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Development of a model for inducing transient insulin resistance in the mare: Preliminary implications regarding the estrous cycle¹,²

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ABSTRACT: Peripheral insulin resistance is the failure of proper cellular glucose uptake in response to insulin. Insulin resistance and hyperinsulinemia are associated with several disease states in the horse and reproductive function disturbances in humans, including polycystic ovarian syndrome. To test the hypothesis that insulin resistance (IR) and hyperinsulinemia disrupt the estrous cycle in mares, two experiments were conducted to first develop a model to induce IR and to then examine the effect of this model on the duration of the estrous cycle. In Exp. 1, a hyperinsulinemic-euglycemic clamp (HEC) procedure was performed on seven mares to determine insulin sensitivity before and immediately following infusion of a heparinized lipid solution. The HEC procedure was repeated 1 wk after lipid infusion. Mares developed IR following the lipid infusion (\(P < 0.05\)), and some individuals maintained IR for up to 1 wk. Mares also exhibited increased blood insulin both immediately following treatment and 1 wk later (\(P < 0.05\)). In Exp. 2, induction of insulin resistance by lipid solution was not accompanied by changes in circulating concentrations of luteinizing hormone, and duration of the luteal phase, compared with the duration of untreated luteal phases. Nonetheless, lipid infusion and the resultant insulin resistance were associated with an increased interovulatory period (\(P < 0.05\)), and peak concentrations of progesterone (\(P < 0.05\)) were higher during the treated vs. untreated luteal phases of the estrous cycle. The results from the preliminary study suggest that infusion of a lipid solution may induce transient insulin resistance and hyperinsulinemia. The resulting insulin resistance and hyperinsulinemia may modify characteristics of the estrous cycle, perhaps at the level of the ovary.

Key Words: Equine, Free Fatty Acids, Insulin, Insulin Resistance, Lipid Infusion, Reproductive Cycle

Introduction

In the horse, obesity is associated with insulin resistance and may predispose individuals to the development of several pathologies, including laminitis (Coffman and Colles, 1983). In addition, obesity and insulin resistance have been associated with disturbances in the duration of the breeding season (Fitzgerald and McManus, 2000) and the duration of the estrous cycle (Fitzgerald et al., 2002).

The mechanisms that lead to disruption of the estrous cycle in obese mares remain to be elucidated; however, increased circulating concentrations of insulin disrupt gonadotropin secretion and consequently reproductive function in many species, including mice (Bruning et al., 2000), pigs (Barb et al., 2001; Mao et al., 2001), and sheep (Bucholtz et al., 2000). In addition, in human females, polycystic ovarian syndrome, a reproductive state characterized by multiple anovulatory follicles (Pettigrew and Hamilton-Fairley, 1997), is associated with insulin resistance and hyperinsulinemia (Nestler, 2000).

There has been limited research on the role of obesity and insulin in the regulation of reproduction in mares. For this reason, the preliminary studies described in this paper were designed to test the hypothesis that transient insulin resistance and the resulting hyperinsulinemia disrupt the estrous cycle. To better understand the significance of insulin resistance in reproductive function in the mare, we developed a model for the induction of transient insulin resistance. This model is based on studies in several species, including humans,
that demonstrated development of insulin resistance following an intravenous infusion of a heparinized lipid solution (Lee et al., 1988; Boden et al., 1995; Paolisso et al., 1995).

Materials and Methods

Horses

Light horse mares (5 to 15 yr of age) were selected according to weight, lean body condition score (Henneke et al., 1983), and low percentage of body fat as estimated using ultrasound of tailhead fat thickness (Kane et al., 1987). The Institutional Animal Care and Use Committee approved all experimental procedures.

