ABSTRACT: Four experiments were conducted 1) to assess the use of glucose responses to insulin injections as a means of estimating insulin sensitivity in horses and 2) to compare the insulin sensitivities of normal horses vs. those displaying hyperleptinemia (HL). In Exp. 1, HL mares and geldings (n = 4 each) and 4 mares and geldings with normal leptin concentrations (NL) were injected intravenously with 20 and 100 mU/kg of BW of bovine insulin on 2 separate occasions in December 2008. In Exp. 2, the experimental protocol was repeated in late April 2009. In Exp. 1, the 20 mU/kg of BW dose of insulin caused a greater \( P < 0.05 \) decline in glucose concentrations in NL mares and geldings compared with HL horses. The response of HL mares to the 100 mU/kg of BW dose was less \( P < 0.05 \) than for the other groups. In Exp. 2, responses of all groups to the 20 mU/kg of BW dose were small and similar among groups \( P > 0.1 \), whereas the greater dose revealed differences \( P < 0.05 \) in sensitivity among groups consistent with those observed with the smaller dose in Exp. 1. Experiment 3 was conducted in June and July of 2009 to further examine the dose-response relationship in mares of potentially different insulin sensitivities in an attempt to standardize the approach for studying a wide range of sensitivities. Recombinant human insulin was used at doses of 8, 20, 50, and 125 mU/kg of BW, as needed, to estimate (by linear regression) the dose of insulin causing a 50% decline in glucose concentrations (ED50). Five mares each of reduced leptin concentrations (LL) and small BCS (3 to 5), LL and larger BCS (6 to 7.5), and increased leptin concentrations and increased BCS were studied. The ED50 was similar \( P > 0.1 \) for LL mares, regardless of BCS, and was less \( P < 0.01 \) than for mares with increased leptin concentrations. It was concluded that a dose of 50 mU/kg of BW of recombinant human insulin could be used safely to start the dose-response curve; smaller or larger doses could then be applied as appropriate to get sufficient data for estimation of ED50. Experiment 4, conducted in October of 2009, assessed the repeatability of the estimates for ED50 obtained in Exp. 3. Six mares with LL vs. increased leptin concentrations received the 50 mU/kg dose of insulin; appropriate larger or smaller doses were used to obtain estimates of ED50. Estimates obtained were highly correlated \( r = 0.91 \) with those obtained in Exp. 3, with an average within-mare CV of 8.9%; this is equal to or better than the repeatabilities of the currently used methods of assessing insulin sensitivity in horses. It was concluded that hyperleptinemic horses, which are also hyperinsulinemic and have exaggerated insulin responses to glucose injection, are indeed less sensitive to insulin than normal horses with reduced leptin concentrations of the same body condition.

Key words: gelding, glucose, hyperleptinemia, insulin sensitivity, mare

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INTRODUCTION

Insulin sensitivity in horses is typically measured via 1 of 2 standard methods: 1) hyperinsulinemic-euglycemic clamp, in which insulin is infused at 1 or more fixed doses and sufficient glucose is infused to maintain euglycemia (CLMP; Kaske et al., 2001; Powell et al., 2002; Rijnen and van der Kolk, 2003), or 2) minimal modeling of the insulin-modified, frequently sampled intravenous glucose tolerance test (FSIGT; Bergman et al., 1987), in which a bolus of glucose is administered at time 0, and then a bolus of insulin is administered 20...
min later (Hoffman et al., 2003; Treiber et al., 2005). Although direct insulin injection has been used in various experimental settings with horses and mules (Silver et al., 1987; Alexander et al., 1997; Forhead and Dobson, 1997), it has not been routinely used as a method of estimating insulin sensitivity, either clinically or experimentally. One concern is insulin overdose, which might lead to hypoglycemic shock (Given et al., 1988). In addition, there is a chance of inducing laminitis with repeated injections (Asplin et al., 2003). However, doses of insulin up to 0.4 USP units (U) per kg of BW were administered to feed-deprived donkeys without any reported serious side effects (Forhead and Dobson, 1997). Similarly, we have noticed no detrimental effects in horses administered a single intravenous (i.v.) dose of insulin of 0.1 U/kg of BW (Gentry et al., 1999).

