Final report

The role of glucagon in the pathogenesis of hyperinsulinemia induced laminitis of the horse

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Part 1: Detailed summary

Syfte:
Syftet med studien var att jämföra plasmakoncentrationerna av glukagon och GLP-1 hos hästar med ekvint metabolt syndrom (EMS) och hos friska hästar under fasta, efter en glukosgiva via munnen (oral glukosbelastningstest) och under en infusion av både insulin och glukos till blodet (euglukemisk hyperinsulinemisk clamp).

Material och Metoder:
Studien avsåg att studera material från en tidigare studie, finansierad av stiftelsen hästforskning. Frysta prover fanns tillgängliga från 20 hästar med EMS och 20 friska hästar med normal insulinreglering. Samtliga hästar hade fasteprover, prover från en oral glukosbelastning och prover från en euglykemisk, hyperinsulinemisk clamp tillgängliga för fortsatta analyser av glukagon och inkretinhormonet GLP-1.

Resultat:
Studien visade att hästarna med EMS och de friska hästarna hade likartade hormonsvar när det gäller GLP-1, ett hormon som bland annat hämmar frisättningen av glukagon. Fastenivåerna av glukagon var dock högre hos hästarna med EMS jämfört med de friska hästarna. Hästarna med EMS hade över 10 gånger kraftigare insulinsvar under ett oralt glukostoleranstest jämfört med de friska hästarna. Trots det kraftiga insulinsvaret hade hästarna med EMS också 2,4 gånger så kraftigt glukagonsvar, som de friska hästarna under glukostoleranstestet. Även under en euglykemisk clamp så hade hästarna med EMS svårt att hämma glukagonfrisättningen, trots mycket höga insulinnivåer. Resultaten visar att hästar med EMS har en störd glukagonreglering och att α-cellerna, som frisätter glukagon, är resistenta för hormonet insulin. Höga glukagonnivåer leder till att leverns produktion av

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glukos fortsätter trots att hästen befinner sig i en fas då glukos tas upp från tarmen och tillförs blodet (postprandiell fas). De ökade mängderna med glukos stimulerar β-cellerna att frisätta mer insulin. Den störda glukagonregleringen bidrar således till den kraftiga hyperinsulinemi, som är kännetecknande för hästar med EMS.

Betydelse för hästnäringen:
Fång är en smärtsam och betydelsefull sjukdom, som oftast orsakas av för höga insulinnivåer i blodet (hyperinsulinemi). Prevalensen av fång i olika hästpopulationer runt om i världen varierar mellan 1,5% och 34 %. Ekvint metabolt syndrom behandlas genom att utfodra med grovfoder, som innehåller låg andel lättlösliga kolhydrater, men denna åtgärd är inte tillräcklig i de fall hästarna har kraftigt utvecklad hyperinsulinemi. I dagsläget finns ingen effektiv medicinsk behandling tillgänglig. Upptäckten att hästar med EMS också har en störd glukagonreglering med höga nivåer av plasmaglukagon under fasta och efter utfodring ger ny kunskap om patofysiologin kring EMS. Den nya insikten om patofysiologin skapar också framtida möjligheter till farmakologisk behandling av den störda regleringen av glukagon.

Rekommendationer:
Framtida behandlingsstudier med läkemedel, som har dokumenterad effekt för att minska glukagonsvaret hos människor med typ 2 *diabetes mellitus*, bör därför utföras på hästar med EMS.

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Part 2: Main report (max. 10 pages)

