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Equine hyperinsulinemia: investigation of the enteroinsular axis during insulin dysregulation

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de Laat MA, McGree JM, Sillence MN. Equine hyperinsulinemia: investigation of the enteroinsular axis during insulin dysregulation. *Am J Physiol Endocrinol Metab* 310: E61–E72, 2016. First published November 3, 2015; doi:10.1152/ajpendo.00362.2015.—Compared with some other species, insulin dysregulation in equids is poorly understood. However, hyperinsulinemia causes laminitis, a significant and often lethal disease affecting the pedal bone/hoof wall attachment site. Until recently, hyperinsulinemia has been considered a counterregulatory response to insulin resistance (IR), but there is growing evidence to support a gastrointestinal etiology. Incretin hormones released from the proximal intestine, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide, augment insulin secretion in several species but require investigation in horses. This study investigated peripheral and gut-derived factors impacting insulin secretion by comparing the response to intravenous (iv) and oral D-glucose. Oral and iv tests were performed in 22 ponies previously shown to be insulin dysregulated, of which only 15 were classified as IR (iv test). In a more detailed study, nine different ponies received four treatments: D-glucose orally, D-glucose iv, oats, and commercial grain mix. Insulin, glucose, and incretin concentrations were measured before and after each treatment. All nine ponies showed similar iv responses, but five were markedly hyperresponsive to oral D-glucose and four were not. Insulin responsiveness to oral D-glucose was strongly associated with blood glucose concentrations and oral glucose bioavailability, presumably driven by glucose absorption/distribution, as there was no difference in glucose clearance rates. Insulin was also positively associated with the active amide of GLP-1 following D-glucose and grain. This study has confirmed a functional enteroinsular axis in ponies that likely contributes to insulin dysregulation that may predispose them to laminitis. Moreover, iv tests for IR are not reliable predictors of the oral response to dietary nonstructural carbohydrate.

insulin; incretin; glucagon-like peptide-1; glucose; horse

THE BIOLOGY OF INSULIN SECRETION and action and the disruption of these processes are substantively investigated in both humans and laboratory animal models due to their expanding impact on human health and economics (1). The consequences of insulin dysregulation for animal production and companion animal health are also emerging as important fields of research (3, 22, 39). Despite its potential relevance to human metabolic syndromes and insulin resistance (IR), equine metabolic syndrome (EMS) rarely proceeds to type 2 diabetes mellitus and is poorly understood. Hyperinsulinemia causes laminitis in horses, a common and often lethal disease affecting the pedal bone/hoof wall attachment site (4, 14, 31, 38). Although there are several triggers for laminitis (6), it is most commonly associated with endocrine dysregulation [such as pituitary pars intermedia dysfunction (PPID)] and/or the grazing of pastures that are rich in nonstructural carbohydrates (NSC) (20).

Understanding why certain horses develop hyperinsulinemia when others do not, even though they are fed the same diet, is the key to preventing laminitis. It is largely accepted in many species that IR develops secondarily to persistent stimulation and subsequent downregulation of insulin receptors during pancreatic overstimulation, usually in association with dietary factors (28, 34). In horses, there is mounting evidence to suggest a similar scenario occurs, with the existence of an EMS phenotype that encompasses regional obesity and insulin dysregulation (19). It is also known that ponies tend to be more IR than horses (26). Furthermore, fasting hyperinsulinemia is an inconsistent finding in horses/ponies diagnosed with EMS, which may support the theory that IR develops as a downstream event of insulin dysregulation and even then only in some animals.

Traditionally, diagnostic tests for IR have employed intravenous glucose tolerance tests (IVGTT) and/or combined glucose-insulin tests (CGIT), which are clinically more practical although less precise, than gold standard tests such as the euglycemic-hyperinsulinemic clamp. However, due to the relative difficulty of performing even these tests in a field environment, modified oral glucose tests (OGTs) have become increasingly popular with clinicians. In addition, demonstration of an excessive insulin response to oral glucose may be more closely aligned with the paddock grazing situation, where ingestion of pasture or grain high in NSC has been repeatedly associated with an increased risk for laminitis (19). However, OGTs do not provide a measure of IR, which is often assumed. Nonetheless, in this paper we question whether IR is, in fact, the most useful indicator of laminitis risk. Instead, we argue that hyperinsulinemia is the critical factor, and that this may originate not from a failure of peripheral glucose uptake but from other, primarily gastrointestinal, causes.

Studies in other species have shown that the incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP, previously gastric inhibitory peptide), are released from the intestine following carbohydrate consumption and work in concert with glucose to augment pancreatic insulin release (5). The relative contribution of incretins to insulin production varies considerably among species, ranging from up to 70% in humans to almost zero in obligate carnivores such as the cat (21, 40). To date, very few studies have investigated enteroinsular signaling in the horse (7, 11, 15, 35). Thus, the present study had two objectives. First, we aimed to compare the insulin response when glucose was delivered orally with the response seen following iv delivery to determine whether the hyperresponsiveness to glucose seen in some animals was dependent on, or independent of, the route of glucose administration. Second, we sought to

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Table 1. *Characteristics of ponies used in 3 experiments to examine the endocrine response to oral and iv D-glucose*

Exp.	Group	*n*	Sex	Age, yr	BW, kg	BCS, /9	CNS, /5	FGIR	2-h Insulin, μIU/ml
1		22	12 F; 10 M	15.9 ± 1.2	227 ± 20	7.1 ± 0.3	3.1 ± 0.2	0.5–42	166 ± 13
2 & 3	NI	4	1 F; 3 M	11.8 ± 2	284 ± 24*	6.5 ± 1.0	1.3 ± 0.8#	> 10	19.6 ± 2.4*
2 & 3	HI	5	3 F; 2 M	13 ± 3.3	172 ± 7	7.4 ± 0.5	2.6 ± 0.5	< 4.5	110 ± 16

Data are means ± SE. BCS, body condition score; BW, body weight; CNS, cresty neck score; FGIR, fasting glucose-to-insulin ratio; F, female; HI, hyperresponsive to oral glucose; M, male; NI, normal insulin response to oral glucose, $*P < 0.05$, $\#P < 0.1$ HI vs. NI groups.

determine the extent to which incretins are associated with the insulin hyperresponsiveness of some horses to oral NSC, provided in the form of free glucose or a high-energy, grain-based diet.

MATERIALS AND METHODS

Sample population and study design. In total, the study used 31 ponies owned by Queensland University of Technology. The group contained various breeds (principally Shetland, Welsh, Connemara, Australian, and mixed-breed), spanned a wide range of ages (8-25 yr) and body weights (90–368 kg), and included both mares and geldings (Table 1). The ponies were given routine vaccinations, dental care, and antiparasitic drugs at least 2 wk prior to the study and were subjected to biochemistry and hematology blood testing. Blood test results were all within species-specific reference ranges for the tests performed (data not shown). Diagnostic tests for PPID were not specifically performed for this study. The study included three separate experiments: *1*) a comparison of the insulin response to oral and iv glucose, using a cohort of 22 ponies; *2*) a more detailed examination of the insulin and incretin responses to oral and iv glucose in separate cohort of nine different ponies; *3*) a comparison of the insulin and incretin response to two commonly fed, commercial grain-based diets, using the same nine animals.

All the ponies were housed individually in dry lots during study periods, and for the 10-day acclimatization period preceding each phase with ad libitum access to water. They received a daily maintenance diet of 0.8% body weight (BW) lucerne chaff, 200 g of wheat bran, and a vitamin and mineral nutrient balancer (Vitam, International Animal Health, Huntingdon, Australia) at 8 AM and 1% BW lucerne hay at 5 PM for 1 wk preceding each experiment and on nontest days during the study period. Nutritional analyses of the dietary components are outlined in Table 2, and test diets varied as outlined below. All treatments were performed at 8 AM after an overnight fast. No feed refusals occurred during the study. Daily access to a larger dry run was provided to pairs of ponies to facilitate free exercise (1 h). Ethical approval for the study was granted by the University Animal Ethics Committee (SVS/QUT-109/13 and 470/14).