The purpose of the present series of experiments was to develop a safe, direct assessment of insulin sensitivity that could be applied experimentally and perhaps clinically, to horses. We hypothesized that hyperleptinemic (HL) horses would have a reduced insulin sensitivity relative to normal horses because they display increased insulin concentrations and exaggerated insulin responses to glucose infusion (Cartmill et al., 2003), which are indicative of insulin insensitivity. To date, there is no report indicating that these horses have reduced insulin sensitivity.

**MATERIALS AND METHODS**

Procedures used were approved by the Institutional Animal Care and Use Committee of the Louisiana State University (LSU) Agricultural Center; all 4 experiments were conducted at the LSU Agricultural Center Horse Unit in Baton Rouge.

**Exp. 1**

Experiment 1 was designed to determine whether the glucose responses to 2 different doses of bovine insulin would differ between normoleptinemic (NL) and HL mares and geldings. Sixteen adult (5 to 19 yr) horses (8 mares and 8 geldings) of light horse breeds with moderate to large BCS (range = 5 to 7.5; determined approximately 7 d before onset of the experiment by 2 technicians; Henneke et al., 1983) were designated as HL or NL based on mean plasma leptin concentrations recorded on 3 separate occasions in 3 different seasons of the same year before performing this experiment. Although leptin concentrations vary with BCS (Buff et al., 2002; Gentry et al., 2002), feeding regimen (Steelman et al., 2006; Storer et al., 2007), and time of year (Fitzgerald and McManus, 2000; Gentry et al., 2002), the HL horses consistently ranked the greatest in the resident herd and had average leptin concentrations outside the 95% confidence interval of those animals considered NL. All horses were kept on pasture, the grasses of which were mainly dormant in December, and were supplied native grass hay (round bales) for ad libitum consumption.

The horses were grouped based on sex, BCS, and mean leptin concentrations: NL mares (BCS = 6.4 ± 0.2; leptin < 2 ng/mL), HL mares (BCS = 6.6 ± 0.1; leptin > 12 ng/mL), NL geldings (BCS = 6.4 ± 0.6; leptin < 5 ng/mL), and HL geldings (BCS = 6.8 ± 0.3; leptin > 12 ng/mL). On d 1 of the experiment (December 15, 2008), 1 of 2 different dosages of bovine insulin (20 and 100 mU/kg of BW; Sigma Chemical Co., St. Louis, MO, catalog No. I550; 27 U/mg) was administered to each horse; doses were randomly assigned such that one-half of the horses received each dose. Three days later, each horse received the opposite dose.

For each treatment day, horses were deprived of feed overnight (approximately 13 h) in a drylot paddock with ad libitum access to water. Between 0630 and 0800 h on the morning of testing, each horse was fitted with a 14-ga indwelling jugular catheter (Becton Dickinson, Nutley, NJ) that was kept patent with aqueous 6% sodium citrate solution. Insulin injections were administered via the catheters as bolus i.v. injections (in saline solution at 0.01 mL/kg of BW) followed by 3 mL of the citrate solution. Blood samples were collected at −20, 0, 20, 40, 60, 90, 120, 180, and 240 min relative to insulin injection, and plasma glucose concentrations were estimated in duplicate in the whole blood with a hand-held glucometer (Precision Xtra, Abbott Laboratories, Abbott Park, IL; Eiler et al., 2005) within 30 s of blood withdrawal. A 5-mL aliquot of blood was also transferred to a 7-mL tube containing potassium oxalate and NaF; plasma was harvested after centrifugation at 1,200 × g for 15 min at 5°C and was stored at −15°C.

Plasma leptin concentrations were determined in the preinjection samples by RIA described by Cartmill et al. (2003). The intra- and interassay CV averaged 6 and 4%, respectively. Sensitivity of the leptin assay, based on a 0.2-mL sample size, was 0.1 ng/mL (Cartmill et al., 2003).