Experiment 1

Hyperinsulinemic-Euglycemic Clamp (HEC). Owing to high repeatability (Soop et al., 2000) an HEC procedure, validated for the horse (Powell et al., 2002), was used to determine peripheral insulin sensitivity in all mares (n = 7, mean BW = 532 ± 51.41 kg; mean percentage of body fat = 13.1 ± 4.7%). Briefly, following a 12-h fast, circulating concentrations of glucose were determined at the beginning of the HEC procedure by use of a hand-held glucose meter (One Touch; Johnson and Johnson, New Brunswick, NJ). Hand-held meters were validated for use in the horse by demonstrating that blood glucose values were similar to those measured using a glucose auto-analyzer (YSI 3000 STAT Plus; Yellow Springs Instrument Co. Inc., Yellow Springs, OH). A bolus injection of insulin (0.4 mU/kg crystalline bovine insulin; Sigma-Aldrich, St. Louis, MO) was administered (i.v.) and followed immediately by a constant infusion of insulin (0.1 mU·kg⁻¹·min⁻¹) for 120 min. Two minutes following the start of insulin infusion a 50% (wt/vol) dextrose solution was infused simultaneously (30 mL/min), and the infusion rate was adjusted to maintain euglycemia. Circulating concentrations of glucose were determined every 5 min throughout the period of infusion. As all mares maintained euglycemia within the 120-min period, the rate of glucose infusion during the final 30 min of the HEC procedure was used to determine insulin sensitivity.

Experimental Design. One week after the initial HEC procedure (control period), each mare was infused (i.v.) with a heparinized (0.2 IU·kg⁻¹·min⁻¹) 20% (wt/vol) lipid emulsion (Liposyn II; Abbott Laboratories, North Chicago, IL) at a rate of 2 mL/min for 4 h. Concurrent with the start of the infusion, a 200 IU bolus of heparin was also administered to facilitate lipolysis into free fatty acids (Orme and Harris, 1997). Immediately following lipid infusion, another HEC procedure was executed to determine insulin sensitivity (treatment period). To confirm the anticipated transient insulin resistant condition, an additional HEC procedure was performed 1 wk after infusion of the lipid solution (recovery period).

Collection of Blood Samples. Before each HEC procedure, blood samples were collected at 10-min intervals for 20 min before the HEC to determine physiological concentrations of insulin and glucose. Thereafter, blood samples were collected at 10-min intervals to determine concentrations of insulin. Additional blood samples were collected at 20-min intervals into evacuated tubes (Vacutainer Systems; Becton Dickson, Franklin, NJ) containing EDTA and used for determination of free fatty acids. Sampling for insulin and determination of free fatty acids occurred for 120 min, encompassing the duration of the HEC procedure. Blood samples were immediately centrifuged and the plasma harvested for subsequent determination of FFA concentrations or allowed to clot overnight at 4°C. The next day, blood samples were centrifuged at 1,900 × g and the serum harvested and stored frozen for subsequent analysis of insulin concentration.

Experiment 2

Experimental Design. Mares were selected at random from the general herd, and the HEC procedure was performed to determine insulin sensitivity. Mares that had a glucose infusion rate of greater than 100 mL/h were considered the most insulin sensitive and were therefore selected for the experiment (n = 7). All mares served as their own controls. Estrous cycles were synchronized (Loy et al., 1981), and mares completed one control cycle (n = 7) approximately the first week of May. To decrease the duration of the experiment and thereby minimize the effects of environmental influences on insulin sensitivity, mares were administered prostaglandin F₂, to initiate premature luteolysis. During this “short cycling,” mares again underwent an HEC the first week of June, to ensure sustained insulin sensitivity. Ovarian follicular development was determined at 2- to 3-d intervals by palpation per rectum and ultrasonography. On identification of an ovarian follicle of 30 mm or greater, each mare received a heparinized lipid infusion. This infusion occurred approximately 2 d (2.00 ± 1.5 d; n = 7) before ovulation. Immediately after cessation of the lipid infusion, an HEC was executed to confirm induced insulin resistance. An additional HEC was performed 1 wk later to determine duration of transient insulin resistance.

Collection of Blood Samples. During the estrous cycle, before and after lipid infusion, blood samples were collected three times per week (Monday, Wednesday, and Friday). Samples were subsequently assayed for concentrations of progesterone and LH. Changes in circulating concentrations of progesterone were used to identify the occurrence of ovulation, the interval between successive ovulations (interovulatory interval) and the duration of the luteal phase in each estrous cycle.