Experiment 1: oral diet challenge vs. iv dynamic testing. Twenty-two ponies were selected from a larger cohort of 50 animals that had undergone a screening test for their oral response to dietary NSC 2–4 wk prior to *experiment 1*. This test involved feeding a highly palatable mixture of oats, molasses, lucerne chaff, and D-glucose, which had been formulated to provide 3.8 g NSC/kg BW in a single meal. This NSC content was selected on the basis of a prior study (unpublished data) that investigated dietary NSC concentrations that cause significant pancreatic stimulation without inducing laminitis. On the basis of their insulin response to this oral diet challenge (>90 μIU/ml 2 or 4 h after feeding), the selected ponies were all considered to be insulin dysregulated (Table 1). During the 3-wk experimental phase of *experiment 1*, the ponies were all initially subjected to a repeat oral diet challenge (as above) in groups of four ponies per day, followed a week later by an intravenous CGIT. A randomized block design was used to ensure an equivalent wash-out period between tests for all ponies. Blood samples were collected via intravenous jugular catheters placed aseptically the evening before, and catheter patency was maintained with heparinized saline. For the CGIT, glucose (150 mg/kg; Baxter Healthcare, Old Toongabbie, Australia) was administered as a bolus via the indwelling catheter followed by insulin (0.1 IU/kg Humulin-R; Eli Lilly, Melrose Park, Australia).

Experiment 2: oral vs. iv D-glucose. The nine ponies used for *experiment 2* received a preliminary OGT 1 wk before the study commenced. This entailed feeding a meal containing 0.3% BW lucerne chaff, 200 g wet wheat bran (as carrier), and 1 g/kg D-glucose powder (Sigma-Aldrich, Castle Hill, Australia) and collecting blood samples for serum insulin and blood glucose analysis before and 2 h after feeding. Based on their fasting glucose-to-insulin ratios (FGIR, a proxy for insulin sensitivity, below 4.5 considered IR) and their insulin responses to the OGT (>80 μIU/ml at 2 h considered excessive) (9, 17), the ponies were classified as either hyperinsulinemic (HI) or normoinsulinemic (NI) responders (Table 1).

During the subsequent experimental phase, a second OGT was performed as described above but using a smaller quantity of D-glucose for palatability reasons (0.75 g/kg), and the time taken for each pony to consume the meal was recorded (*part 1*). Seven days later, the same dose of D-glucose (Baxter Healthcare, 0.75 g/kg) was administered iv via an indwelling catheter (Milacath, Vetquip, Castle Hill, Australia) placed aseptically in the left jugular vein (*part 2*). The rate of glucose infusion was individualized for each pony to match their rate of feed intake during *part 1*. The ponies were concurrently fed the same base diet without added glucose to ensure that the total energy intake was equivalent in the two parts of the study. A series of blood samples was collected for glucose, insulin, and incretin analysis as described below.

Experiment 3: grain-based diets. The same nine ponies were used for *experiment 3*, which involved the feeding of 0.3% BW lucerne chaff mixed with one of two commercial grain-based diets at 0.2% BW: oats or Workhorse mix (Riverina Stock Feeds, Australia). A 2-wk acclimatization period preceded *experiment 3* to allow gastrointestinal adjustment to the grain-based diets and avoid neophobic responses. During the study period, each pony was fed each concentrate twice, using a randomized, cross-over design, and the average of the data values recorded for each test was used for data analysis. Blood sampling for hormone analysis was as described below.

Table 2. *Feed analyses of dietary components*

Diet	Lucerne Chaff	Lucerne Hay	Wheat Bran	Whole Oats	Workhorse Mix	Molasses
DE, MJ/kg DM	10.1	9.6	12.7	11.8	13.2	—
WSC, %	8.0	4.2	—	3.0	6.4	23.6
Starch, %	<2.0	<2.0	16	33	32	34
CP, %	24.4	24.6	16.6	11.8	13.0	6.6

DE, digestible energy; DM, dry matter; WSC, water-soluble carbohydrate; CP, crude protein.

Samples. In *experiment 1*, blood samples (5 ml) for the oral diet challenge were collected just before feeding and then after 2 and 4 h for immediate blood glucose and retrospective insulin analyses. Blood samples (0.1 ml) for the CGIT were collected at 0, 1, 5, 15, 25, 35, and 45 min and then every 15 min for up to 2.5 h for immediate blood glucose analysis. Serum insulin concentration was analyzed retrospectively in samples (5 ml) obtained at 0, 45, and 75 min. In *experiments 2* and *3*, blood samples (5 ml) were collected at the following time points after the provision of each test diet: 0, 15, 30, 60, 90, 120, 180, 240, and 360 min for immediate blood glucose analysis and retrospective insulin and incretin analyses.

The blood samples were divided equally between plain and prechilled EDTA-coated tubes. Plain tubes were left to clot for 30 min, while EDTA tubes were placed on ice for 10 min prior to centrifugation (10 min, 1,500 *g*). Aliquots (1 ml) of serum and plasma were frozen rapidly and stored at −80°C until analyzed for insulin (serum) and incretin (plasma) concentrations. Blood glucose concentration was analyzed immediately using a hand-held glucometer[g] previously validated by the investigators for equine blood against the hexokinase method (data not shown).

Sample handling: incretins. It has been reported that GLP-1 and GIP are less stable than many other hormones (5), so our processing methodology was examined to determine the likelihood of any significant degradative activity by the enzyme dipeptidyl peptidase-4 (DPP-4) in the plasma (EDTA) samples. Blood samples (24 ml) were obtained from 10 of the ponies used in *experiment 1* 60–90 min after feeding, and each sample was divided equally among eight prechilled tubes: four tubes containing EDTA and four containing EDTA plus a proprietary cocktail of protease inhibitors, including a DPP-4 protease inhibitor (P800 tubes; Becton-Dickinson, North Ryde, Australia). All eight tubes were placed on ice immediately, and one tube of each type was removed from the ice after 10, 30, 60, and 120 min. Once removed, the tubes were immediately centrifuged (10 min, 1,500 *g*), and the plasma was harvested, rapidly frozen, and stored at −80°C until analyzed for GLP-1.

Assays. Serum insulin concentrations were measured by a commercial diagnostics laboratory using a chemiluminescent assay (Immulite 200; Siemens, Bowen Hills, Australia) (*experiment 1*) or in-house using a validated ELISA (Mercodia, Uppsala, Sweden) (*experiments 2* and *3*). Equine-specific assays are not available for any incretin hormone. Based on sequence homology, ELISAs (EZHGIP54K, EZGLPHS35K, EZGLP1T36K; Millipore, Abacus ALS, Meadowbrook, Australia) with anti-human capture antibodies against active GLP-1 (7–36 amide), total GLP-1, and GIP were selected for evaluation (Table 3). Assay specificity could not be determined accurately due to a lack of equine-specific standards. Pooled equine samples expected to contain incretin hormone concentrations in the high and mid-to-low range (based on fed state and metabolic status) were assayed in duplicate for all ELISAs. The assays were assessed for cross-reactivity with equine plasma, precision (intra-assay CV for 6 replicates of the pooled samples), and accuracy [linear dilution (r^2) of pooled samples diluted with assay buffer 1:1, 3:4, 1:2, and 1:4]. Interassay CV was calculated for the validated assays. Recovery after the addition and overnight incubation of a midrange assay standard was assessed for each pooled sample and was used as an indicator of potential assay interference.

Glucose kinetics analysis. The results obtained in *experiment 2* demonstrated marked variability in blood glucose concentrations between different animals fed the same diet. To determine whether this reflected a difference in glucose absorption/distribution or clearance rates, the clearance rates were estimated from the data obtained after iv glucose infusion. This was done employing a population pharmacokinetic (PK) analysis of the glucose concentration-time data, using a nonlinear, mixed-effects modeling approach.

Glucose concentrations were modeled as a nonlinear function of horse-specific PK parameter values whose distribution around the population mean was specified as a level of hierarchy in the model. No prior information was included in the data analysis. The PK analysis was undertaken using the SAEMIX package of the R software program (13). This package computes the maximum likelihood estimator of the population parameters using the stochastic approximation expectation maximization algorithm (30). Models were parameterized in terms of the natural logarithm of the parameter values [e.g., *ln*(*CL/F*)].