Glucose concentrations were analyzed by ANOVA using the GLM procedure (SAS Inst., Cary, NC) for a replicated Latin square design with repeated measures; the main effects of sex and leptin status (NL vs. HL) were arranged in a 2 × 2 factorial. Dose was considered the first application of repeated measures, and sampling times was considered second repeated measures within each dose. Appropriate error terms were selected to test each main effect and interaction.

The glucose data were also used to calculate the percentage decline in glucose concentration for each horse on each occasion. The least glucose concentration in the first 90 min after insulin injection was subtracted from the mean preinjection concentration and then divided by that mean preinjection concentration; the result was multiplied by 100 to give a percentage. These data were analyzed by ANOVA with a 2 × 2 factorial arrangement of main effects (sex and leptin status) for each in-
sulfur dose; differences among means were assessed with the LSD test. Mean preinjection leptin concentrations and BCS were also analyzed by ANOVA with a 2 × 2 factorial arrangement of main effects (sex and leptin status); differences among means were assessed with the LSD test.

Exp. 2

A repeat of the procedures in Exp. 1 was conducted in late April of 2009 with the aim of assessing the repeatability of the results. The same horses were used; with one exception (1 NL gelding had to be replaced for reasons unrelated to the previous experiment). The horses had been maintained on winter (annual) ryegrass pasture from approximately mid-January. At the time of Exp. 2, native grasses and clovers had also become active and were being grazed along with the ryegrass.

Based on the results of Exp. 1 and our previous report (Gentry et al., 1999), the blood sampling schedule was reduced to 4 samples relative to insulin injection: −10, 0, 40, and 50 min, and samples were collected via 20-ga needles by jugular venipuncture. Doses of insulin and procedures for treatments and handling of blood samples were as described in Exp. 1. The percentage decline in glucose concentrations were calculated and then analyzed for each dose by ANOVA with a 2 × 2 factorial arrangement of main effects (leptin status and sex); differences among means were assessed with the LSD test.

Exp. 3

Given the differences in apparent insulin sensitivities observed between December and April in Exp. 1 and 2, Exp. 3 was designed with the goal of developing a repeatable protocol of insulin injections for assessing insulin sensitivity across a wide range of sensitivities. Starting in June of 2008, 15 mares from the resident herd were selected with the following characteristics: reduced leptin concentrations and small BCS (LL/HBCS; n = 5), reduced leptin concentrations and larger BCS (LL/HBCS; n = 5), and HL and HBCS (HL/HBCS; n = 5). The mares were maintained on native grass pastures, which were predominantly Bermuda-grass, bahiagrass, and dallis grass.

The original plan was to conduct the experiment as a Latin square design with 3 doses of human recombinant insulin (20, 50, and 125 mU/kg of BW; Sigma, catalog No. J2643, 27.5 U/mg) after overnight feed deprivation. Human recombinant, rather than pancreatic bovine, insulin was chosen for 2 reasons: potentially greater long-term availability and greater consistency of the product over time. After treating 1 replicate of mares, in which a LL/HBCS mare was treated with the 125 mU/kg of BW dose and displayed signs of lethargy, it was decided that a gradual ramping up from the smallest dose (20 mU/kg of BW) would be necessary from a safety standpoint. Over the next several weeks, mares were injected in small groups with doses of insulin of 8, 20, 50, or 125 mU/kg of BW, depending on their responses. That is, all mares received the 20 and 50 mU/kg of BW dose, and the percentage declines were calculated. The 125 mU/kg of BW dose was administered only to mares not experiencing at least a 50% decline in glucose to the smaller doses. The 8 mU/kg of BW dose was added primarily for mares experiencing a 50% decline or greater to the 20 and 50 mU/kg of BW doses, but was subsequently administered to all mares.

