The equine glucose-dependent insulinotropic polypeptide receptor: a potential therapeutic target for insulin dysregulation[1]

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Running head: Equine GIP receptor physiology

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ABSTRACT: Metabolic disease is a significant problem that causes a range of species-specific co-morbidities. Recently, a better understanding of glucose-dependent insulinotropic polypeptide (GIP) biology has led to the suggestion that inhibiting its action may attenuate obesity in several species. In horses, antagonism of GIP may also reduce hyperinsulinemia, which leads to insulin-associated laminitis, a painful co-morbidity unique to this species. However, little is known about GIP in horses. The aims of this study were to examine the tissue distribution of equine GIP receptors (eGIPR), determine whether eGIPR can be blocked using a GIP antagonist not tested previously in horses, and to establish whether there is any association between GIP concentrations and body mass in this species. Archived tissues from healthy horses were used to establish that eGIPR gene expression was strong in pancreas, heart, liver, kidney and duodenum, and absent in gluteal muscle. Pancreatic islets were isolated from fresh horse pancreas using collagenase digestion and layering through a density gradient. Islet viability was confirmed microscopically and by demonstrating that insulin production was stimulated by glucose in a concentration-dependent manner. Insulin release was also shown to be concentration-dependent with GIP up to 0.1μM, and the response to GIP was decreased ($P = 0.037$) by the antagonist (Pro3)GIP. As for the relationship between body mass and GIP *in vivo,* post-prandial GIP concentrations in archived plasma samples were positively correlated with body condition and cresty neck scores ($P < 0.05$). Thus, the eGIPR is a potential therapeutic target for insulin dysregulation and obesity in horses.

Key words: equine metabolic syndrome, glucose-dependent insulinotropic polypeptide receptor, horse, incretin, insulin, laminitis

INTRODUCTION

Obesity and metabolic disease precipitate attributive health problems that differ between species (Geor and Frank, 2009; Grundy, 2012). Horses develop a variant of metabolic syndrome (**EMS**), but rarely progress beyond a pre-diabetic state (Frank and Tadros, 2014). Principally, they exhibit insulin dysregulation, which can manifest as pulsatile (usually post-prandial) or persistent hyperinsulinemia (Frank et al., 2010; de Laat et al., 2016). The primary sequela to insulin dysregulation is laminitis, a painful hoof disease of ungulates (McGowan, 2008).

Gastrointestinal hormones play an important role in the development of insulin dysregulation (Nguyen et al., 2012; Campbell and Drucker, 2013). In particular, incretin hormones enhance insulin release after nutrient intake and are therapeutic targets for metabolic disease in other species (Tiwari, 2015; Wu et al., 2015). However, the incretin effect differs between species, being more pronounced in humans (Nauck et al., 1986) than horses (de Laat et al., 2016). Thus, a species-specific approach to investigating incretin-based therapies is required (Renner et al., 2016).

Glucose-dependent insulinotropic polypeptide (**GIP**) is an incretin that stabilises blood glucose concentration, promotes the proliferation and survival of pancreatic β-cells and modulates obesity (Drucker, 2013; Ceperuelo-Mallafre et al., 2014). Currently our understanding of GIP physiology in horses is limited. For example, there are no published data on the distribution of equine GIPR (**eGIPR**) and our first aim was to address this. Secondly, it is not known whether GIP action can be inhibited in the horse. Our second aim tested the hypothesis that GIP action can be attenuated using the antagonist (Pro3)GIP, which required us to isolate equine pancreatic islets and demonstrate their functionality *in vitro*. Our

final aim was determine if any relationship exists *in vivo* between circulating GIP concentrations and body condition in the horse.