Introduction

Background

Laminitis is a serious and extremely painful disease of the equine foot, resulting in lameness and pathology with long-lasting functional defects (Heymering, 2010). Laminitis can be acute, chronic or recurrent and, at worst necessitate euthanasia if the horse is in considerable pain or if the condition is recurrent (Hunt, 1993). If deviation of the coffin bone occurs, one year of frequent hoof trimming is necessary to realign the hoof to the coffin bone (Pollitt, 2004). Laminitis is associated with systemic disease (endocrine disease and systemic inflammatory response syndrome) or altered weight bearing. Endocrine disease is recognized as the most common cause of natural occurring laminitis in the horse (Karikoski *et al*., 2011). Laminitis is thus an important and serious disease for the equine industry and there is a need for extensive research in this area in order to define underlying pathophysiological mechanisms for development of endocrinopathic laminitis. Endocrinopathic laminitis is caused by hyperinsulinemia or pituitary *pars intermedia* dysfunction (PPID) (Patterson-Kane *et al*., 2018). However, laminitis occurs variably in horses with PPID. A recent study showed that the magnitude of hyperinsulinemia correlated with laminitis in PPID horses, suggesting that hyperinsulinemia, and not PPID per se, is the causal factor for laminitis in PPID horses (Tadros *et al*., 2018). Excessive postprandial hyperinsulinemia is thus the most important factor for laminitis in the horse. Therefore, strategies to reduce excessive postprandial hyperinsulinemia need to be developed and evaluated in order to be able to prevent and treat laminitis.

Insulin dysregulation, a key feature of the equine metabolic syndrome, is a nonspecific term used to describe horses with abnormalities in their insulin metabolism encompassing fasting hyperinsulinemia, excessive insulin response to sugars, and/or insulin resistance (Frank and Tadros, 2014). Weight reduction has been suggested to increase insulin sensitivity and thereby reduce the postprandial insulin response after feeding (Ungru *et al*., 2012). However, a recent study by our research group found that the improvement in insulin sensitivity and postprandial insulin response was limited after weight reduction in severely insulin dysregulated horses (Bröjer unpublished data 2020). This management factor thus has limited impact on decreasing the risk of developing laminitis for all insulin dysregulated horses. Another way of attenuating the excessive postprandial insulin response is to feed a limited amount of non-structural carbohydrates (NSC). This is commonly recommended to horse owners as an important management of horses with insulin dysregulation (Carslake *et al*., 2018). A recent publication by our research group demonstrated that feeding roughage with low NSC content indeed attenuates the insulin response compared to roughage with normal or higher NSC content, but the decrease in postprandial insulin response is not of such a magnitude to be sufficient for the horse with severe insulin dysregulation (Lindåse *et al*., 2018). We also found that the upregulated β-cell response as a compensatory response to the insulin resistance in these horses is by far the most important factor for affecting the excessive insulin response (Lindåse *et al*., 2017; Lindåse *et al*. 2018).

Compared to humans, horses with insulin dysregulation rarely develop type 2 *diabetes mellitus* (T2DM) (Frank and Tadros, 2014). The horse usually remains in a stage with insulin resistance with concurrent compensation of the insulin producing β-cell resulting in excessive postprandial hyperinsulinemia (Frank and Tadros, 2014; Lindåse *et al*., 2017). In this context, the horse has a rather unique β-cell function, which helps control the postprandial glucose levels (Lindåse *et al*., 2017) but at the same time has detrimental effects by inducing laminitis

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(Patterson-Kane *et al*., 2018). Recently, there has been a major interest in evaluating the incretin effect in the horse by measuring the plasma incretins after feeding (Bamford *et al*., 2015; deLaat *et al*., 2015). Incretins are gut derived hormones that contribute to the postprandial glucose regulation by controlling the postprandial insulin response (Campbell and Drucker, 2013; de Graaf-Roelfsema, 2013). One theory that has been put forward is that horses with insulin dysregulation have an upregulated release of incretins, which contributes to the excessive postprandial insulin response (Bamford *et al*., 2015; deLaat *et al*., 2015). These theories are based on studies where horses have been fed non-physiologically high levels of glucose or starch. Whether feeding more physiological levels of glucose induce a high incretin effect is not known for the horse.

To identify novel treatment strategies for preventing laminitis we compared insulin dysregulation in the horse with that observed in humans. In children with obesity we have observed excessive postprandial hyperinsulinemia during oral glucose tolerance tests (OGTTs) as well as elevated fasting insulin levels i.e. already early in life (Staaf et al., 2016). Not all obese children have accentuated insulin secretory responses, however. Importantly, in obese children with excessive insulin levels rise in inflammatory markers as well as development of glucose intolerance and T2DM are seen much more frequently than in obese children with near-normal insulin levels (Staaf et al., 2016). In the obese children hypersecreting insulin, elevated levels of glucagon and decreased levels of the incretin glucagon-like peptide-1 (GLP-1) are observed (Manell *et al*., 2016).