Blood sampling in Exp. 3 was via jugular venipuncture through 20-ga needles; approximately 3 mL of blood was collected into 5-mL syringes at −10, 0, 40, 60, 90, 120, 180, and 240 min relative to insulin injection. After approximately 1 mL of blood was expressed from the syringe, a drop of whole blood was used to estimate plasma glucose concentration with the glucometer. Estimates were generally based on 1 glucometer strip reading; replicate readings were conducted whenever a value seemed unreasonable (about 5% of readings). An earlier assessment (after Exp. 2 but before Exp. 3) of the glucometer (Precision Xtra, Abbott Laboratories) for duplicate readings of 15 blood samples between 77 and 335 mg/dL resulted in a regression equation: second estimate = 1.03 × (first estimate) + 3.4 mg/dL (r = 0.98).

The percentage decline in glucose concentrations was calculated for all injections and plotted against the natural log (ln) of the insulin dose for each mare. In general, these plots resembled a typical dose-response curve, with a linear portion between 20 and 60%. Linear regression analysis was used to calculate the regression equation for each mare [x = ln(dose) and y = % decline], and the ln of the dose of insulin resulting in a 50% decline in glucose concentration [ln(ED50)] was estimated from that equation; ED50 was calculated by taking the antilog of ln(ED50). Estimates of ln(ED50) and ED50 were based on at least 3 doses of insulin. In 4 of the 5 HL/HBCS mares, all percentage declines were less than 50%; thus the estimate of ln(ED50) was an extrapolation to 50%.

Glucose concentrations were analyzed separately for each leptin status-BCS group by 1-way ANOVA with repeated sampling, with dose as the main effect and blood sampling times as the repeated effect. From that analysis, comparisons of postinjection glucose concentrations were compared (LSD test) with the mean at time 0 to determine when they were no longer different; this was considered to be the time of recovery. Body condition scores and the ln(ED50) and ED50 estimates were analyzed by 1-way ANOVA, and differences among groups were assessed with the LSD test.

Exp. 4

Experiment 4 was conducted with 2 main objectives: 1) to determine if the insulin injection scheme could be streamlined and standardized and 2) to determine the repeatability of the ln(ED50) estimates obtained in
Exp. 3. Twelve mares previously tested (6 LL/HBCS and 6 HL/HBCS) were retested during October of 2009, with 1 d of no treatment between each day of insulin injection. Although the quality and quantity of pasture grasses in October would not be expected to be identical to those in the summer, it was assumed that the relative insulin insensitivity displayed by hyperleptinemic mares in Exp. 3 would persist because we have observed that the hyperleptinemic condition itself persists over years.

The standard approach was to treat each mare with the 50 mU/kg of BW dose of recombinant human insulin on the first day. The mares were deprived of feed overnight (with ad libitum access to water) and treated the next morning between 0700 and 0900 h. Blood samples were collected via jugular venipuncture (as described for Exp. 3) at −10, 0, 40, and 60 min relative to i.v. injection of human recombinant insulin. Plasma glucose concentrations were estimated with the glucometer as described in Exp. 3.

Depending upon the percentage decline in glucose concentration for a given mare, the second injection 2 d later was 32 mU/kg of BW for those mares exhibiting a 50% decline or greater to the 50 mU/kg of BW or 79 mU/kg of BW for those exhibiting less than a 50% decline. The dose on the third day was 20 or 125 mU/kg of BW. The goal was to bracket the approximate 50% point; if the first 2 injections were on each side of 50%, the selected third dose chosen was on the lower, rather than higher, end of the dose-response curve. The third dose was administered 2 d after the second, so that the entire 3-injection protocol was completed in 5 d.

The ln(ED50) and ED50 values were calculated for each mare as described in Exp. 3. These data were analyzed by 1-way ANOVA to test the effect of leptin status. In addition, the percentage decline in glucose values in response to the 50 mU/kg of BW dose of insulin and the calculated ln(ED50) values from Exp. 3 were compared with those obtained in Exp. 4 by linear regression analysis as an assessment of the repeatability of the estimates.