Model selection was based on the deviance value (−2 log likelihood). In the absence of any prior information, models with smaller deviance are preferred. However, some penalty for model complexity was also considered. To facilitate this, for models with the same random effects structure, the Akaike information criterion (2) and the Bayesian information criterion (36) were evaluated and used to decide between a one- or two-compartment model and between an additive, proportional, and combined (additive with proportional) error structure. For covariate modeling, scatter plots showing the relationship between estimated random effect values for relevant parameters and covariates (such as weight) were visually inspected to determine potential for covariates being included into the model.

Given the small sample size, body weight (kg) was the only covariate considered for inclusion in the model. If a trend with PK parameters was seen, then weight was considered for inclusion into the final model using the following relationship: $\theta\_i = \theta 1 \times z\_i \hat{} \theta 2 \times \exp(\eta\_i)$; where θ_i are the individual values of clearance, $\theta 1$ represents the population value of the parameter, and $\theta 2$ denotes the effect of z_i being horse-specific covariate values normalized to have a mean of 0 and a variance of 1. The decision about whether a covariate should appear in the final model was based on the decrease in the deviance value and also the decrease in the (unexplained) between-horse variability (BHV) of the relevant PK parameter. Decreases in both of these would need to be of reasonable size to warrant the inclusion of a given covariate.

Statistical analyses. All remaining data were analyzed using parametric tests, as the data were distributed normally (Shapiro-Wilk test). Concordance between values for GLP-1 obtained in samples collected

Table 3. *Assay characteristics of 3 ELISA kits used to measure incretins GLP-1 (total and active) and GIP in equine plasma*

Assay	Capture Antibody	Precision CV, %	Accuracy, %	Linearity (r^2)	Interassay CV, %	Limit of Detection, pM
tGLP-1	Anti-GLP-				1.6	0.95
High	1 human	10.1	88	0.99		
Low	polyclonal	10.1	71.4	0.97		
aGLP-1	Anti-GLP-				9.7	0.14
High	1 human	6.0	88.0	0.99		
Low	polyclonal	10.7	80.0	1.00		
GIP	Anti-GIP				9.1	1.83
High	human	5.9	106	0.98		
Low	monoclonal	8.1	93.8	0.99		

Limit of detection (LOD) as stated by the manufacturer, not the actual LOD for equine plasma. Suggested conservative LOD for GIP kit is 18.3 pM for equine plasma. GLP-1, glucagon-like peptide-1; GIP, glucose-dependent insulinotropic peptide; CV; coefficient of variation.

with or without a DPP-4 inhibitor was determined using the Bland-Altman method. Data from the iv and oral tests were compared with a paired *t*-test. AUC was calculated using the trapezoidal method. Given that glucose clearance and administered dose (iv and oral) did not differ, the percent glucose bioavailability was calculated as $F = 100 \times AUC_{oral}/AUC_{iv}$. Correlation coefficients were estimated using Pearson's test, and linearity was determined by simple linear regression. The independent effects of mode of glucose administration and metabolic status of the ponies, and the existence of any interaction between these two factors, were determined for each hormone using a two-way ANOVA. The contribution of several independent variables (glucose and incretin concentrations) to insulin secretion observed during the oral test was examined with multiple linear regression. Differences in hormone response between diet types were assessed with one-way ANOVA. Data were analyzed using SigmaPlot v. 12.5 and are reported as means ± SE. Significance was set at $P < 0.05$.

RESULTS

Experiment 1: oral diet challenge vs. dynamic testing. The 22 ponies used in this study were considered to be insulin dysregulated (or HI) based on excessive insulin responses to oral NSC. All the ponies had insulin concentrations of >94 μIU/ml 2 or 4 h after feeding a high-energy meal, with a mean insulin concentration at 4 h of 214 ± 24 μIU/ml. When this group was subjected to a CGIT, mean values for insulin at 45 and 75 min were relatively high (105 ± 22 and 44 ± 13 μIU/ml, respectively), and the AUC insulin (AUCi) showed a positive correlation with the AUCi obtained from the oral diet challenge ($r^2 = 0.58$, $P = 0.004$).

However, when the ponies were examined on an individual basis, applying standard cut-off values (18) for the diagnosis of IR (positive phase glucose duration >45 min, serum insulin not returned to baseline by 75 min), only 15 ponies in this cohort were deemed to be IR. Thus, seven ponies showed a dysregulated response to oral NSC but would not have been diagnosed as IR and, hence, at risk of laminitis, if no oral test had been performed. These results are consistent with the hypothesis that insulin dysregulation may precede IR or may occur independently of IR.

Experiment 2: insulin and glucose responses to D-glucose, oral vs. iv administration. In *experiment 2*, insulin and glucose responses to iv D-glucose administration were more marked than following oral consumption (Fig. 1, *A* and *B*). This was readily apparent when the AUCs were compared for the iv and oral routes for insulin (442 ± 40 vs. 288 ± 64.6) and glucose (73.6 ± 3.78 vs. 43.6 ± 3.15), respectively ($P < 0.05$). Mean oral glucose bioavailability was 60 ± 4.9%. Of particular note is the strong correlation between the AUCs for insulin and glucose following the OGT ($P < 0.05$), which was not the case following iv D-glucose administration (Fig. 1, *C* and *D*).

Although the number of ponies used in this experiment was small, dividing the group into HI and NI animals based on FGIR and a previous OGT was very revealing (Fig. 2). When the D-glucose was administered orally, insulin concentrations were much higher in the HI group ($P < 0.05$), as expected (Fig. 2*A*). However, when the glucose was administered iv, all the ponies showed a similar glucose and insulin response (Fig. 2, *A* and *B*).

The reason for the marked difference in insulin response between HI and NI ponies following oral glucose can be explained in part by examining the blood glucose response. In particular, HI ponies demonstrated a larger AUC for glucose and a higher insulin-to-glucose ratio than NI ponies following this test (Fig. 2*C*). The oral bioavailability of glucose was also higher in the HI group than in the NI ponies (67.4 ± 6.9 vs. 51.1 ± 3.6%; $P = 0.048$). Overall, insulin and glucose concentrations were both affected by the method of D-glucose administration ($P = 0.01$ and $P < 0.001$, respectively) and metabolic status ($P < 0.01$ and $P = 0.07$, respectively), with no significant interaction between these factors.

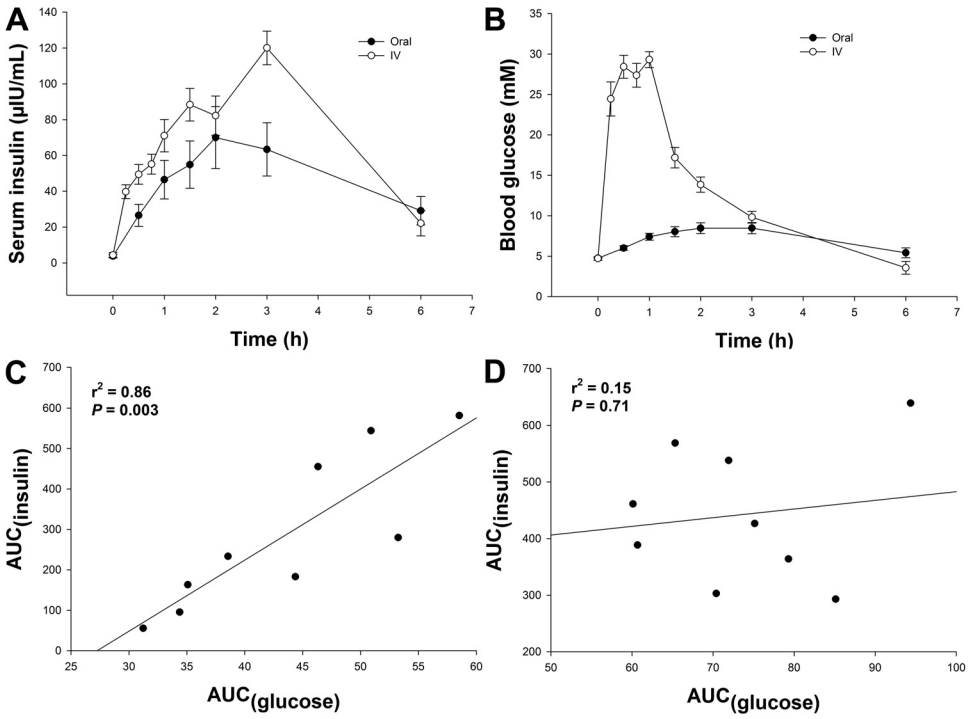

Fig. 1. Insulin and glucose responses to oral and intravenous (iv) D-glucose in 9 ponies. Serum insulin (*A*) and blood glucose (*B*) concentrations were higher when D-glucose (0.75 g/kg) was administered iv route (○) than the oral route (●). AUC for insulin correlated with AUC glucose for the oral test (*C*) but not for the iv test (*D*).

