Rapid hepatic metabolism blunts the endocrine action of portally infused GLP-1 in male rats

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Abstract

Glucagon-like peptide-1 (GLP-1) is an enteral peptide that contributes to the incretin effect. GLP-1 action is typically described as endocrine, but this mechanism has been questioned because rapid inactivation in the circulation by dipeptidylpeptidase 4 (DPP4) results in a short half-life, limiting the amount of the hormone that can reach the pancreatic islet. An alternative mechanism for GLP-1 to regulate insulin secretion through neuroendocrine signaling originating from sensors in the portal vein has been proposed. We hypothesized that portal infusion of GLP-1 would cause greater glucose-stimulated insulin secretion than equimolar administration into the jugular vein. To test this, hyperglycemic clamps with superimposed graded infusions of GLP-1 into the jugular or portal veins of male rats were performed. These experiments were repeated with pharmacologic DPP4 inhibition to determine the effect of GLP-1 metabolism in the jugular and portal venous beds. Contrary to our hypothesis we found a higher insulinotropic effect with jugular compared to portal GLP-1, which was associated with higher plasma concentrations of intact GLP-1. The greater insulinotropic effect of jugular venous GLP-1 persisted even with pharmacological DPP4 inhibition. These findings do not support an important role of portal vein GLP-1 signaling for the incretin effect but highlight the hepato-portal bed as a major site of GLP-1 degradation that persists even with pharmacological inhibition. Together, these results support rapid inactivation of enterally released GLP-1 in the liver as limiting endocrine actions on the β-cell and raise questions about the conventional endocrine model of pharmacologic effects of DPP4 inhibitors.

Introduction

Glucagon-like peptide-1 (GLP-1) is a physiological incretin in humans and other mammalian species (7). Unlike the other known incretin Glucose-dependent insulinotropic polypeptide (GIP), GLP-1 retains some of its insulinotropic effect in patients with type 2 diabetes (T2D) (27). Hence, the glucose-lowering actions of GLP-1 have led to the development of incretin-based therapies for T2D, namely GLP-1 receptor (GLP-1r) agonists and dipeptidylpeptidase 4 (DPP4) inhibitors (12).

The conventional model of GLP-1 action is endocrine, with mediation of effects on target tissues through the circulation. However, GLP-1 undergoes rapid cleavage by the ubiquitous endovascular enzyme DPP4 (24) that inactivates its insulinotropic activity (36). Metabolism by DPP4 results in a half-life of GLP-1 in the circulation of 60-90 s and plasma concentrations of the active peptide are very low relative to GIP (8) even after stimulation by meals (10, 11). The narrow range of plasma GLP-1 concentrations is contrasted by its wide dynamic range of action. In healthy humans, GLP-1 infusion reaching supraphysiologic concentrations five- to six-fold higher than postprandial levels causes an almost exponential insulinotropic effect (3). In addition, experimental data suggests that infusion of GLP-1 at a dose mimicking postprandial levels has only minimal effects to stimulate insulin secretion in a canine model (19), and a similar experiment in humans also had equivocal results (26).

The rapid metabolism of GLP-1 by DPP4, and low plasma concentrations that may be sub-stimulatory has led to a questioning of an endocrine mode of action (8, 16, 17). Several studies have proposed that a neuroendocrine signal originating in the hepatoportal region is responsible for mediating the glucose lowering actions of GLP-1, as this venous bed is exposed to the highest GLP-1 concentrations in the circulation (4, 5, 25). We have previously reported that the GLP-1r is expressed in afferent neurons in the portal vein, and local antagonism of the GLP-1r in this region impaired glucose tolerance in rats (37). However, few studies published so far have directly compared the insulinotropic effect of portal GLP-1 to systemic administration of the peptide. In this paper we report experiments to test two hypotheses. First, we hypothesized that portal infusion would cause greater insulin secretion than an equimolar dose of GLP-1 infused

into the jugular vein. As a corollary to this hypothesis, we proposed that differences in the insulinotropic effect of portal and jugular venous GLP-1 is due to differential metabolism by DPP4.

Material and methods

Animals and placement of catheters

Experiments were performed on male Long-Evans rats with a mean body weight of 270-300 g, purchased from Harlan Laboratories Inc. (Indianapolis, IN – USA). We have previously demonstrated expression of the GLP-1r on nerve endings in the portal vein of this strain of rat (37). The animals had *ad libitum* access to food and water and were fed a pelleted chow diet (Teklad; Harlan, Madison, WI – USA). They were housed in individual cages in a vivarium with constant temperature (22°C) and were on a 12/12-hour light/dark-cycle. All experiments were approved by the University of Cincinnati Internal Animal Care and Use Committee and carried out in accordance to the Association for Assessment and Accreditation of Laboratory Animal Care-approved facilities conforming to National Institutes of Health and U.S. Department of Agriculture regulations.