MATERIALS AND METHODS

eGIPR Gene Expression

For gene expression studies, samples of pancreas were obtained from five, healthy, mixed-breed horses (*Equus caballus*, < 15 years old) immediately following euthanasia at a commercial abattoir in South East Queensland, Australia and snap-frozen. Archived samples of the left ventricle of the heart, kidney, digital lamellae, tongue, skeletal muscle and duodenum obtained from four healthy horses during a previous study (SVS/013/08/RIRDC) were also used. Tissue use approval was granted by the Animal Ethics Committee of Queensland University of Technology (1400000039). Tissue samples (50-100 mg) were homogenised (Omni International, Kennesaw, GA, USA) prior to total RNA extraction using Trizol reagent (1 mL/100 mg tissue) according to the manufacturer's instructions (Invitrogen, Scoresby, Vic, Australia). The RNA pellet for each sample was treated with RNAse-free DNAse I (Invitrogen, Scoresby, Vic, Australia) to eliminate genomic DNA contamination. The integrity and concentration of the RNA was determined for each sample using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) prior to cDNA synthesis.

The cDNA was synthesised from Dnase-treated RNA in a total volume of 20 µL, according to the Tetro cDNA Synthesis Kit protocol (Bioline, Alexandria, NSW, Australia). Each reaction contained 1 µg of cDNA, 1 µL Oligo $(dT)_{18}$, 4 µL of 5x RT Buffer, 1 µL RiboSafe Rnase Inhibitor, 1 µL Tetro Reverse Transcriptase (200 µg/µL) made up to 20 µL with DEPC-treated water. Harvested cDNA was stored at -80°C until polymerase chain reaction (**PCR**) analysis. The eGIPR primers (forward: 5'-TGGAAAGTTACTGCTAGGGAGC-3',

reverse: 5'-CCCACTTCTCCCTCTCCATCT-3') were designed using Primer-BLAST (Ye et al., 2012) and primer concentration and PCR conditions were optimised prior to use. The reference gene selected was GAPDH (GenBank Accession Number: AF157626; forward primer: 5'-GATTGTCAGCAATGCCTCCT-3', reverse primer: 5'-AAGCAGGGATGATGTTCTGG-3'). No template, negative controls containing water instead of cDNA were included in each experiment.

After optimisation, PCR was performed using the One*Taq* Hot Start Quick-Load 2X Master Mix protocol (New England BioLabs Inc., Ipswich, MA, USA). Each 25 µL reaction contained 50 ng cDNA, 12.5 µL One*Taq* Master Mix Buffer, 10 pm/µL of each primer (forward and reverse), and 10.5 µL deionised water (dH_2O). The PCR cycle protocol involved 94°C for 30 sec, 40 cycles of 94°C for 30 sec, 52°C for 15 sec, and 68°C for 1 min, with final extension at 68°C for 5 min. Following cycling, PCR products were visualised with 2% agarose gel electrophoresis. The PCR products were purified using an ISOLATE II PCR and Gel Kit (Bioline, Alexandria, NSW, Australia) and sequenced using a standard protocol on an ABI 3500 sequencing platform (Applied Biosystems, Carlsbad, CA, USA). Sequencing confirmed amplification of the desired target and reference genes.

Pancreatic Islet Isolation

Following collection at the abattoir, fresh pancreatic tissues were immediately irrigated with 0.9% NaCl (Baxter, Old Toongabbie, NSW, Australia), blotted and placed in 500 mL of oxygenated (Carbogen) Krebs-Ringer buffer (KRB: NaCl 129 mM, $NaHCO_3$ 5 mM, KCl 4.8 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 2.5 mM, HEPES 10 mM, BSA 0.1% at pH 7.4) for transportation (10 min) to the laboratory. The technique developed for equine pancreatic islet isolation was based on a validated protocol used in rats (Carter et al., 2009a), with some