RESULTS

Exp. 1

The mean BCS of the mares and geldings in Exp. 1 were similar \( (P > 0.10) \) among the 4 groups (Figure 1A). Mean leptin concentrations in HL horses were approximately 4 to 6 times greater \( (P < 0.001) \) than in NL horses (Figure 1B).

The changes in plasma glucose concentrations after injection of bovine insulin and the mean percentage decline are presented in Figure 2. After injection of insulin at 20 mU/kg of BW (Figure 2A and 2B), plasma glucose concentrations declined by an average of 45% in NL horses, which was greater \( (P < 0.01) \) than the average decline in HL horses \( (16\%; \text{SEM} = 8.2\%) \). At the greater dose of insulin \( (100 \text{ mU/kg of BW}; \text{Figure 2C and 2D}) \), the difference between HL and NL horses was less obvious, with HL mares exhibiting a lesser \( (P < 0.05) \) percentage decline in glucose concentrations than NL mares and HL geldings; the response of NL geldings was less \( (P < 0.05) \) than that in HL geldings.

Exp. 2

When the insulin doses were repeated in April 2009, the mean percentage decline in glucose concentration after the 20 mU/kg of BW dose (Figure 3A and 3B) was less \( (P < 0.05) \) in HL horses compared with NL mares and HL geldings. In contrast, injection of the greater \( (100 \text{ mU/kg of BW}; \text{Figure 3C and 3D}) \) resulted in differences \( (P < 0.01) \) in percentage decline in glucose concentrations similar to those observed in December of 2008 for the 20 mU/kg of BW dose: HL horses had lesser percentage decline (20 to 25%) than NL horses (approximately 45 to 50%).
Exp. 3

Glucose concentrations in response to various doses of recombinant human insulin in mares with LL/LBCS, LL/HBCS, and HL/HBCS are presented in Figure 4. In mares with LL/LBCS (Figure 4A), injection of 8, 20, and 50 mU/kg of BW produced mean dose-dependent declines ($P < 0.001$) in glucose concentrations of 23.4, 43.1, and 64.3%, respectively (SEM = 6.2%). Likewise, injection of the same doses in mares with LL/HBCS (Figure 4B) produced mean dose-dependent decreases ($P < 0.001$) in glucose concentrations of 26.8, 41.2, and 54.8%, respectively (SEM = 7.3%). Injection of doses of 8, 20, 50, and 125 mU/kg of BW to mares with HL/HBCS (Figure 4C) produced mean dose-dependent declines ($P < 0.01$) in glucose concentrations of 9.0, 16.6, 32.6, and 47.5%, respectively (SEM = 5.4%).

In addition to the initial percentage decline in glucose concentrations (40 or 60 min after injection), there were differences among groups in the recovery of glucose concentrations back to preinjection concentrations. Mares with LL/LBCS displayed delayed recovery ($P < 0.05$) at the 50 mU/kg of BW dose; in general, the recoveries were similar for other doses in all 3 groups.

Mean BCS (Figure 5A) of mares with LL/HBCS and HL/HBCS were similar ($P > 0.1$), but were both greater ($P < 0.05$) than mean BCS of mares with LL/LBCS. Mean ln(ED50) and ED50 were similar for mares with LL/LBCS and LL/HBCS (Figure 5B and 5C); both were less ($P < 0.01$) than the respective means for mares with HL/HBCS.

Exp. 4

Estimates of ln(ED50) and ED50 based on the standardized approach in October of 2009 in the 12 mares that had been assessed during the previous summer are presented in Figure 6A and 6B. As in the summer, LL/HBCS mares had lesser ($P < 0.001$) values in both cases relative to HL/HBCS mares. All mares were first administered the 50 mU/kg of BW dose of recombinant human insulin; the percentage decline in glucose concentrations for those injections were highly correlated ($P < 0.01$; $r = 0.92; \text{decline in October} = 0.72 \times \% \text{ decline in June and July} + 6.98\%$) to the responses obtained earlier (Figure 7A). Estimates of ln(ED50), calculated after the subsequent injection of greater (79 and 125 mU/kg of BW) or lesser (20 and 32 mU/kg of BW) doses, as appropriate, were also highly correlated [$P < 0.01; r = 0.91; \text{ln(ED50) in October} = 0.77 \times \text{ln}(\text{ED50}) \text{ in June and July} + 1.4$] with those obtained earlier (Figure 7B).
DISCUSSION

