle) rather than those fuels being partitioned into fat stores when feed is restored (Peters et al., 2004). It could be argued that this type of control would aid in the survival of the individual by delaying satiety and thus sustaining feeding behavior. A mechanism of this nature would seem to be more critical in lean animals than in obese animals after a feed restriction.

Concentrations of insulin were dynamically responsive to the effects of feed restriction and the reciprocal change. A decline in insulin was observed in the FRF mares after restriction and an increase followed the return of feed, but did not return to prerestrication concentrations. A similar response was observed in the RFR group, in which peripheral insulin increased after the initial feeding, but not to the same magnitude as in the FRF group. The changes in insulin profiles are similar to the profiles of leptin, because a blunted response was observed in the FRF group after refeeding. This may be a result of a metabolic interaction between leptin and insulin (Schwartz et al., 2000). The elevated concentrations of insulin in the FRF group could be due to an insuliningenic adaptation response, because these animals were on grass pasture before experimentation and then fed alfalfa hay ad libitum thereafter. These observations provide evidence that insulin secretion may be influenced by chronic patterns of feeding, because feed concentrations after restriction did not rise to prerestricition concentrations.

Concentrations of GH also responded inversely coincident with availability of feed supplies. These results agree with previous observations in obese pony mares (Buff et al., 2005), in which mean concentrations, pulse frequency, and pulse amplitude of GH were greatest in feed-deprived obese pony mares. In the current study, GH pulse frequency and area under the GH curve was greater in the RES mares, indicative of their response to feed deprivation. The response to feed restriction or feeding did not differ between treatments, thus providing evidence that GH may be influenced by an acute response to feed restriction rather than patterns of feeding.

Seasonal adaptation of the horse to forage availability may have provided this species a key mechanism to ensure survival during extreme changes in nutrient availability. We assert that the hormonal responses presented herein offer insight into the mechanisms of appetite regulation and body fat management in the horse. The delayed or blunted increase in leptin and insulin, respectively, in response to refeeding may be inherently suppressed or delayed so as to ensure increased feed consumption in preparation for forage scarcity. Understanding these hormonal responses to feed restriction will allow us a better understanding of the homeostatic mechanisms in the horse that are critical to the management of obesity.

LITERATURE CITED


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