modifications. Two lobes of pancreatic tissue from the same horse (300-550 g) were transferred from the transport buffer and placed together into sterile (0.22 µm syringe filters; Millex-GV, Merck, Bayswater, Vic, Australia) G-solution [1x Hanks balanced salt solution (HBSS; Gibco, Life Technologies, Mulgrave, Vic, Australia), 0.35 g NaHCO3/L and 1% BSA] for 5 min. The tissue was then distended with 5-6 mL of C solution [1.4 mg/mL collagenase-P (Roche, Mannheim, Germany) in G-solution] using a 25 gauge needle and left for 5 min at room temperature (**RT**). Following this initial digestion, the tissue was dissected (0.5 cm^3) and 4 pieces were placed in one 15 mL falcon tube containing 5 mL of C solution (six tubes were prepared). The tubes were incubated for 15-20 min at 37°C in a shaking water bath (Ratek, Boronia, Vic, Australia). The tissue was then further gently broken up using a transfer pipette and/or hand agitation for 5 sec. The reaction was stopped by adding 10 mL ice-cold G solution prior to washing 3 times with 10 mL G solution for 10 min at RT and 290 x *g* with gentle sieving (Rowe Scientific, Sumner Park, Qld, Australia). The tissue was then layered through a density gradient using 10 mL Histopaque 1.1 g/mL (25 mL of 1.077 and 35 mL of 1.119 g/mL; Sigma Aldrich, Castle Hill, NSW, Australia) and centrifuged (RT; 20 min; 290 x *g*). The supernatant (containing the islets) from 3 tubes (of the same pancreas) was combined and diluted in 25 mL of G solution. Islets were retrieved by centrifugation (RT; 4 min; 493 x *g*) and resuspension of the pellet in 10 mL of G solution, followed by washing 3 times (RT; 3 min; 290 x *g*). The final pellet was resuspended in 1 mL of G solution and examined microscopically (Premiere, Cumming, GA, USA) for islet integrity, purity and approximate yield. Isolations with < 80% live islets were discarded.

In order to assess the viability of the isolated islets, equal aliquots of pelleted islets were re-suspended (0.5 mL) and added to a 24-well-plate containing 0.5 mL of KRB and allowed to pre-incubate for 10 min prior to the addition of glucose to achieve a final glucose

concentration of 0, 1, 2, 4, 8, or 16 mM. An identical viability experiment was performed in parallel using tissue explants in order to enable a direct comparison between pancreatic tissue (the currently available *in vitro* technique) and islets in terms of their responsiveness to glucose.

For the tissue incubations, pancreatic explants (~50 mg) were placed in 1 mL KRB with a glucose concentration of 0, 1, 2, 4, 8 or 16 mM. Both isolated islets (in triplicate; mean CV 12%) and tissue explants (in quadruplicate; mean CV 14.6%) were incubated for 60 min at RT. After incubation, the tissue was removed, or the incubation medium centrifuged (RT; 16,400 x *g*, 30 s), to pellet the islets and the supernatant was retained for both islet and tissue incubations. The protein concentration of each sample/pellet was determined in triplicate (mean intra-assay CV = 3.8%; mean inter-assay CV = 5.6%) using the bicinchoninic acid assay (Pierce, Rockford, IL, USA). The supernatant was stored at -80° C until analysed for insulin concentration using a validated equine ELISA kit (Mercodia, Uppsala, Sweden). The mean intra- and inter-assay CVs for this assay were 8.3% and 9.1%, respectively. The insulin production for each reaction was normalised to protein concentration and corrected for basal insulin secretion.

Stimulation of Pancreatic Islets with GIP Analogues

A series of concentration-response experiments was performed using freshly isolated equine pancreatic islets to determine the insulin response to human GIP (G2269; Sigma Aldrich, Castle Hill, NSW, Australia). This product was used because an equine-specific product was not available. Aliquots (0.5 mL) of pancreatic islets were added to wells containing 0.5 mL G-solution (final glucose concentration 2.75 mM) and allowed to pre-incubate for 10 min prior to the addition of increasing concentrations of GIP ($1x10^{-12}$, $1x10^{-11}$, $1x10^{-10}$, $1x10^{-9}$ and