In the course of these experiments, more than 200 insulin injections were given to mares and geldings. The only indication of any side effects to treatment was that described for the LL/HBCS mare that was treated with the 125 mU/kg of BW dose of human insulin early in Exp. 3. Based on that experience, it was decided to modify the originally planned procedure such that all horses first received a moderate dose of insulin (20 mU/kg of BW in Exp. 3 and 50 mU/kg of BW in Exp. 4), and subsequent injections were based on the response of an individual horse to that dose. Again, as a precaution, no horse was administered a dose above 125 mU/kg of BW, even when that dose did not reduce glucose concentrations at least 50%. This forced us to extrapolate beyond the actual data to calculate ln(ED50) for the least sensitive mares. Because of this extrapolation, our estimate of an actual ED50 greater than 125 mU/kg of BW could be expected to be less precise than for those estimates less than 125 mU/kg of BW; however, a horse with an ED50 greater than 125 mU/kg of BW would be considered insensitive, regardless. Moreover, the repeatability of the ln(ED50) estimates in Exp. 3 and 4 would indicate that this is likely not a serious limitation to the estimation procedure.

The glucose response to insulin injection seems to be composed of 2 phases, the second of which is only noticeable at larger insulin doses relative to the sensitivity of the horse. The first and immediate component is the decline from time of injection to the occurrence of the nadir in glucose concentrations, usually within the first 60 min. This is assumed to be primarily due to the uptake of glucose by peripheral tissues, mostly skeletal muscle, but also liver and adipose tissue. After small doses of insulin, glucose concentrations rapidly recover and return to preinjection concentrations by approximately 90 to 120 min postinjection. The second phase, observed after the largest doses of insulin, is a slow recovery, such that glucose concentrations stay depressed longer and return to baseline 60 to 90 min later than after the smaller insulin doses. This latter effect likely reflects continued suppression of liver output of glucose from glycogenolysis, gluconeogenesis, or both. Because the 2 standard methods of assessing insulin sensitivity (CLMP and FSIGT) supposedly measure primarily or solely the first component (muscle, liver, and adipose tissue uptake), we finally settled on the response in the first 40 to 60 min after injection as the best indicator of that event. The occurrence of the glucose nadir at 40 min was about equal to that at 60 min (48 vs. 52% of all responses, respectively).

Figure 3. Mean glucose concentrations (panels A and C) and percentage decline in glucose concentrations relative to preinjection concentrations (percentage decline; panels B and D) in mares and geldings with normal leptin concentrations (NL) and hyperleptinemia (HL) in Exp. 2. Horses received bovine insulin intravenously at time 0 at 20 mU/kg of BW (panels A and B) or 100 mU/kg of BW (panels C and D) on 2 occasions in late April 2009 in a replicated Latin square design. a,b Means without a common letter differ (P < 0.05). Pooled SEM were 4.3 mg/dL for glucose concentrations and 5.6% for the percentage decline in glucose concentrations.
Although Exp. 2 was designed as a test of repeatability of the results obtained in Exp. 1, the responses in April indicated that all horses were less sensitive to insulin (smaller declines in glucose concentrations to the same dose of insulin). We suspect that this difference was due to the differences in nutrient intake of the horses. The main source of intake in December was native grass hay in round bales. As noted by Storer et
(2007), when horses were fed grass hay in a drylot situation, they had consistently less plasma insulin and leptin concentrations than when they were housed on native grass pasture. This was likely due to the difference in readily absorbable carbohydrates in the two feeding regimens because horses fed high-carbohydrate diets have been reported (Hoffman et al., 2003; Treiber et al., 2005) to have decreased insulin sensitivity relative to those fed a diet based on fat and fiber. Although the relative insulin sensitivities of all the horses were less in April than December, the differences between HL and NL persisted.