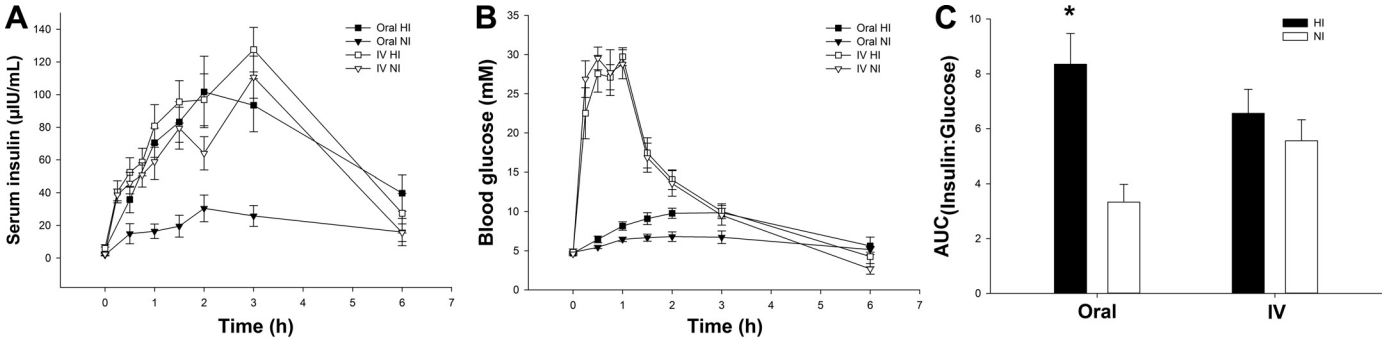

Fig. 2. Insulin and glucose responses to oral and iv D-glucose in 9 ponies differed according to metabolic status and route of D-glucose administration. Insulin (*A*) and glucose (*B*) responses to iv D-glucose were similar in ponies classified as hyperinsulinemic (HI; □) and those that were normoinsulinemic (NI; △) ponies. However, HI ponies (■) demonstrated a greater insulin (*A*) and glucose (*B*) response to oral D-glucose, than NI ponies (▼). *C*: AUC for the insulin-to-glucose ratio was also greater in HI (filled bars), than NI (open bars) ponies for the oral test (OGT) but not the iv test (IVGT). $*P < 0.05$.

Experiment 2: modeling glucose kinetics. As glucose bioavailability seemed to be a distinguishing feature between HI and NI ponies, glucose kinetics were investigated further to establish whether this might have been due to differences in glucose absorption and/or glucose clearance rates. Initially, a one-compartment infusion model with the three error structures was fitted to the data. The combined error structure provided the smallest values of deviance (Table 4); however, significantly smaller deviance values were observed for the two-compartment models, with the proportional and combined error structures giving small deviance values (Table 4). As the combined error structure had an additional parameter compared with the proportional error structure, the latter model was selected and used for further analysis.

Covariate relationships were investigated for all parameters and the covariate weight. There appeared to be linear relationships between the random effect values and weight for all population parameters. Hence, weight was considered as a covariate to reduce the BHV of all parameters. Having weight on CL/F and V1/F significantly reduced the deviance and yielded similar values (Table 4). Consequently, weight on both of these parameters was considered, which again significantly reduced the value of deviance.

Table 4. *Deviance values for 11 different models describing the pharmacokinetics of glucose following iv infusion of D-glucose (0.75 g/kg) in 9 ponies*

Model	Deviance
1-cpt infusion with additive residual error	1,031.41
1-cpt infusion with proportional residual error	1,438.66
1-cpt infusion with combined residual error	1,028.66
2-cpt infusion with additive residual error	958.44
2-cpt infusion with proportional residual error	929.48
2-cpt infusion with combined residual error	929.22
2-cpt infusion with proportional residual error + weight on CL/F	912.08
2-cpt infusion with proportional residual error + weight on Q/F	919.70
2-cpt infusion with proportional residual error + weight on V1/F	913.16
2-cpt infusion with proportional residual error + weight on V2/F	920.77
2-cpt infusion with proportional residual error + weight on CL/F & V1/F	896.06

CL/F; oral clearance, Q/F; bioavailable intercompartmental clearance, V/F; compartmental volume of distribution after oral administration.

To investigate whether weight should be included in the model on these parameters, the reductions in BHV were also considered. With and without weight in the model, the BHV of CL/F was 0.019 ± 0.016 and 0.20 ± 0.098, respectively, similarly, for V1/F: 0.21 ± 0.138 and 4.8e-6 ± 0.028. Therefore, including weight reduced the unexplained BHV for CL/F and V1/F and was thus included in the final model. The updated data revealed no trends with weight for either of these parameters. Trends were still observed for Q/F and V2/F. However, these were not investigated due to the small sample size considered in this study.

In summary, the model providing the best fit for these glucose data was a two-compartment infusion model with a proportional error structure and with the inclusion of weight as a covariate predictor for individual values of CL/F and V1/F. The estimated PK parameters from the final model are shown in Table 5. Individual predicted and observed concentrations and individual predicted versus observed concentrations are shown in Figs. 3 and 4, respectively. A plot of the distribution of the residuals is shown in Fig. 5.

The relationship between individual values of CL/F and the HI/NI grouping was investigated based on individual estimates of clearance from the final model for the PK data. No trends for CL/F and V1/F were detected. However, potential trends were observed for Q/F and V2/F. Acknowledging that there was a difference in mean body weight between the HI and NI groups, and after adjusting for this factor in the model, we concluded that no difference was seen in glucose clearance rates between the HI and NI groups in this study. Accordingly, we conclude that the higher glucose concentrations reached in HI ponies were either a result of enhanced glucose absorption, or due to differences in the first-pass effect of the liver.

Experiment 2: incretin assay validation. Three incretin assays demonstrated sufficient cross-reactivity to be used with equine serum, presumably due to the conserved nature of incretin structure. Values for assay precision and recovery were acceptable, and all the assays showed good linearity (Table 3). The kits showed good sensitivity relative to the incretin concentrations measured in the pooled samples collected after feeding, but for prefeeding baseline samples, concentrations of GIP were below the limit of detection in several

Table 5. *Parameters estimated from a 2-compartment model used to describe the pharmacokinetics of glucose following iv infusion of D-glucose in 9 ponies*

Parameter	Estimate	SE	CV, %
CL/F (dl/h)	0.0915	0.00504	5.51
Q/F (dl/h)	0.3824	0.06479	16.94
V1/F (dl)	0.0023	0.00016	16.94
V2/F (dl)	0.2130	0.0320	15.04
θ2_CL, θ2_V1	0.4319, 0.4919	0.0567, 0.0748	13.12, 15.20
BHV	2.1e-2, 2.1e-1, 4.8e-6, 1.9e-1	0.014, 0.121, 0.028, 0.096	66.12, 57.14, >1000, 50.87
Proportional error	0.1721	0.00022	0.13

BHV, between-horse variability.

instances. Diluting the standards supplied with the kit in an attempt increase the detection range was not successful. Furthermore, when samples in the low range were assayed, validation parameters for the GIP kit were not acceptable. Thus, for the GIP kit, we suggest a conservative limit of detection of ~18 pM.

When plasma samples were collected without a DPP-4 inhibitor, the mean GLP-1 concentrations were numerically lower at all time points. However, this apparent difference was small and not statistically significant. Furthermore, there was no significant decrease in GLP-1 concentrations over time (Fig. 6), suggesting that the results obtained from samples collected using EDTA tubes are valid.