166 $1x10^{-7}$ M). Samples were incubated in triplicate (mean CV was 6.4%) for 60 min at RT prior
167 to centrifugation (RT; 7 min; 16,400 x g) to separate the supernatant from the cells. The
168 supernatant was immediately frozen and stored at -80°C until analysis. The islets were
169 solubilised prior to protein extraction and quantification as outlined above.
170
171 Subsequently, the ability of the GIPR antagonist (Pro3)GIP (Biocore, Alexandria, NSW,
172 Australia) to prevent GIP-stimulated insulin production from islets was assessed under the
173 same incubation conditions, with the addition of (Pro3)GIP to the incubation media prior to
174 adding the islets. Antagonism was tested at $1x10^{-10}$ M of GIP (n = 4 horses) using a
175 (Pro3)GIP concentration of $1x10^{-9}$ M (Gault et al., 2003).
176
177 Given that GIP has been shown to promote β-cell proliferation, the islets were analysed for
178 evidence of any effect of the antagonist (Pro3)GIP on the amount of cellular proliferative and
179 apoptotic activity occurring during the incubation described above with the agonist, GIP.
180 Caspase 3 and proliferating cell nuclear antigen (**PCNA**) were used as markers of apoptosis
181 and proliferation, respectively. Colorimetric equine-specific ELISA kits (MyBioSorce, San
182 Diego, CA, USA) were used to determine caspase 3 and PCNA concentrations. The mean
183 intra-assay CVs for the caspase 3 and PCNA assays were 3.2% and 7.7%, respectively.
184 Absorbance was measured at 450 nm using a plate reader (Glomax Explorer, Promega, WI,
185 USA).
186
187 ***Correlation of Circulating GIP and Body Condition***
188 The study *in vivo* used archived plasma samples collected during a previous investigation of
189 incretin action in nine, mixed-breed ponies (de Laat et al., 2016). The collection and use of
190 these samples was approved by the Animal Ethics Committee of the University of

Queensland (SVS/QUT/109/13/QUT). Plasma samples were collected from nine (4 female, 5 male; 14.1 ± 2.5 years), mixed-breed ponies 30, 60, 90 and 180 min after a voluntarily-consumed dose of dextrose (0.75 g/kg bwt). The blood samples were collected into pre-chilled EDTA tubes and immediately placed on ice for10 min, prior to centrifugation for 10 min at 1,500 x *g*, separation of the plasma, and immediate freezing at -80˚C of a 1 mL aliquot. This blood collection protocol has been previously validated for incretin measurement in horses (de Laat et al., 2016). Plasma GIP concentration was measured in duplicate (mean intra-assay CV was 6.2%) with an ELISA (Merck Millipore, Germany) validated for use in horses (de Laat et al., 2016).

Each pony was examined by a veterinary surgeon experienced in assigning body condition (**BCS**) and cresty neck (**CrNS**) scores to ponies as markers of generalised and regionalised adiposity, respectively, according to published, commonly-utilised scales (Henneke et al., 1983; Carter et al., 2009b). The pony cohort was assessed for metabolic disease using both physical parameters and an oral glucose test, and consisted of both normal ($n = 4$) ponies, and ponies with EMS ($n = 5$).

Statistical Analyses

The data were distributed normally (Shapiro-Wilk test). The average amount of insulin produced by the tissue explants was compared to islet insulin production using an unpaired t-test. The GIP-stimulated insulin production for each horse was compared with and without the addition of (Pro3)GIP using a paired t-test. Caspase 3 and PCNA concentrations in the islet extracts were compared in the presence and absence of (Pro3)GIP using a paired t-test. The maximum (**Cmax**), post-dextrose GIP concentration for each pony was correlated with BCS and CrNS using Pearson's correlation test. Significance was set at $P < 0.05$ and the data

are reported as mean ± s.e.m. Data analyses were performed with SigmaPlot v.12.5 (Systat software, San Jose, CA, USA).

RESULTS

eGIPR Gene Expression

As expected, gene expression of the eGIPR was consistent for all horses and was confirmed in the pancreas, with >98% sequence identity to *E. caballus* mRNA sequence available from GenBank (XM_001917029). The pancreas was used as a positive control for the remaining PCR runs. Electrophoresis of the PCR products from the other tissues detected eGIPR gene expression in the heart, liver, kidney and duodenum. Faint banding was detected in the digital lamellae and the tongue. There was no evidence of eGIPR gene expression in the gluteal skeletal muscle (Fig. 1). The GAPDH gene transcript was amplified in all tissues (Fig. 1).