Based on the similarities in insulin dysregulation between horses developing laminitis and obese children with insulin hypersecretion researchers at SLU (Johan Bröjer and coworkers) and Uppsala University (Peter Bergsten and coworker) have initiated a research collaboration. Our collaboration focusses on translating the role of glucagon in insulin hypersecretion in the obese children to the horse. Glucagon is a peptide hormone secreted from the α-cells in the pancreas and the hormone is counter regulatory to insulin, stimulating hepatic glucose production (Ahrén 2015). Glucagon secretion is stimulated by hypoglycemia but also by the incretin hormone glucose-dependent insulinotropic peptide (GIP). At the same time, GLP-1 decreases glucagon secretion and has insulinotropic effects (Godoy-Matos, 2014). Glucagon is thus a hormone that plays an important role in glucose homeostasis and diabetes pathophysiology in humans. There is evidence for an inappropriate increase in α-cell function in both adults and adolescents with T2DM (Faerch *et al*., 2015; Manell *et al*., 2016). Indeed, we have observed hyperglucagonemia both at fasting state and during OGTT especially in the obese children with insulin hypersecretion. Such elevated glucagon levels, which cause glycogenolysis and elevation of glucose levels, may in turn aggravate insulin hypersecretion.

Objectives

The major objective of the study was to evaluate the glucagon response to oral sugars during physiologic post-prandial endogenous insulin response as well as during supraphysiologic constant rate infusion of exogenous insulin in horses with insulin dysregulation and in control horses with normal insulin regulation. Another objective was to investigate the postprandial levels of GLP-1 following administration of oral sugars.

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Material and methods

Materials

We have used previous taken samples from a research project funded by the Swedish-Norwegian Foundation for Equine Research (H1347212). In this project, 20 horses with ID as well as a control group of 20 horses with normal insulin regulation (NIR) were evaluated by the oral sugar test (OST) and the euglycemic hyperinsulinemic clamp (EHC). The study was approved by the Swedish Animal Ethics Committee. All horse owners provided informed written consent. Aliquots of plasma samples obtained from the previous study have been stored at –80°C and were available for new analyses and thus a new practical experiment was not needed as stated in the research application.

Methods

Previously performed oral sugar test:

The day before the OST, an intravenous catheter (Intranule, 2.0 x 105 mm. Vygon, Ecouen, France) was induced into one of the jugular veins under local anaesthesia (EMLA, AstraZenica AB, Södertälje, Sweden). The OST was performed as described previously (Lindåse et al. 2016). Briefly, Dan Sukker Glucossirap (Nordic Sugar A/S, Copenhagen, Denmark) was administered per orally at a dosage of 0.2 mL/kg body weight. Blood samples were collected from the jugular catheter into evacuated tubes (Vacuette 9 ml, Greiner Bio-One GmbH, Kremsmünster, Austria) containing lithium heparin or EDTA before and at 30, 60, 90, 120, 150 and 180 min after administration of glucose.

Previously performed euglycemic hyperinsulinemic clamp:

A second IV catheter (Intranule, 2.0 x 105 mm. Vygon, Ecouen, France) for infusions was inserted under local anesthesia (EMLA, AstraZenica AB, Södertälje, Sweden) into the contralateral jugular vein in the afternoon on the day preceding the EHC. Blood samples for determination of baseline concentrations of plasma glucose and plasma insulin concentrations were drawn from the sampling IV catheter immediately before the start of the EHC at 7 am. The EHC procedure has previously been described for use in horses (Pratt et al. 2005; Lindåse et al. 2016). A continuous infusion rate of regular insulin (Humulin Regular, Eli Lilly Sweden AB, Solna, Sweden) was maintained throughout the 180 min clamp procedure at 3 mIU/kg/min. Blood glucose was kept at 5 mmol/L using a variable continuous infusion rate of glucose (Glucose Fresenius Kabi 500 mg/ml, Fresenius Kabi AB, Uppsala, Sweden). Adjustment of the glucose infusion was made based on the results of measured blood glucose concentration performed every 5 minutes (Accu-Check Aviva, Roche Diagnostics Scandinavia AB, Bromma, Sweden). Serial blood samples (evacuated tubes containing heparin or EDTA; Vacuette 9 ml, Greiner Bio-One GmbH, Kremsmünster, Austria) were obtained every 10 minutes during the clamp for later analysis of plasma glucose to permit calculation of whole body glucose uptake, i.e. metabolic rate of glucose (M index). The steady-state period of the clamp was defined as the last 60 minutes. The M index was defined as the infusion rate of exogenous glucose administered during the steady state after correction of the glucose space (Pratt et al. 2005; Lindåse et al. 2016).