As indicated by the results from Exp. 1 and 2, the proper dose of insulin is important for gaining meaningful information about apparent insulin sensitivity. That is, insulin doses too small or too large on the dose-response curve, if administered as a single dose, are less able to differentiate between horses of decreased and increased insulin sensitivities. As data collection progressed in Exp. 3, it became obvious that points between 20 and 60% decline in glucose concentrations provided the most reliable regression lines. Because of this, smaller increments in the insulin doses (the 32 and 79 mU/kg of BW doses) were added for assessments in Exp. 4. These doses allowed for closer bracketing of the 50% decline point for horses exhibiting percentage decline in glucose concentrations close to 50% after administration of the 50 mU/kg of BW dose (starting dose).

Figure 6. Mean natural log (ln) of the dose of insulin that caused a 50% decline in glucose concentrations (ED50; panel A) and ED50 (panel B) for 6 mares with low leptin concentrations (LL) and high BCS (HBCS) vs. 6 mares with high leptin concentrations (HL) and HBCS originally assessed for insulin sensitivity in Exp. 3 and reassessed in October 2009, in Exp. 4. *a,bMeans without a common letter differ (P < 0.05). Pooled SEM were 0.22 for the ln(ED50) and 74 mU/kg of BW for ED50.

Figure 7. Regression analysis for the percentage decline in glucose concentrations (panel A) and the natural log (ln) of the dose of insulin that caused a 50% decline in glucose concentrations (ED50) for data collected in Exp. 3 vs. 4 from 6 mares with decreased leptin concentrations and high BCS and 6 mares with increased leptin concentrations and high BCS. In each case, the data were highly correlated (r > 0.90; P < 0.001).
In addition to the horses used in these 4 experiments, another 9 mares and 9 geldings were tested as part of a comprehensive assessment of the LSU herd. Throughout all the tests, the greatest percentage decline in glucose concentrations observed was 78% in a gelding administered insulin at 125 mU/kg of BW. The percentage decline >70% was obtained on a few other occasions, and we suspect that the upper limit, without noticeable side effects, may be around 80%. We decided on ED50 as our standard based on its common use in classical dose-response (sigmoidal curve) analyses. However, it is based on the assumption that percentage decline in glucose concentrations can range from 0 to 100%, which is unlikely from a physiological standpoint. A truer ED50 point might be based on a decline in 40% of preinjection values (i.e., 50% of 80%); however, a retrospective recalculation of our data from Exp. 3 and 4 based on a 40% decline as the reference point did not alter the results (differences among groups or correlations) in any meaningful way.

The calculation of \( \ln(ED50) \) and ED50 in these experiments was based on regression analysis of the ln of the insulin dose and percentage decline in glucose concentrations, and in general, 3 doses of insulin provided linear regression equations with large coefficients of determination. In the process of developing a standardized procedure for estimating ED50, we tested whether the first 2 doses of insulin would be predictive of the final estimates based on 3 doses. Our conclusion was that 2 doses provided good estimates in most cases in which the ED50 was low, but were less adequate for horses of reduced insulin sensitivity. Data from a single dose of insulin (50 mU/kg of BW) seem to provide a close approximation of the sensitivity to insulin of an animal; however, to be applicable across a wide range of sensitivities, we feel the procedure with 3 insulin doses provides the most reliable and repeatable information.

Pratt et al. (2005) assessed the repeatability of the CLMP and FSIGT methods of estimating insulin sensitivity in horses by administering each test twice to 6 horses in a 4-wk period. The interday CV of insulin sensitivity estimates averaged 14.1% (range, 7 to 20%) and 23.7% (range, 9 to 35%) for the CLMP and FSIGT tests, respectively. For comparison, a similar calculation for the data in Figure 7B (actually a month or more apart) resulted in an average within-horse CV of 8.9% (range = 2.3 to 18.8%). Pratt et al. (2005) concluded that the interday CV for the CLMP was less than for the FSIGT; thus, the repeatability of our method is at least equivalent, if not better, than that of the CLMP.