Validation of Islet Isolation Method

Functional equine pancreatic islets were isolated successfully for the first time (to our knowledge). Insulin secretion by the islets was increased by glucose in a concentration-dependent manner up to a glucose concentration of 4 mM, but with a possible glucotoxic effect and less ($P = 0.02$) insulin production at 16 mM (Fig. 2A). Overall, the isolated islets secreted 73% more insulin (± 0.07%; $P = 0.047$) than the tissue explants (per mg of protein) over the range of glucose concentrations tested. Insulin production by the explants peaked at 8 mM glucose and was lower ($P < 0.05$) than the islets (per mg of protein) at 4, 8 and 16mM glucose (Fig 2A). Based on these data, isolated islets were used for all future experiments and a basal glucose concentration of 2.75 mM was selected for the incubation medium for the GIP analogue experiments. This glucose concentration was expected to support basic cell function, without stimulating insulin secretion markedly.

The Response to GIP Analogues

Basal insulin secretion in the absence of GIP (130 ± 10 μIU/mg protein) was consistent with that expected in the presence of 2.75 mM glucose, based on the glucose stimulation experiment (Fig. 2B). Insulin release was stimulated by GIP in a concentration-dependent manner, with insulin concentrations reaching 299 μIU/mg protein at the maximum concentration (0.1μM) of GIP tested (Fig. 2B). The insulin secretory effect of GIP was inhibited by 30% ($P = 0.037$) in the presence of (Pro3)GIP (Fig. 2C). When islets were incubated with GIP in the absence or presence of the antagonist (Pro3)GIP, there was no effect on caspase 3 or PCNA concentration (Table 1).

Correlation of Circulating GIP and Body Condition

Due to the range of phenotypes and variation in the metabolic status of the ponies included in the study, the BCS ranged from 4/9 to 9/9 and the CrNS ranged from 0/5 to 4/5. The post-prandial plasma GIP Cmax also varied considerably between individuals, with concentrations ranging from 18.3 pM to 58.9 pM. Circulating GIP Cmax was positively correlated ($P < 0.05$) with both BCS and CrNS in the cohort of ponies examined (Fig. 3).

DISCUSSION

This study has confirmed the presence and functionality of the eGIPR. The eGIPR transcript was present in the pancreas, as expected based on findings in other species (Fujita et al., 2010) and prior studies indicating a functional GIP axis that responds to oral carbohydrates in horses and ponies (Dühlmeier et al., 2001; de Laat et al., 2016). Although the eGIPR protein was not studied, the results suggest that the pancreatic eGIPR transcript detected in normal horses responded to native GIP *in vitro*, by stimulating insulin release in a concentration-

dependent manner, as has been demonstrated with pancreatic islets isolated from other species (Fujita et al., 2010). In establishing this outcome the current study has also presented a valid methodology for the isolation of functional equine pancreatic islets, thus providing a valuable technique for further study of equine incretin biology.

The principal hypothesis of this study was supported. It was demonstrated that not only was insulin secretion from equine islets dose-dependent with GIP, but that GIP-stimulated insulin secretion could be attenuated with the use of the GIPR antagonist (Pro3)GIP. This finding suggests that inhibition of GIP action may be achievable *in vivo* in horses through the administration of GIPR antagonists, and the pharmacokinetics and pharmacodynamics of these compounds is worth investigating. The GIPR antagonist (Pro3)GIP is, to date, the most thoroughly investigated compound for inhibition of GIP action, although small molecule receptor antagonists and neutralizing antibodies against GIP have also been studied (Finan et al., 2016). Recent studies have indicated that (Pro3)GIP appears to have species-specific and dose-dependent actions, with a switch from being an antagonist to a weak partial agonist at high doses in humans and mice, but not rats (Sparre-Ulrich et al., 2016). The current study has demonstrated that (Pro3)GIP functions as an antagonist in horses at the concentration tested, but further studies would be required to determine if this effect is intensified, or indeed nullified, at lower or higher doses.