New analyses in the current project

All blood samples were immediately placed on ice for 5 minutes before centrifugation (10 min, 2700 × g). EDTA plasma was separated, frozen rapidly and stored at -80 °C until later analysis of plasma glucagon and plasma GLP-1. Plasma glucagon was measured using a commercialized glucagon ELISA – 10 µL (Mercodia, Uppsala, Sweden). Plasma GLP-1 was

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measured using a commercialized GLP-1 NL-ELISA (Mercodia, Uppsala, Sweden). All analyses were performed in duplicate.

Data analysis:
All data were analyzed using JMP Pro 15.0 (SAS institute Inc, Cary, North Carolina, USA). Data were analyzed for normal distribution by visual inspection of Q-Q plots. Non-normally distributed data were log-transformed prior to analysis and then back transformed after analyses and expressed as geometric means and 95% confidence interval (CI). Data are presented as least square means ± SEM, geometric means with 95% CI or as mean ± SD (described in the text). Data were analyzed using a two-tailed t-test or a linear mixed model. The linear mixed model used sampling time and group of horses (ID vs NIR) as fixed effects and horse as a random effect. Tukey´s test was used to identify simple effect differences. $P < 0.05$ were considered as statistically significant.

Results and discussion

Validation of glucagon and total GLP-1 assays
There is an absence of validated assays as well as of equine-specific antibodies for glucagon and total GLP-1 in horses. We therefore used assays developed for other species, which is very common in equine research, due to lack of specific assays for the horse. The human ELISA performed very well with the equine samples. The inter- and intra-coefficient of variation (CV) for glucagon was 7.1% and 8.6% respectively for low readings (mean 2.1 pmol/L) and 3.4% and 10.4% respectively for higher readings (mean 12.2 pmol/L). Mean ± SD recovery on dilution was 99.7 ± 6.1 %. The regression line for the dilution study was $y = 0.92x + 0.45$ ($r^2 = 0.98$, $P < 0.001$). The human ELISA for total GLP-1 performed adequately with equine samples. The inter- and intra-coefficient of variation (CV) for GLP-1 was 7.3 % and 10.6 % for intra- and inter-coefficient of variation respectively. Mean ± SD recovery on dilution was 106 ± 8.2 %. The dilution demonstrated linearity ($r^2 = 0.92$).

Horses
Twenty horses of different breeds were diagnosed as ID based on the EHC (M index < 2.4 mg/kg/min; mean age ± SD was 13 ± 5 years) and 20 horses were diagnosed as NIR (M index > 2.4 mg/kg/min; mean age ± SD was 9 ± 3 years).

Previous results for plasma insulin and new results for glucagon and GLP-1 after OST and EHC in horses with ID and NIR
The fasting plasma insulin concentration was higher in ID compared NIR horse, 34.2 (25.2 – 46.5) µIU/mL and 3.8 (2.6 – 5.6) µIU/mL respectively (geometric means (CI); $P < 0.001$). Administration of oral sugars resulted in a minimal insulin response over time in NIR horses. In contrast, this insulin response over time was exaggerated in the ID horses (Fig 1A). The insulin responses were significantly higher in the ID horses compared to the NIR horses at all time points ($P < 0.001$). The fasting plasma glucagon concentration was higher in ID compared to NIR horses, 10.3 (7.9 – 13.4) pmol/L and 4.1 (3.0 – 5.8) respectively (geometric means (CI); $P = 0.002$). Administration of oral sugars did not cause a change in plasma glucagon concentrations in the NIR horses whereas it caused a decrease in plasma glucagon concentrations in the ID horses (Fig 1B). The total insulin response as well as the total glucagon response over time expressed as the AUC was higher in the ID compared to the NIR horses ($P < 0.0002$).