Experiment 2: incretin responses to D-glucose, oral vs. iv administration. Total GLP-1 concentrations declined from baseline during both iv and oral D-glucose tests, returning to pretest levels within 6 h only for the iv test (Fig. 7*A*). Furthermore, tGLP-1 concentration was lower 30–120 min after iv D-glucose compared with oral consumption. By contrast, aGLP-1 increased from basal values in both tests, with a bimodal (30 and 90–120 min) pattern of secretion (Fig. 7*B*). The ratio of active to total GLP-1 had increased tenfold by 90 min after oral D-glucose (2.3% vs. 23%).

A positive GIP response was also observed, although a bimodal pattern of secretion was not detected and GIP release following the iv test appeared negligible (Fig. 7*C*). Based on a comparison of AUCs following oral and iv glucose, both the aGLP-1 and GIP responses were greater for the oral test (Fig. 7*D*). Neither AUC for aGLP-1 nor GIP was correlated with AUCi for the iv test. However, $AUC_{aGLP\text{-}1}$, but not AUC_{GIP}, was correlated with AUCi for the oral test ($r^2 = 0.66$, $P = 0.05$). Overall, the independent contributions of glucose ($P < 0.01$), aGLP-1 ($P = 0.05$), and GIP ($P = 0.56$) to the variability in insulin secretion for the oral test were 75.5, 22.7, and 1.7%, respectively.

The pattern of aGLP-1 secretion also differed between pony subgroups for the two modes of D-glucose administration (Fig. 8*A*). The $AUC_{aGLP\text{-}1}$ was greater ($P \leq 0.05$) in HI ponies than in NI ponies for both the oral (21.7 ± 6.3 vs. 7.6 ± 2.3) and iv tests (11.0 ± 3.1 vs. 1.4 ± 0.2), respectively. However, for GIP, although a similar trend was apparent between groups, the results were not significant (Fig. 8*B*). Overall, aGLP concentration was affected by metabolic status ($P = 0.01$) and arguably the route of glucose administration ($P = 0.06$), whereas GIP levels were influenced by route of administration only ($P < 0.01$). No significant interactions were found between metabolic status and route of glucose administration.

Experiment 3: responses to grain. Insulin and aGLP responses over time were not affected significantly by the type of grain supplied in the two different diets (Fig. 9, *A* and *B*), and based on the AUC they were similar to the response seen with oral D-glucose, showing a marked difference ($P < 0.05$) between HI and NI ponies (Fig. 9, *D* and *E*). For GIP secretion, there was still no significant difference between the diets across all nine ponies (Fig. 9*C*), no difference between the HI and NI groups following oral D-glucose, and only a trend toward a difference between these groups ($P < 0.1$) for both diets (Fig. 9*F*).

DISCUSSION

This study has demonstrated that IR is not a prerequisite for, nor necessarily a precursor to, the hyperinsulinemia that may predispose horses to laminitis. Instead, our results are consistent with the hypothesis that gastrointestinal factors are key to insulin dysregulation, due in part to incretin hormone release (chiefly aGLP-1), but also to the fact that ponies with excessive insulin responses to oral NSC absorb more glucose from their diet or metabolize less during first pass through the liver than normal ponies.

Data to support the theory that insulin dysregulation in horses/ponies can occur independently of tissue resistance to insulin was obtained in *experiment 2*, where the insulin response to iv glucose did not discriminate between ponies that were clearly HI or NI based on the oral test. Data from *experiment 1*, where only 68% of HI ponies were also IR may further support this theory. Although the correlation between insulin data for the two tests used in *experiment 1* might indicate that oral tests are a good early predictor of insulin insensitivity and is consistent with findings of correlation between oral and iv tests in other studies (8), it may also suggest that the CGIT simply has lower specificity than the OGT. The current data suggest that oral tests should be an important part of field-based diagnostics for veterinarians, with less emphasis placed on simple iv tests for insulin sensitivity. However, iv tests will remain useful for evaluation of more advanced disease.

If it is accepted that insulin dysregulation may have a gastrointestinal etiology, then it is important to elucidate the factors that contribute to this, as these factors ultimately demarcate HI ponies from normal ones. Insulin and glucose concentrations and glucose clearance were closely aligned in all ponies following iv D-glucose irrespective of metabolic status. However, during the oral test, whereas HI ponies responded with comparable magnitude to the oral and iv doses

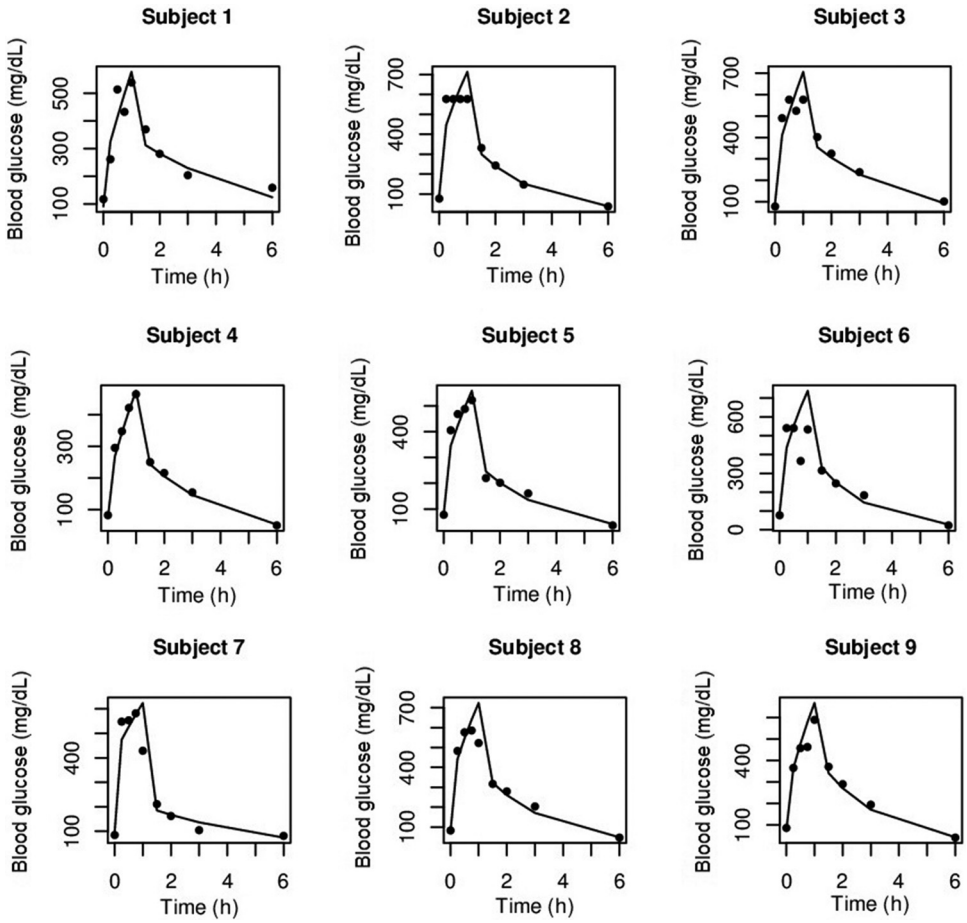

Fig. 3. Individual predicted (-) and observed (●) concentrations of glucose for 9 ponies given a D-glucose (0.75 g/kg) infusion. Predicted values were based on a 2-compartment model with a proportional error structure. Body weight was included in the model as a predictor for individual values of CL/F (oral clearance) and V1/F (compartmental volume of distribution after oral administration).

of D-glucose, NI ponies barely registered a response to oral D-glucose. The mechanism underlying the different insulin responses to oral D-glucose between groups was demonstrated to be due, in part, to an incretin effect. Both aGLP-1 and GIP contributed to insulin secretion in an additive manner to glucose, with glucose remaining the primary driver of insulin secretion. The importance of glucose was established four ways in the present study: by a simple comparison of blood glucose concentrations in HI and NI ponies following the OGT; by comparison of glucose bioavailability drawing on