Since the GIPR has been shown to undergo reduced recycling of the receptor to the cell membrane in response to ongoing ligand stimulation (Mohammad et al., 2014; Al-Sabah, 2016), the effectiveness of GIPR antagonists may differ *in vivo*. Further, whether pancreatic eGIPR expression (density) differs between horses or ponies that are insulin-dysregulated and those that are not, as it does in humans, is important to investigate as this would further affect

pharmacodynamics *in vivo* (Ceperuelo-Mallafre et al., 2014). However, the insulin-dysregulated ponies studied by de Laat et. al. (2016) did not show a significantly larger GIP response to an oral glucose load, when compared to normal control ponies, which suggests that its role in metabolic dysfunction requires further investigation. It is possible that GIP release may be more responsive to dietary fat in horses, and future studies on the role of lipids may be relevant (Schmidt et al., 2001).

In rodents the GIPR is expressed on both the β and α-cells of the pancreatic islets, where GIP not only stimulates insulin secretion, but increases β-cell mass due to enhanced proliferation and decreased rates of apoptosis which reflect improved cell survival (Fujita et al., 2010). We examined whether β-cell fate was altered during incubation with GIP and (Pro3)GIP using markers for cell proliferation (PCNA) and apoptosis (caspase 3). Despite detecting no effect of GIP on these markers we suggest that the short incubation time may have prevented these effects from being sufficiently advanced to enable detection with the assays used. In comparison to GLP-1, the physiology of GIP in the pancreas is still poorly understood in all species, and further study will no doubt improve our capacity to fully determine the range of effects of this incretin hormone (Campbell and Drucker, 2013).

Extra-pancreatic distribution of the eGIPR was also described in this study, which suggests that GIP may exert a multitude of effects in horses. Numerous extra-pancreatic effects, including effects beyond metabolic function, have been reported, and the GIPR is similarly expressed in a variety of tissues, in humans (Yamada et al., 2006; Renner et al., 2016). The presence of the eGIPR in the equine myocardium and small intestine is consistent with findings in rodents and humans (Usdin et al., 1993). The cardiovascular actions of GIP in other species include an increase in cardiovascular triglyceride metabolism, and the current

316 results suggest that GIP may affect cardiovascular function in horses which has identified an
317 avenue for future investigation (Campbell and Drucker, 2013). The role of GIP in the equine
318 liver and kidney also awaits investigation, considering that data regarding GIPR expression in
319 these tissues is sparse (Usdin et al., 1993). Unfortunately, data on the extrapancreatic effects
320 of GIP are scant in comparison with the detailed descriptions of the actions of another
321 incretin, glucagon-like peptide-1 (GLP-1), in a range of tissues (Yamada et al., 2006; Ussher
322 and Drucker, 2012; Campbell and Drucker, 2013).
323
324 One extra-pancreatic effect of GIP that has received recent attention is its ability to promote
325 obesity, and systemic GIP concentration is positively correlated with body mass index in
326 humans (Calanna et al., 2013). Similarly, we have demonstrated that maximally-stimulated,
327 circulating GIP concentrations were positively correlated with both BCS and CrNS in ponies,
328 which suggests that GIP may promote obesity in ponies, as it has been shown to do in rodents
329 (Zhou et al., 2005; Naitoh et al., 2008). However, whether the findings from the current study
330 that used ponies can be applied to horses, which may differ in their predisposition to
331 metabolic disease, requires investigation. The GIPR has been associated with the obesogenic
332 actions of GIP as impaired receptor function has been correlated with insulin resistance in
333 humans (Ceperuelo-Mallafre et al., 2014), and resistance to weight gain in GIPR knock-out
334 rodent models following high-fat feeding (Miyawaki et al., 2002). If GIP modulates obesity
335 in horses, then antagonism of endogenous GIP at the receptor may be expected to have
336 positive effects on adiposity and metabolic dysfunction in this species.
337
338 The CrNS is a commonly used marker of regionalised adiposity in horses, and nuchal crest
339 adipose tissue has been shown to be metabolically active, not unlike visceral adipose deposits
340 in humans (Burns et al., 2010; Pedersen et al., 2015). The stronger correlation between

maximum post-prandial GIP concentrations and CrNS, compared to BCS, which is not necessarily a marker of metabolic dysfunction (Bamford et al., 2016; Ipsen et al., 2016), supports a role for GIP/R in equine metabolic dysfunction. A more in-depth investigation of the relationship between metabolic disease, regional adiposity and plasma GIP in horses, using a larger sample size, is warranted.