Beginning one week after arrival at our facility rats had surgery to implant vascular catheters. Polyethylene tubing (Instech Solomon, Plymouth Meeting, PA) was used for carotid catheters and silicone tubing (Braintree Scientific Inc., Braintree, MA) for cannulation of the portal and jugular veins.

Rats were anesthetized with standard isoflurane inhalation (99%Iso/ml, Abbott Laboratories, North Chicago, IL), shaved over the neck, abdomen and back and carotid and jugular catheters were placed as previously described (35). The portal vein was not clamped or obstructed during catheterization to avoid damage to the vessel and the surrounding nervous plexus. All three catheters were tunneled subcutaneously to the back and externalized between the shoulder blades. Catheters were flushed with heparinized saline and closed with steel rods until use.

During post-surgical recovery (8-14 days) rats were weighed and monitored daily until they reached their pre-surgical body weight. During that time they were handled on a daily basis to habituate them to contact by investigators. Experiments were performed only on fully recovered, healthy rats.

Clamp experiments

Before the clamps rats were fasted overnight having free access to water. After weighing, the animals were brought to a room to which they had been habituated and where they were able to readjust to the new environment for >30 minutes. All catheters were connected and flushed with heparinized saline. The clamp procedure was only started if all 3 catheters would allow both injection and blood withdrawal. During experiments the rats were conscious and freely moving.

Syringe pumps (Harvard Apparatus, Holliston, MA) for the variable infusion of glucose and GLP-1 were connected to the jugular and portal catheters through a 2-channel Swivel (Instech Solomon). The GLP-1(7-36)amide infusion was prepared from a frozen stock solution with 2.5 µg/ml (Bachem, Torrence, CA) stored at -20°C and 200 µl of blood from the respective rat for protein coating of the large plastic surfaces. In previous studies we did not observe degradation of GLP-1 when prepared in this fashion.

After removal of fasting samples (-10, 0 min) a bolus infusion of glucose (D25% Baxter, Deerfield, IL) was started into the jugular vein to create a square wave of hyperglycemia. Blood glucose was monitored every 5 min taking samples from the carotid catheter, and a variable glucose infusion was adjusted by ad-hoc algorithm to maintain constant hyperglycemia of 100 mg/dl over basal. Constant hyperglycemia was maintained for a total of 120 min. In addition, a constant infusion of 4 mg/kg/min glucose was given into the portal vein to maintain steady glycemia and comparable activation of glucose sensors (13, 18) in the hepato-portal bed to all animals.

After 60 minutes of constant hyperglycemia a graded GLP-1 infusion was either given into the portal or jugular vein. The three infusion doses were 1.5 µg/kg/h (60-80 min), 2.5 µg/kg/h (80-100 min), and 5 µg/kg/h (100-120 min). These doses were based on previous dose-finding experiments in our laboratory (data not shown).

Blood samples for measurement of insulin (0.3 ml) were taken at 0, 10, 30, 40, 50, 55, and 60 minutes during the first hour of the clamp. After starting the GLP-1 infusion additional samples were taken at 70, 75, 80, 90, 95, 100, 110, 115, and 120 minutes (3 samples for each GLP-1 dose). Plasma was immediately obtained by spinning the samples for 2 minutes at 6000 rpm in a mini centrifuge (Research Products International

Corporation, Mount Prospect, IL)) and stored on ice. To avoid progressive anemia throughout the clamp the red blood cells (RBC) were re-suspended with saline 0.9% and reinfused after the next blood draw.

Clamp experiments with DPP-4 inhibition

To test the hypothesis that differences in GLP-1 activation of insulin secretion was accounted for by differential DPP4 activity in the portal and systemic circulation, identical clamp experiments were performed after administration of a DPP4 inhibitor. Rats were given 10 mg vildagliptin (kindly provided by Dr. Bryan Burkey of Novartis, Cambridge, MA) suspended in 1 ml of saline intraperitoneally (i.p.) 60 min before the clamp.