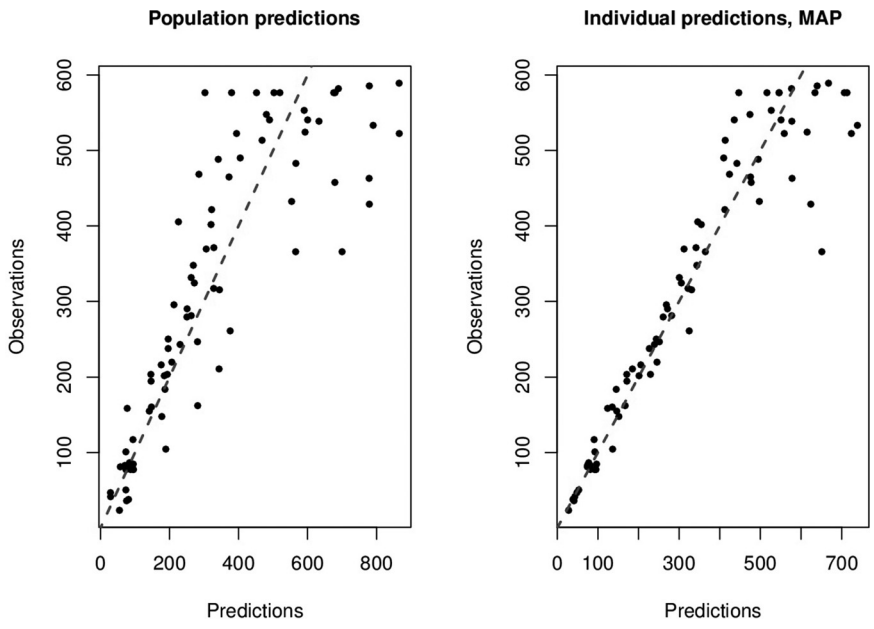

Fig. 4. Population and individual predicted values of glucose as measured in 9 ponies after a D-glucose infusion, based on a 2-compartment pharmacokinetic model with a proportional error structure and body weight as a predictor of individual values of CL/F and V1/F vs. observed concentrations.

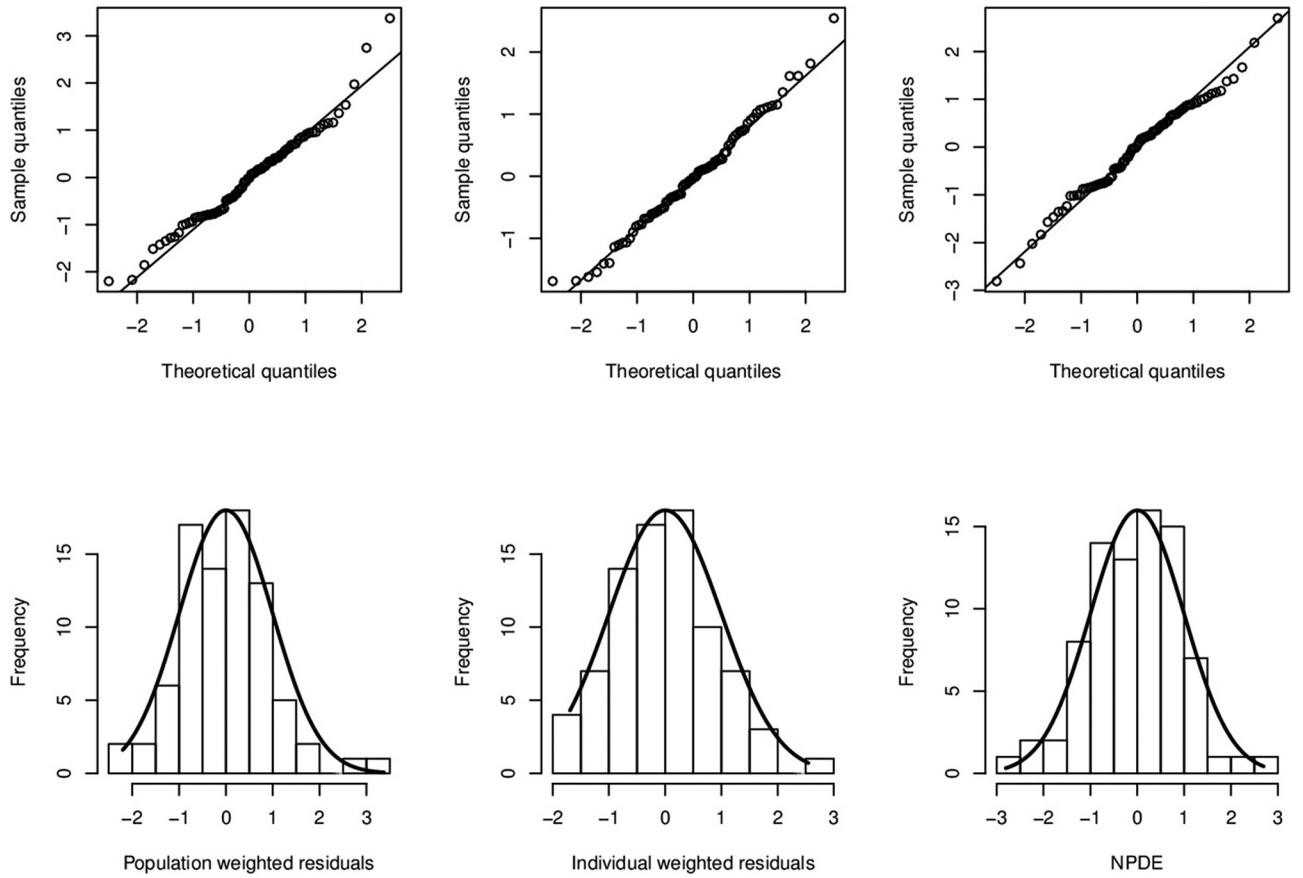

Fig. 5. Distribution of population weighted residuals, individual weighted residuals, and normalized prediction distribution errors for a 2-compartment pharmacokinetic model of glucose metabolism in 9 ponies.

OGT and iv data; by multivariate analysis, which included incretin hormones in the model; and by a detailed glucose kinetics analysis, which estimated glucose clearance rates. We acknowledge that these data should be treated with some caution due to the small number of observations and the potential for the results to be confounded by time due to the sequential design of the experiment. Furthermore, studies to examine the first-pass hepatic clearance of glucose are required to elucidate the effect of hepatic IR on glycemia in ponies. Nevertheless, our data suggest that a focus on enteroendocrine signaling is warranted in the quest to treat or prevent equine hyperinsulinemia.

It should be noted that when performing the iv experiment we attempted to mimic both the total energy provided to the animals (by providing the base diet as part of the test) and the rate of glucose delivery into the blood by pacing the infusion rate to match the rate of feed consumption. However, despite this, the iv infusion resulted in much higher blood glucose levels than oral delivery, which masked the incretin effect. Thus, a significant amount of orally administered D-glucose was either not absorbed or, as is the case in other species, was substantially removed during its first pass through the liver before reaching the systemic circulation (12, 25). Either way, the result was an unexpectedly low bioavailability of oral glucose.

Glucose bioavailability can be affected by gastric emptying rate, intestinal absorption, incretin biology, and hepatic extraction (25). Studies have demonstrated that rates of glucose transport across the intestine can be affected by metabolic dysfunction, with increased sodium-glucose cotransporter density associated with increased glucose absorption in obese people (32). Similarly, glucose uptake and cotransporter expression were upregulated following 2 mo on a high-energy diet in horses compared with a pasture-based diet (16). Furthermore, slower rates of glucose absorption enhanced the effectiveness of insulin and glucose disposal (27), which could explain the subdued glucose responses to oral D-glucose in NI ponies. Thus, the potential exists for intestinal glucose transport to play an important role in equine metabolic disease. As such, further studies in horses to investigate glucose uptake and sensing in the proximal intestine, incretin action, and feedback mechanisms, as well as enteroendocrine cell distribution, are warranted.