This study has provided an important first step in proposing and investigating the feasibility of a novel therapeutic strategy for equine insulin dysregulation, and potentially even for the management of obesity. Given that horses with insulin dysregulation are often obese, it is possible that GIP antagonism may have two benefits. Firstly, attenuating post-prandial insulin secretion could reduce the considerable morbidity and mortality associated with insulin-associated laminitis, a disease that currently lacks an effective treatment strategy. Secondly, GIP antagonists may be useful for addressing obesity in this species.

Overall, this study has provided the first detailed examination of GIPR physiology in the horse, and has demonstrated a relationship between adiposity and circulating GIP in ponies. It has also detailed valuable, novel methodologies that can be used, and adapted, to further enhance islet biology research in horses. A commercially available agonist and antagonist of the GIPR were tested, with both compounds demonstrated to be functional at the eGIPR. These data may be useful in guiding future studies about the efficacy and appropriateness of manipulating the GIP axis for the treatment of metabolic disease in this species.

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TABLES

Table 1. Isolated equine pancreatic islets incubated with the incretin GIP did not differ in mean ± s.e.m. caspase 3 or PCNA concentration compared to islets incubated with both GIP and the GIP antagonist (Pro3)GIP

Marker	Cellular process	GIP	GIP + (Pro3)GIP
Caspase 3, pM	Apoptosis	47.7 ± 1.47	45.4 ± 2.21
PCNA, ng/mL	Proliferation	3.63 ± 0.68	3.69 ± 0.73

GIP: glucose-dependent insulinotropic polypeptide; PCNA: proliferating cell nuclear antigen

FIGURE LEGENDS

Figure 1. The equine glucose-dependent insulinotropic polypeptide receptor (eGIPR) gene was expressed in the pancreas (positive control, +) as well as the heart (H), liver (L), kidney (K) and proximal small intestine (SI) of healthy horses (n = 4). There was also weak amplification of eGIPR in the digital lamellae of the left front foot (LF) and the tongue (T). However, there was no eGIPR gene expressed in the gluteal skeletal muscle (SM). The reference gene, GAPDH, was expressed in all tissues. The figure depicts representative results from one horse.

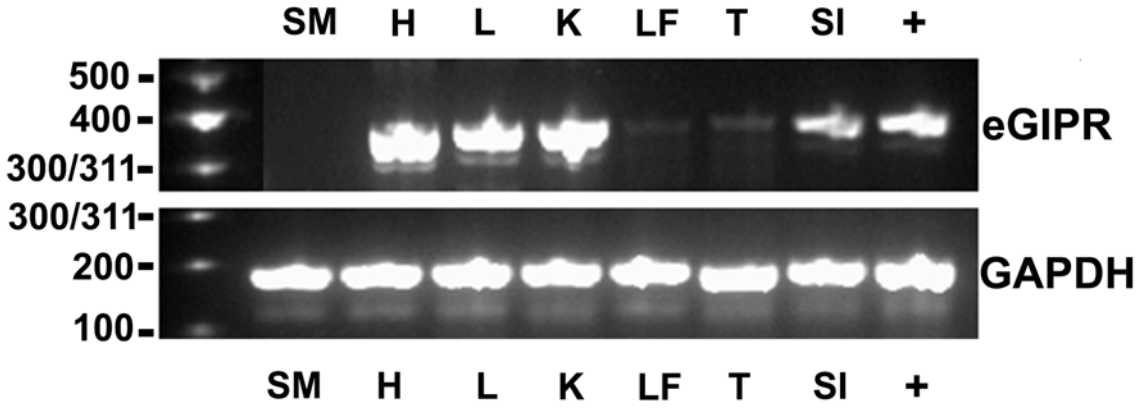
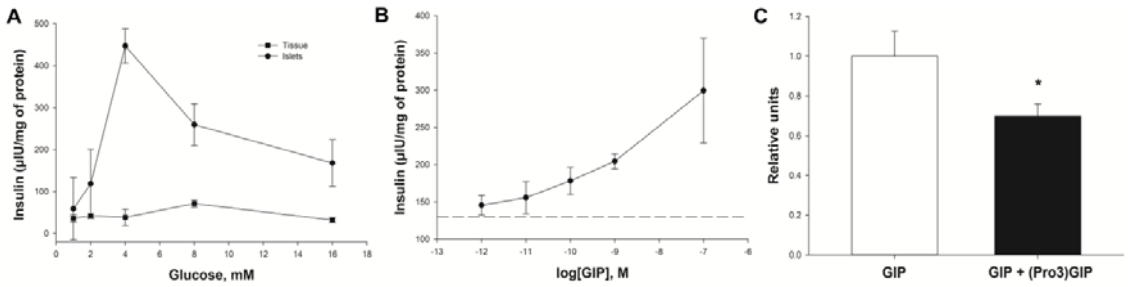