Hormone and FFA Determination

Circulating concentrations of insulin were determined by RIA (Coat-A-Count; Diagnostic Products
Figure 1. Changes in mean glucose infusion rates (±SEM; n = 7) during a hyperinsulinemic-euglycemic clamp procedure performed for the control (○), immediately following treatment (□), and recovery (●) periods for Exp. 1. The period immediately following treatment differed from both the control and recovery periods (P < 0.05).

Statistical Analyses

Data are presented as means ± SEM. For Exp. 1, mean glucose infusion rates (GIR), and pre-HEC concentrations of insulin and free fatty acid were analyzed using repeated measures ANOVA employing the procedure of Satterthwaite for degrees of freedom using SAS (SAS Inst. Inc., Cary, NC). For Exp. 2, glucose infusion rates and concentrations of insulin were also evaluated using repeated measures ANOVA. Interovulatory intervals, luteal phase durations, peak and mean concentrations of progesterone, and mean concentrations of LH were compared between control and treated cycles by Student’s paired t-test. In all instances with the use of the repeated measures ANOVA, time was the fixed effect, and the mares were the random effect; consequently, these were the only sources of variation in the model. There was no interaction specified in the model because there is only one fixed effect. The error term used to test the main effects was residual error.

Results

Experiment 1

Glucose Infusion Rates. As depicted in Figure 1, there was a significant difference in glucose infusion rates between treatment periods (P < 0.05). Analysis of differences of least squares means indicated that changes were observed between the control and treatment HEC procedures (2.30 ± 0.29 mg·kg⁻¹·min⁻¹ vs. 1.41 ± 0.25 mg·kg⁻¹·min⁻¹; P < 0.05). Although lipid infusion significantly modified group insulin sensitivity, the GIR in one mare was greater than pretreatment values.
Overall, mean glucose infusion rates did not differ between control and recovery periods (2.02 ± 0.54 vs. 2.29 ± 0.30 mg·kg⁻¹·min⁻¹). However, 1 wk after infusion of the lipid solution, three of six mares that were previously identified as insulin resistant immediately following treatment were found to have maintained an insulin-resistant state compared with pretreatment. One mare that did not display decreased insulin sensitivity immediately following lipid treatment exhibited decreased insulin sensitivity 1 wk after treatment. Collectively, therefore, infusion of a heparinized lipid solution was accompanied by development of insulin resistance in all seven animals between 1 to 7 d after treatment.

Concentrations of Insulin. Concentrations of insulin increased following treatment as illustrated in Figure 2 (P < 0.01). Mean concentrations of insulin tended to be higher for the treated vs. control period (10.11 ± 1.46 vs. 7.15 ± 2.73 μIU/mL; P = 0.18). Circulating insulin values at the time of the recovery were markedly higher than pretreatment concentrations (18.78 ± 3.20 vs. 7.15 ± 2.73 μIU/mL; P < 0.01).

Concentrations of Free Fatty Acids. Mean (±SEM) concentrations of free fatty acids were significantly higher following treatment compared with control values (1.97 ± 0.24 vs. 0.57 ± 0.22 mmol/L; P < 0.001; Figure 3A). However, at the time of the recovery period, HEC mean concentrations of free fatty acids did not differ from control values (0.61 ± 0.11 vs. 0.57 ± 0.22 mmol/L). Mares that maintained decreased insulin sensitivity at the time of the recovery HEC also showed a decreased ability of insulin to stimulate FFA uptake, whereas mares that were sensitive at the time of the recovery showed an increased response to insulin (Figure 3B).

Figure 2. Changes in mean concentrations of insulin (±SEM; n = 7) in blood samples collected immediately before a hyperinsulinemic-euglycemic clamp procedure performed during the control, treatment, and recovery periods during Exp. 1. Means for each period with different letters differ (P < 0.05).

Figure 3. Changes in mean concentrations of plasma FFA during Exp. 1 (n = 7). Panel A illustrates mean (±SEM) concentrations during the hyperinsulinemic-euglycemic clamp for the control (○), treatment (□), and recovery (●) periods. The treatment period differed from both the control and recovery periods (P < 0.001). Panel B illustrates concentrations of FFA in mares identified to be insulin sensitive (▼, n = 2) or resistant (▲, n = 5) during the recovery period during Exp. 1.