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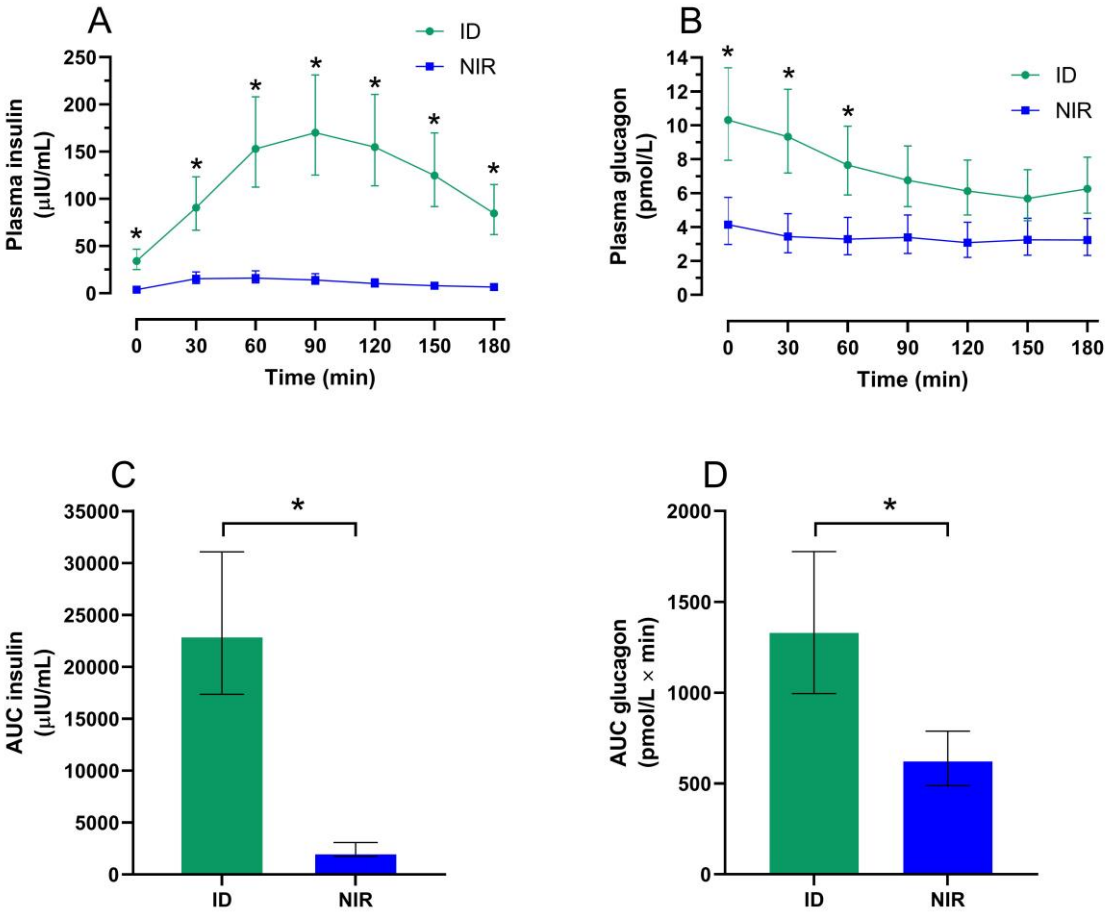

Fig 1. Postprandial concentrations of plasma insulin (A), and plasma glucagon (B) after an oral sugar test in insulin dysregulated (ID) horses (green circle; n=20) and in normal insulin regulated (NIR) horses (blue square; n=20). * indicates significant ($P < 0.05$) difference between groups (ID vs NIR) within a time point. Insulin (C) and glucagon (D) response expressed as the area under the curve (AUC) for ID (green) and NIR (blue) horses. * indicates significant ($P < 0.05$) difference between groups. All data were non-normally distributed and they were therefore log transformed before analyses. Data were then back transformed after analyses and expressed as geometric means and 95% confidence interval.

There was no difference in total GLP-1 response between ID and NIR horses (main effect $P = 0.75$) but there was a main effect of time ($P = 0.02$) (Fig 2A). The GLP-1 response increased after 30 min in both groups to a plateau during the oral sugar test.