Figure 2. A: The mean ± s.e.m. insulin production by isolated pancreatic islets was concentration-dependent with glucose up to 4 mM glucose and significantly greater than insulin production by pancreatic tissue explants at 4, 8 and 16 mM glucose. **B:** The mean ± s.e.m. insulin secretion by pancreatic islets was concentration-dependent with glucose-dependent insulinotropic polypeptide (GIP) in horses (n = 5) up to 0.1μM ($1x10^{-7}$ M). The basal insulin production (dotted line) was consistent with that expected in the presence of 2.75 mM glucose in the incubation medium. **C:** The insulin secretory response to GIP at

499 $1x10^{-10}$ M was inhibited by 30% ($P = 0.037$) by the GIP receptor antagonist (Pro3)GIP at

500 $1x10^{-9}$ M.



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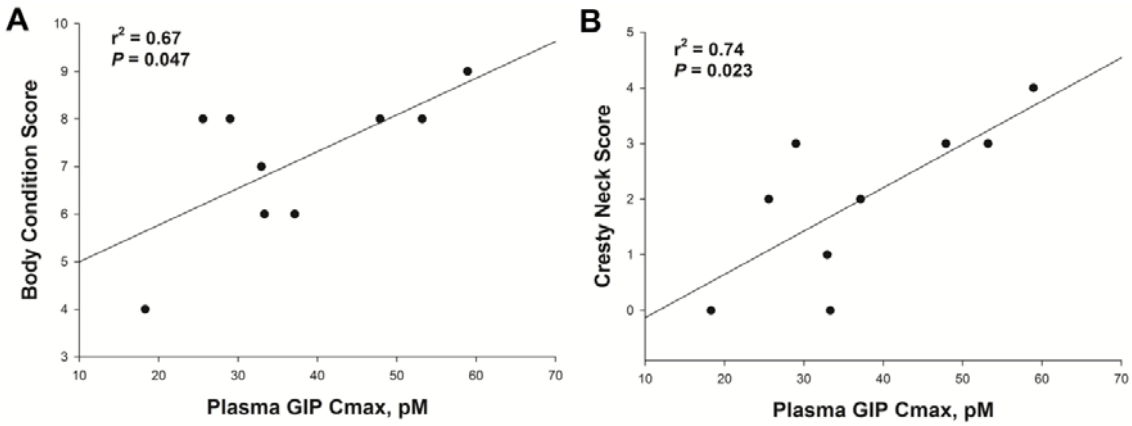
502 **Figure 3. A:** The maximum circulating glucose-dependent insulinotropic polypeptide (GIP)

503 concentration (Cmax) reached in ponies ($n = 9$) following oral dextrose (0.75 g/kg) was

504 positively correlated (Pearson's co-efficient) with their body condition score. **B:** Similarly,

505 the post-prandial GIP Cmax was positively correlated with cresty neck score in the same

506 ponies.



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