In addition to responding to glucose, pancreatic insulin response is also augmented by incretin hormone release in several species (5). The present study has demonstrated a strong association between aGLP-1 and the insulin response to oral NSC, estimating that almost 23% of the variation in insulin concentrations can be explained by the variation in aGLP-1 concentrations. Thus, this study has achieved the second objective of demonstrating, and partly quantifying, an incretin response. The pattern of aGLP-1 secretion following oral D-glucose in ponies was similar to that seen in humans, where a comparable dose of glucose also resulted in the biphasic release of aGLP-1 over a similar time

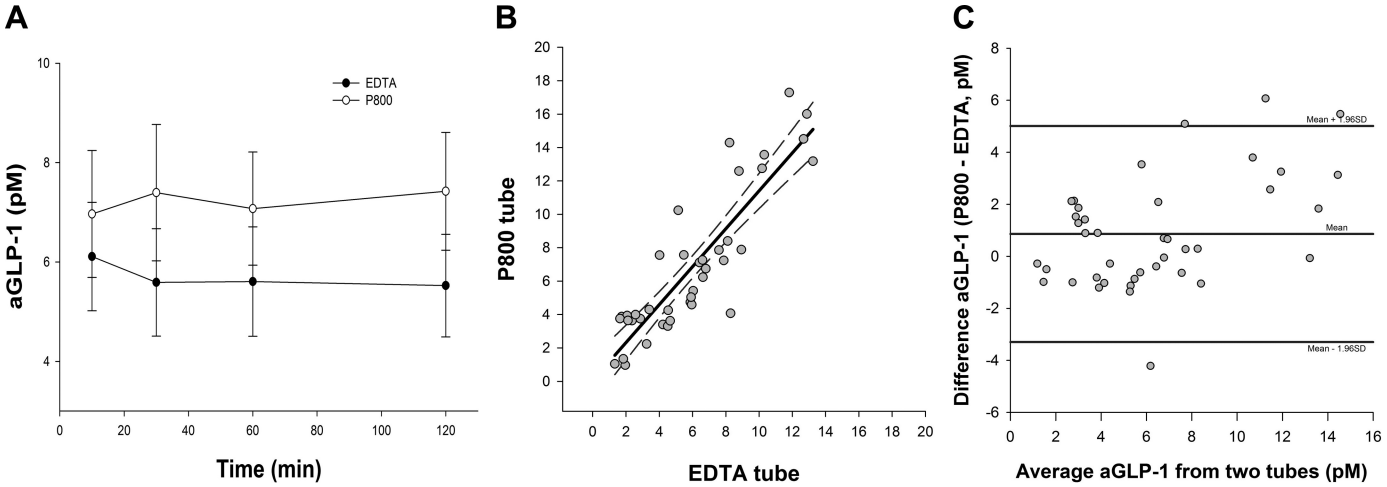

Fig. 6. Active glucagon-like peptide-1 (a)GLP-1 concentrations in samples of pony plasma collected in tubes containing either EDTA alone or EDTA plus a proprietary cocktail of protease inhibitors, including a dipeptidyl peptidase (DPP)-4 inhibitor (P800 tube). *A*: concentrations of aGLP-1 did not differ significantly between collection tubes or over time if samples were kept on ice and processed rapidly. *B*: comparison of both sampling methods using linear regression. *C*: Bland-Altman analysis revealed no meaningful difference between the 2 methods: bias = 0.856, SD = 2.12, limits of agreement = −3.297, 5.009, bias 95%, CI = 0.179–1.533, lower LOA 95% CI = −4.469 − 2.124, upper LOA 95% CI = 3.836 − 6.182.

frame (24). There was no aGLP-1 response to iv glucose in humans, and the small response observed in the present iv study can be explained by the concurrent provision of a small, low-glycemic meal to the ponies.

Although GLP-1 showed a significant association with postprandial insulin concentrations in horses, the influence of incretins in this species may not be as great as in humans, where they can account for up to 70% of postprandial insulin release (41). Furthermore, the ponies used in the present study showed a higher insulin response to iv D-glucose than orally consumed D-glucose, whereas the converse has been shown to occur in humans (23). There are limited data to draw on from other equine studies, but GLP-1 was reported to have a smaller effect on insulin secretion than glucose in a single pony (7).

The negative response of tGLP-1 was potentially related to an increased rate of amide degradation and the conversion of tGLP-1 to aGPL-1. The ratio of active to inactive forms of the peptide increased tenfold as a consequence of oral D-glucose ingestion. The mean values for basal concentrations of aGLP-1 in ponies covered a larger range than that seen in healthy humans (0.1 to 1.5 vs. 0.4 to 1.4 pM), with the low fasted values seen in NI ponies being comparable to those seen in patients with type 1 diabetes (0 to 1.9 pM) (24, 44). However, peak aGLP-1 concentrations in ponies following oral D-glucose were lower than those recorded in

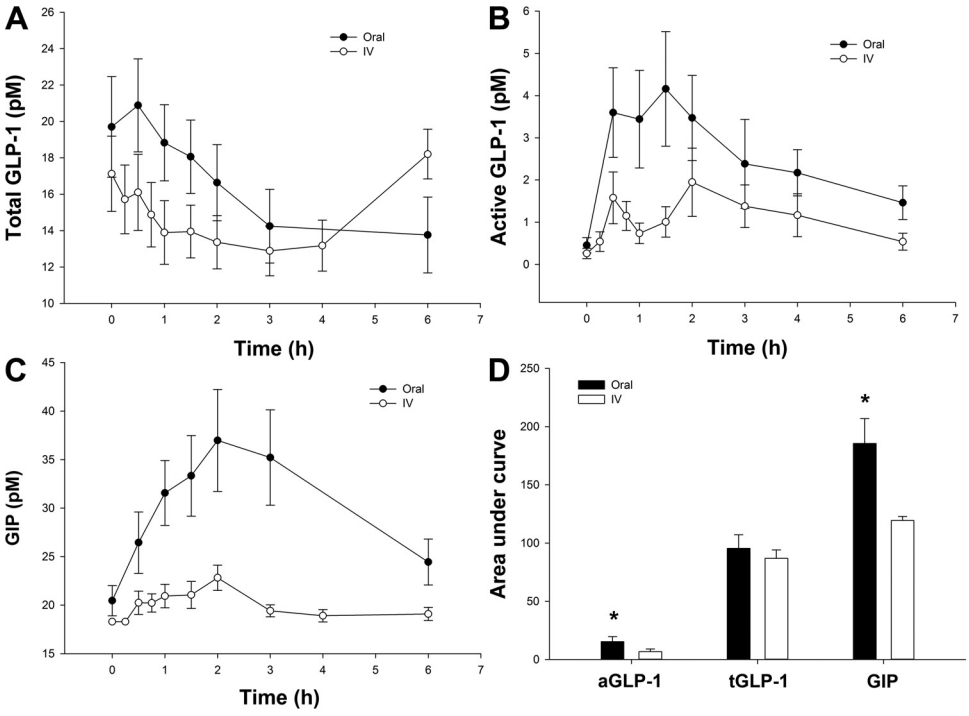

Fig. 7. Incretin responses to oral and iv D-glucose in 9 ponies. *A*: mean total GLP-1 (tGLP-1) decreased from basal values following both oral (●) and iv (○) D-glucose (0.75 g/kg) administration. *B*: however, mean aGLP-1 concentrations increased from basal values, with a larger increase 30–90 min after oral (●) D-glucose vs. increase after iv (○) D-glucose. *C*: mean glucose-dependent insulinotropic peptide (GIP) concentrations were increased at all time points after oral (●) D-glucose consumption vs. small increase 30–120 min after iv (○) D-glucose administration. *D*: accordingly, mean AUC was greater over the 6 h after oral (filled bars) D-glucose consumption for aGLP-1 and GIP but not tGLP-1 vs. iv (open bars) D-glucose administration. $*P < 0.05$.