During the first 120 min of an EHC, plasma insulin concentrations increased over time and reached a plateau (Fig 2B). The insulin concentrations during the EHC differed between ID and NIR between time points 80 – 120 minutes ($P < 0.02$). In contrast, the plasma glucagon concentration decreased during the EHC (main effect $P < 0.0001$) (Fig 2C). The decrease was faster in NIR horses compared to the ID horses (main effect of interaction $P < 0.0001$). The plasma glucagon concentration was higher in the ID horses compared to the NIR horses between time points 0 – 80 minutes.

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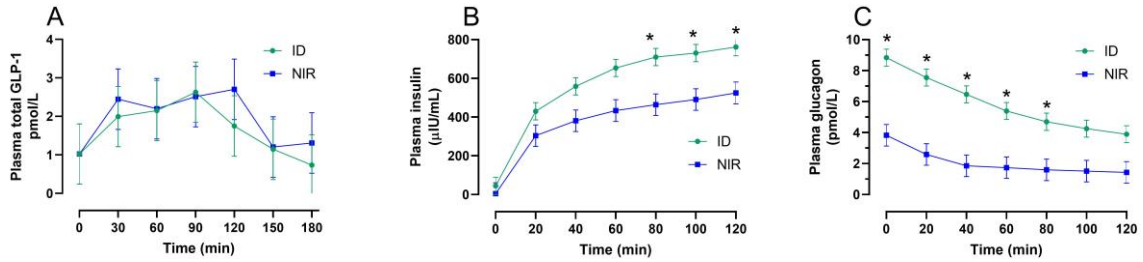

Fig 2. Postprandial concentrations of total GLP-1 (A) after an oral sugar test in insulin dysregulated (ID) horses (green circle; n=20) and in normal insulin regulated (NIR) horses (blue square; n=20). Plasma insulin (B) and glucagon (C) concentrations during the first 120 min of a euglycemic hyperinsulinemic clamp in insulin dysregulated (ID) horses (green circle; n=20) and in normal insulin regulated (NIR) horses (blue square; n=20). * indicates significant ($P < 0.05$) difference between groups (ID vs NIR) within a time point. All data are presented as least square means ± SEM.

Short discussion of the results

The present study demonstrated that ID horses on average had 2.4 times higher fasting plasma glucagon concentrations compared with NIR horses. In addition, the glucagon response during an OST, expressed as the AUC, was more than twice as high for ID horses compared to NIR horses despite the fact that the ID on average had over 10 times higher insulin response compared to the NIR horses during the same time period. Taken together, these results strongly support our theory that ID horses not only are insulin resistant with an upregulated compensatory β-cell response but also have α-cell dysregulation resulting in inappropriate high levels of glucagon during both fasting and during the post-prandial phase.

In human patients with T2DM, regulation of glucagon secretion is disturbed, resulting in elevated plasma glucagon concentrations in the fasting state as well as defective postprandial glucagon suppression. The hyperglucagonemia contributes to increased hepatic glucose production, which is characteristic for adult patients with T2DM (Godoy-Matos, 2014). In contrast, adolescents with T2DM have insulin hypersecretion in response to an oral glucose tolerance test. Adolescents with T2DM also have hyperglucagonemia both at fasting state and during intake of sugars (Faerch *et al*., 2015; Manell *et al*., 2016). Thus, adolescents with T2DM and horses have the same type of β-cell and α-cell disturbances with upregulated β-cell response to oral sugars and a defective excessive glucagon secretion during both fasting and after intake of oral sugars. Although the mechanisms responsible for elevated glucagon levels in adults and adolescents with T2DM are not fully understood, one theory is that pancreatic α-cells are resistant to the glucagon suppressive effects of glucose and insulin. A similar explanation is also possible for the horse. If the inhibitory effect of insulin on the α-cells had been intact one would not expect hyperglycagonemia during the early phase of the OST given the excessive postprandial insulin secretion in ID horses. Elevated glucagon levels, which cause glycogenolysis and elevation of glucose levels, may in turn aggravate insulin hypersecretion in both adolescents with T2DM and in horses with ID.