Experiment 2

Glucose Infusion Rates. Glucose infusion rates for the control cycle, treated cycle pre-infusion and treated cycle HEC 1 wk posttreatment were not different (Figure 4). The control cycle, treated cycle pre-infusion, and 1 wk after heparinized lipid infusion glucose infusion rates were 2.04 ± 0.19 mg·kg⁻¹·min⁻¹, 2.12 ± 0.26 mg·kg⁻¹·min⁻¹, and 2.10 ± 0.39 mg·kg⁻¹·min⁻¹ respectively. However, as expected, the GIR immediately following treatment was lower than preinfusion values (1.49 ± 0.29 vs. 2.12 ± 0.26 mg·kg⁻¹·min⁻¹; P < 0.05).

Concentrations of Insulin. Pre-HEC concentrations of insulin were different following treatment with heparinized lipid (P < 0.05). However, this varied from the results observed in Exp. 1, in that the greatest increase in concentrations of insulin was observed immediately following treatment compared with control values (4.92 ± 0.90 vs. 3.53 ± 0.73 μIU/mL) rather than at the time of
Mean glucose infusion rates (±SEM) during the hyperinsulinemic-euglycemic clamp for the control cycle (○), treated cycle prelipid infusion (□), treated cycle immediately following treatment (●), and 1 wk following treatment (▲) for Exp. 2. Glucose infusion rates for the period immediately following treatment differed from the control period, the treated cycle preinfusion, and the period 1 wk following treatment (P < 0.05).

Duration of the Luteal Phase and Circulating Concentrations of Progesterone. A trend toward a lengthened luteal phase was observed in the estrous cycle following infusion with a heparinized lipid solution compared with the untreated cycle. The duration of the luteal phase of the cycle was lengthened in three of seven mares after treatment compared with the control cycle (mean durations 19.57 ± 2.66 vs. 15.57 ± 0.97 d, P = 0.09, n = 7). However, of the seven treated estrous cycles, three displayed lengthened interovulatory intervals (IOI) compared with the control cycles and the mean duration of treated cycles was longer than control cycles (26.0 ± 2.41 vs. 20.29 ± 0.78 d, n = 7; P < 0.05).

Mean concentrations of progesterone are depicted in Figure 5. There was no significant difference in mean concentrations of progesterone between treated and control cycle (7.46 ± 1.99 vs. 5.64 ± 1.39 ng/mL; n = 7). Also depicted in Figure 5 is peak progesterone during the luteal cycle. During the estrous cycles of treatment mares, significantly higher peak progesterone values were demonstrated compared with control cycles (12.87 ± 4.03 vs. 8.78 ± 2.23 ng/mL; n = 7; P < 0.05).

Luteinizing Hormone. Mean concentrations of LH did not differ for the duration of the estrous cycle that followed lipid infusion compared with the control cycle (3.95 ± 0.34 vs. 3.29 ± 0.13 ng/mL). Variation existed among mares as to differences in concentrations of LH between the control and treated cycles (Figure 6). One mare (Mare 59) exhibited a markedly higher rise in LH before ovulation during the cycle following treatment.