One limitation of the present approach to estimating ED50 is the time involved. The final 3-injection regimen established takes 5 d to complete, given the 1-d rest (nontreatment) period between injections. Whether the injections could be done in 3 successive days, or even closer together, needs to be determined. The potential carryover from 1 injection to the next also needs to be studied. Also, horses used in the current experiments had preinjection glucose concentrations within the normal range for feed-deprived horses; it is not known whether our approach would be applicable to horses with severe hyperglycemia (e.g., glucose concentrations of ≥200 mg/dL). Third, our assessments of possible detrimental effects were limited to external signs and would not detect microscopic changes in hoof lamellar tissues, such as those reported by Asplin et al. (2007). For comparison, the ponies treated by Asplin et al. (2007) had mean insulin concentrations of 1,036 mU/L over a 72-h perfusion period. Peak concentrations expected in our horses at the 125 mU/kg of BW dose would be approximately 1,900 to 2,500 mU/L in the first 10 min after injection (assuming a 5 to 7% of BW plasma volume), which would decay back to normal within a few hours (Gentry et al., 1999; Cartmill, 2004). Using area under the curve (concentration × hours) as an index of exposure to insulin, our greatest dose produced ≤7,500 area units, whereas the ponies in Asplin et al. (2007) experienced an average of 74,592 area units, or 10 times more than our largest dose. Although much of the data reported herein concerns the development of the approach of direct assessment of insulin sensitivity by i.v. administration of insulin, all 4 experiments confirmed our original speculation that HL horses have reduced insulin sensitivity (greater ED50) relative to horses with normal or decreased leptin concentrations. These experiments also confirmed the persistence of the relative insensitivity in HL horses from December through the following October. Earlier attempts to measure insulin sensitivity with the CLMP and FSIGT techniques in similar horses (HL and NL) were variable and indicated no difference between horses in different leptin classifications (Cartmill, 2004), sex, or BW, even though insulin concentrations in response to glucose infusion in the FSIGT were exaggerated, indicative of insulin resistance. The reason for this lack of detection of differences is unclear, but may be in part due to technician experience, to variation among the horses used in those trials, or to relative sensitivities of the detection methods. A comparison of various techniques for assessing insulin action in normal, obese, and type 2 diabetic humans (Davis et al., 1993) showed a similar variation in the ability of the techniques to differentiate among groups.

Cartmill et al. (2003, 2005) reported that HL horses had increased insulin concentrations, and Storer et al. (2007) confirmed that this increase in insulin concentrations persisted in HL horses even when they were maintained solely on grass hay. Given that leptin can be stimulated directly by insulin infusion (while maintaining glucose concentrations within normal limits; Cartmill et al., 2005), we suspect that the hyperleptinemic condition is a result of reduced insulin sensitivity, which equates to long-term increases in insulin concentrations and hence a long-term stimulation of adipose tissue output of leptin. Although most of the HL horses we have studied over the years have had large BCS, Huff et al. (2008) reported that 11 of 24 HL mares (postfoaling and lactating) had BCS between 4 and 5.5. Thus, the
hyperleptinemic condition is not always associated with obesity (BCS of 7 and above), and as Huff et al. (2009) reported, is not associated with alteration of the base sequence of exon 2 of the equine leptin gene.

In conclusion, dose-response analysis of glucose responses to direct insulin injections seems to be a useful approach for assessing insulin sensitivity in horses with relatively normal preinjection glucose concentrations. Based on this approach, it was concluded that hyperleptinemic horses, which are also hyperinsulinemic and have exaggerated insulin responses to glucose injection, are indeed less sensitive to insulin than normal horses with decreased leptin concentrations of the same body condition.

LITERATURE CITED