The incretin GLP-1 decreases glucagon secretion from the α-cells and stimulates insulin secretion from the β-cells (Godoy-Matos, 2014). A blunted GLP-1 response to oral sugars has been shown in both adults and adolescents with T2DM and it has been suggested as a possible factor contributing to the α-cell dysregulation. In contrast, the ID and NIR horses in the present study had similar GLP-1 responses, suggesting another mechanism for the α-cell dysregulation. Interestingly, a recent study in adolescents with T2DM could not confirm a correlation between the early GLP-1 response with the early insulin or glucagon response (Manell *et al*., 2016). The α-cells in horses with NIR responded to hyperinsulinemia without concurrent hyperglycemia or release of incretins as demonstrated in the present study by the

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decrease in plasma glucagon during an EHC. In fact, the horses with ID also responded to the high concentrations of plasma insulin during the EHC, but the response was much slower. These results confirm that the α-cells of ID horses are resistant to increased concentrations of plasma insulin.

The connection between hyperglucagonemia and hyperinsulinemia in the fasting phase as well as the post-prandial phase in horses with ID open up for new possible treatment strategies to decrease glucagon secretion and thereby controlling the excessive insulin secretion. Possible potential classes of drugs available for decreasing glucagon secretion are GLP-1 analogs, inhibitors of dipeptidyl peptidase-4 (DPP-4 inhibitors) and the amylin agonists pramlintide (Godoy-Matos, 2014). In addition, several new drugs attempting to blunt glucagon secretion are currently under development (Haedersdal *et al*., 2018).

Conclusions

Horses with insulin dysregulation have disturbed glucagon secretion, resulting in elevated plasma glucagon concentrations in the fasting state as well as defective postprandial glucagon suppression. The α-cell dysregulation contributes to the hyperinsulinemia by counteracting the physiologic effect of insulin. The incretin GLP-1 appears not play a role in the α-cell dysregulation in horses.

Relevance for the practical horse sector incl. recommendations

Laminitis is a common and extremely painful hoof disorder, resulting in lameness and pathology with long-lasting functional defects. The frequency of laminitis in different horse populations has been reported ranging from 1.5% to 34%. Excessive postprandial hyperinsulinemia is the most important factor casing laminitis in the horse. Therefore, strategies to reduce the excessive postprandial hyperinsulinemia need to be developed and evaluated in order to be able to prevent and treat laminitis. Weight reduction and feeding low sugar diets have limited beneficial effects in severely ID horses. Pharmacological options for treating hyperinsulinemia and thereby preventing laminitis are therefore highly needed. A pharmacological treatment of insulin dysregulation is highly warranted and would be a major breakthrough. It will decrease the incidence of laminitis, increase use and life expectancy for the horses as well as increase welfare.The awareness that horses with ID have disturbed glucagon secretion identifies an important target for pharmacological treatment of these horses. The results from this study highlight the need for future studies where specifically the effect of drugs on decreased glucagon secretion in ID horses are investigated.

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Part 3: Result dissemination

State all result dissemination from the financed project into the appropriate section, including information as indicated. Additional rows can be added to the table.

Scientific publications, *published*	
Scientific publications, *submitted*	
Scientific publications, *manuscript*	Lindåse S, Nostell K, Bergsten P, Forslund A, Bröjer J. Hyperglucagonemia in horses with insulin dysregulation. Intended for submission to Journal of Veterinary Internal Medicine.
Conference publications/ presentations	Hyperglucagonemia in horses with insulin dysregulation. Round table presentation at the Global Equine Endocrinology Conference, Bavaria, Germany, January 6th to 10th, 2020.
Other publications, *media etc.*	Störd glukagonreglering som orsak till ekvint metabolt syndrom. Manuscript submitted to www.hastsverige.se
	Störd glukagonreglering som orsak till ekvint metabolt syndrom. Manuscript submitted to SLU´s "Forskningsnytt djurshälsa och djurvälfärd"
Oral communication, *to horse sector, students etc.*	Veterinärkongressen 2020 – Insulin dysregulation in horses. J. Bröjer invited speaker (fall 2020)
	Horse nutrition and metabolic diseases – S. Lindåse invited speaker for VeTA (Live-stream, June, 2020)
Student theses	
Other	End-seminar has been cancelled due to Covid-19. Might be replaced by a digital presentation in fall 2020.

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