Discussion

The current study demonstrates that infusion of a heparinized lipid solution resulted in decreased insulin sensitivity in adult horses. In agreement with findings in other species (Boden et al., 1995; Paolisso et al., 1995), development of insulin resistance was accompanied by reduced glucose infusion rates during the HEC procedure and increased insulin concentrations immediately preceding the HEC. However, the timing of the development of insulin resistance appeared variable because one mare displayed insulin resistance only after 7 d after treatment and not immediately after treatment. In human studies, a 3- to 4-h delay in the development of insulin resistance has been reported (Boden et al., 2001). To our knowledge, this is the first report in any species that the development of insulin resistance may occur several days after infusion of a lipid solution and, furthermore, that resistance may be sustained for 1 wk after treatment. An explanation for the latter observation is not forthcoming; however, it is unlikely that the maintenance of insulin resistance was caused by increased circulating concentrations of free fatty acids 1 wk after lipid infusion. At this time point, circulating concentrations of FFA were similar to control values. It has been suggested that in humans the timing of insulin resistance following infusion of lipids coincides with an increase in skeletal muscle hexo-phosphates, whereas there is a decrease of glucose-6-phosphate formation, indicating that the FFA effect on peripheral insulin action is through an increased flux of UDP-N-acetyl-hexosamines into the glucosamine pathway (Hawkins et al., 1997). It is conceiv-
Figure 6. Luteinizing hormone profiles for the control (□, n = 7) and treated (●, n = 7) estrous cycles for Exp. 2. Luteinizing hormone was determined in blood samples collected every 2 to 3 d. The date of ovulation is designated time 0. Infusion of the lipid solution occurred 2 d before ovulation.
patients with polycystic ovarian syndrome. The mechanism by which this occurs may reflect an arrest of follicular development. Insulin can play a role in this phenomenon by enhancement of the effects of FSH on follicles that have acquired LH receptors. The prevailing hypothesis is that small follicles that have just attained LH receptors, in the presence of insulin, display enhanced estradiol production equal to mature follicles, thereby inhibiting further growth and arrest of the follicles in the immature stage (Diamanti-Kandarakis and Bergiele, 2001). It is possible that some mares show longer follicular phases because follicular development has been slowed via this mechanism. In the current study, however, circulating concentrations of LH were not significantly different between the follicular and luteal phases of treated and untreated cycles. This latter observation might suggest that in the short-term any action by insulin is unlikely to involve a change in the hypothalamic-pituitary axis.

An alternative site of action by insulin might be the ovary. In this regard, hyperinsulinemia accompanied by increased steroid secretion by the ovary is observed in humans as noted in women with polycystic ovarian syndrome (Nestler, 2000). Observations from Exp. 2 support these findings because concentrations of progesterone were slightly increased following treatment. Peak concentrations of progesterone were significantly higher during the cycle following treatment with FFA and development of insulin resistance compared with the control cycle. However, because samples were taken only three times per week, the values representing peak progesterone may not represent the true peak, as would have been detected by sampling daily. Because these studies were preliminary, it was determined to be sufficient to collect samples three times per week. Another explanation for the differences observed in concentrations of progesterone could be due to environmental conditions, such as photoperiod, varying from the control to treated cycle. However, the administration of prostaglandin F2α to "short-cycle" the mares was employed to minimize these effects. The observation that insulin resistance and hyperinsulinemia was accompanied by increased steroid secretion by the ovary is consistent with observations made in other species. One such study was conducted in pigs. In gilts, feed restriction during follicular development depressed plasma progesterone; however, when animals were supplemented with insulin during feed restriction, the decreased concentrations of progesterone did not occur (Mao et al., 2001). Another study conducted (Cox et al., 1994) found that estradiol was decreased in diabetic pigs. Similar to the findings of Exp. 2, there was no change in LH secretion, indicating that the absence of insulin decreased steroidogenesis. This observation in pigs and other species support the proposal that insulin exerts a stimulatory effect on progesterone production by the corpus luteum, and this effect is mediated by changes that occur in the developing follicle before ovulation.

**Implications**

Infusion of a heparinized lipid solution is a safe, reliable mean for inducing insulin resistance and hyperinsulinemia in mares. The resulting hyperinsulinemia may modify follicular development and luteal function at the ovarian level rather than the hypothalamic pituitary level, as evidenced by no changes in luteinizing hormone. However, further studies are needed to determine whether a critical period exists in follicular development, during which increases in insulin are influential, and by what mechanism insulin exerts its effects. Finally, this model of inducing transient insulin resistance provides an opportunity to investigate the association between insulin resistance and many diseases. Current animal models of insulin resistance demonstrate cytokine and hormone production disturbances associated with insulin resistance. Similar studies in horses may provide insight into the relationship between insulin resistance and development of laminitis, osteochondrosis dessicans lesions, and other disorders.

**Literature Cited**