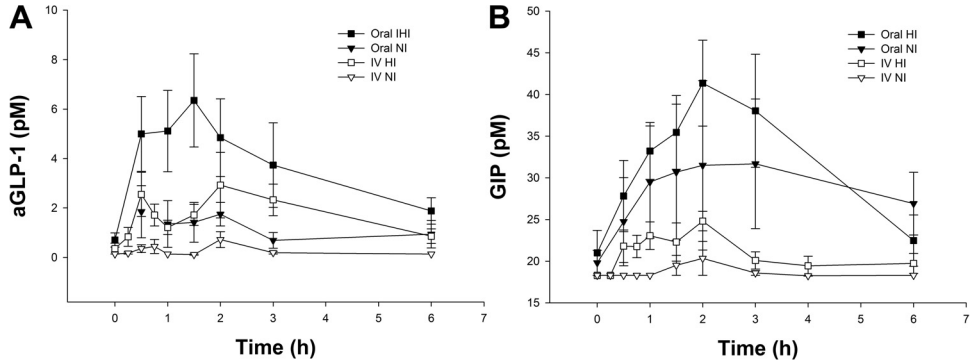

Fig. 8. Incretin responses to oral and iv D-glucose in 9 ponies differed according to metabolic status and route of D-glucose administration. *A*: mean aGLP-1 secretion was greater in ponies classified as HI (■) than NI (▼) ponies for 30–180 min after oral D-glucose (0.75 g/kg) consumption. Similarly, the aGLP-1 response to iv D-glucose was higher in HI (□) ponies vs. a negligible response in NI ponies. Incretin responses following iv D-glucose are most likely associated with consumption of the accompanying low-glycemic meal. *B*: mean values for GIP were not different between HI (■) and NI (▼) ponies following oral D-glucose. However, HI (□) ponies did have a larger GIP response to iv D-glucose for 30–60 min vs. NI ponies.

humans (4.16 ± 1.36 vs. 10 to 12 pM) and in two previous studies using horses (15 to 25 pM), albeit following a much larger oral NSC load (7, 24).

There was a marked GIP response following oral glucose in the present study, but a comparison of GIP dynamics between species is not as closely aligned. We did not observe the biphasic mode of secretion with an early peak at 4–45 min reported in humans (24), although our lower sampling frequency could account for this. Moreover, the GIP concentration range observed in the present study (20 to 37 pM) was lower than that reported in a previous study with horses and ponies (44–133 pM), where a porcine radioimmunoassay was used for GIP measurement (15). Nevertheless, our results do confirm observations in other species (33) that GIP is secreted in higher concentrations than GLP-1 following a meal.

Interestingly, despite the large GIP response, this incretin accounted for much less of the variation in insulin levels than aGLP-1. This is not consistent with the theory that both aGLP-1 and GIP contribute equally to the incretin effect (5). The difference between the two incretins in the present study was that GIP showed a marked though highly variable response to oral glucose in the NI ponies, whereas aGLP-1 and insulin did not. Again, the small number of observations requires caution when interpreting these data, but they are nevertheless consistent with a study that compared ponies (generally HI) with racehorses (generally NI) and found no

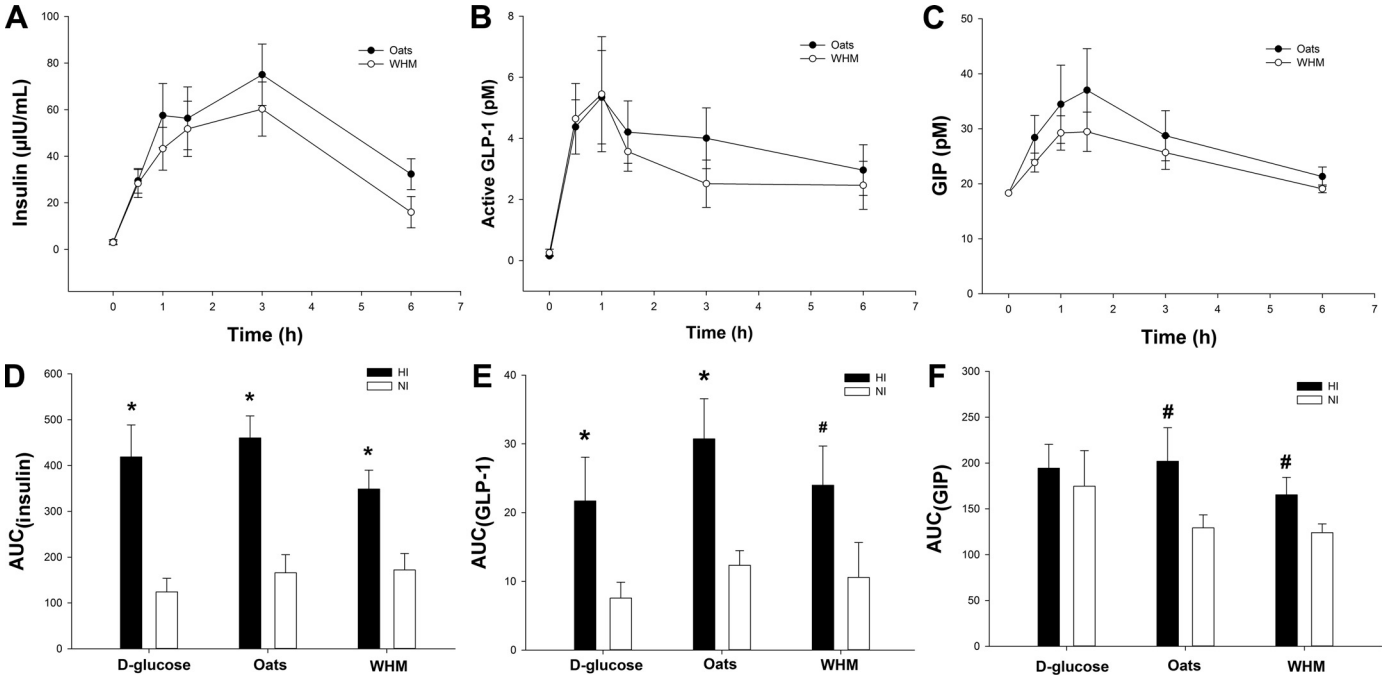

Fig. 9. Incretin responses to oral D-glucose vs. the 2 diets, oats or a commercial grain mixture (Workhorse mix, WHM) in 9 ponies. *A*: mean insulin concentrations after consumption of oats (●) and WHM (○) did not differ over time, and the responses of aGLP-1 (*B*) and GIP (*C*) were similar for the 2 diets. When subdivided according to metabolic status, HI ponies (filled bars) had a larger AUC for insulin (*D*) and aGLP-1 (*E*) following consumption of grain-based diets vs. NI ponies (open bars). Furthermore, these responses to grain were similar to their previous responses to D-glucose (shown for reference). *F*: by comparison, GIP showed no difference in response to D-glucose and only a trend toward a greater response in HI ponies to the grain-based diets. $*P < 0.05$; $\#P < 0.07$.

difference in their GIP response to oral glucose (15). Furthermore, GIP has been reported to be less potent than GLP-1 in stimulating insulin release in humans (29). Given that GIP and GLP-1 do not appear to be cosecreted in other species but have distinct secretion patterns (37), GIP may have other important roles in horses, such as influencing fat metabolism (42), or it may respond differently during insulin dysregulation (10, 40).

As ponies rarely consume free glucose under normal husbandry conditions, it was important to verify our results by feeding more complex, high-energy diets and examining the incretin response. This was also important as incretins are known to respond to dietary components other than glucose, including certain amino acids and fats (42, 43). The incretin responses to the grain-based diets were consistent with the results seen using free D-glucose, as a strong association was seen between insulin and aGLP-1 concentrations, with a weaker association for GIP. Although there was no clear difference in response between the Workhorse mix and the oats, further studies on different dietary components fed to both horses and ponies could be enlightening, particularly as recent studies have demonstrated differential responses to a grain-based diet between horses and ponies (7, 35).

As with metabolic syndromes in other species, EMS is complex and multifactorial. This study has demonstrated that some ponies can be hyperresponsive to oral NSC without attendant evidence of altered peripheral glucose uptake (IR) or clearance rates. In particular, this study has provided evidence for a functional enteroinsular axis in ponies, where alterations in glucose absorption and the secretion of GLP-1 may account for the difference between healthy and dysregulated ponies. A "foregut theory" for equine insulin dysregulation has been proposed and requires ongoing investigation, as further studies into the mechanisms that control glucose absorption and those that stimulate incretin release in the horse may prove beneficial in preventing or treating laminitis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.A.d.L. and M.N.S. conception and design of research; M.A.d.L. performed experiments; M.A.d.L. and J.M.M. analyzed data; M.A.d.L., J.M.M., and M.N.S. interpreted results of experiments; M.A.d.L. and J.M.M. prepared figures; M.A.d.L. and J.M.M. drafted manuscript; M.A.d.L. and M.N.S. edited and revised manuscript; M.A.d.L., J.M.M., and M.N.S. approved final version of manuscript.

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