Arterial plasma concentrations of active GLP-1(7-36) after site specific infusion

Because of the limited amount of blood available during the clamp experiments a separate cohort was used to measure arterial plasma concentrations of active GLP-1 during the infusions. After a baseline sample, GLP-1 was infused either into the portal or jugular vein. An infusion of GLP-1(7-36)amide of 2.5 µg/kg/h was given for 20 min (0-20 min) followed by a rate of 5 µg/kg/h (20-40 min). One ml of blood was taken at 0, 20, and 40 min of the experiment and immediately placed in chilled Eppendorf tubes prepared with a proteinase-inhibiting cocktail (100 µl per tube, EDTA (0.5 M), heparin (800 U/ml), aprotinin (0.28 mM), and diprotin A (0.066 mM)) to avoid peptide degradation. Tubes were kept on ice until the end of the experiment and then immediately spun. Plasma samples were stored at -80°C until they were assayed.

Analytical methods

Glucose was measured in duplicate with a commercial bedside glucometer (Freestyle Flash, Abbott Diabetes Care, Alameda, CA). Insulin assays were performed using a commercially available RIA (Millipore Corporation, Billerica, MA, Cat. # HI-14K) following the manufacturer's instructions except the use of our own specific rabbit insulin antibody as previously described (2). GLP-1(7-36) plasma concentrations were measured using a commercially available ELISA for active GLP-1 (Millipore Corporation, Cat. # EGLP-35K) according to the manufacturer's instructions.

Statistical analysis

We designed the study to detect 30% lower insulin secretion with jugular compared to portal GLP-1 infusion. Based on preliminary studies with GLP-1 infusion into rats showing a standard deviation of 25% in the insulin responses, we estimated sample sizes of 10 per group with an alpha of 0.05 and 80% power. Comparison of the cohorts and the parameters of the hyperglycemic clamps were done by a student's t-test for unpaired samples with normal variance (table 1). The effects on hyperglycemia, glucose infusion rate and insulin concentrations during the hyperglycemic clamp in response to the dose of GLP-1 and infusion site (portal vs. jugular) were compared by 2-way ANOVA for repeated measures. If there was a significant effect of the infusion site, Bonferroni post-tests were performed to compare the effect of portal vein vs. jugular vein infusion. A p-value of <0.05 was considered statistically significant. The results are expressed as mean ± standard error (SE) for the different cohorts. Analysis and graph plotting was done using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA).

Results

Test animals and hyperglycemic clamps

Hyperglycemic clamps were performed in 10 rats with portal vein (pv) and 10 rats with jugular vein (jv) infusion of GLP-1. The body weight at the day of the clamp was similar in both cohorts (pv: 315.1 ± 5.9 g, and jv: 314.8 ± 5.2 g). Similarly, concentrations of fasting glucose, average glucose during the hyperglycemic clamp, and glucose increment over basal did not differ significantly between the cohorts (table 1). Mean blood glucose during the clamp was 212.1 ± 3.5 mg/dl and 206.3 ± 2.5 mg/dl for the pv and jv groups, with coefficients of variation (CV) for blood glucose over the course of the hyperglycemic clamps that were comparable (pv: 8.7 ± 0.6%, and jv: 8.8 ± 0.5%; table 1).

The fasting and clamp parameters of rats given portal and jugular GLP-1 did not differ significantly in the experiments with vildagliptin (table 1). Successful clamps were performed in 9 rats with infusion of GLP-1 into the portal vein and in 12 rats with infusion of GLP-1 into the jugular vein. Mean blood glucose during the clamp was 201.2 ± 1.4 mg/dl and 202.7 ± 1.1 mg/dl for the pv and jv groups, with coefficients of variation (CV) of 8.6 ± 0.7 % and 9.4 ± 0.7 % respectively (p=0.38).

Portal infusion of GLP-1 is less potent to elicit insulin secretion than an equimolar jugular infusion

Glucose concentrations decreased significantly in both cohorts (pv 216.2±4.0 mg/dl to 201.4±7.4 mg/dl; jv 212.4±3.2 mg/dl to 198.8±3.3 mg/dl) at the end of the hyperglycemic clamp with higher doses of GLP-1 ($p<0.0001$ for dose) but with no significant difference between portal and jugular vein infusion ($p=0.1568$ for infusion site) (figure 1A). Consistent with these changes in glycemia, the glucose infusion rate (GIR) to maintain constant hyperglycemia increased significantly (pv 27.7±3.4 mg/kg/min to 34.5±3.8 mg/kg/min; jv 31.2±2.6 mg/kg/min to 54.8±3.6 mg/kg/min) with higher doses of GLP-1 infusion ($p<0.0001$). Maintenance of the glucose clamp with portal vein GLP-1 infusion required a lower GIR than jugular vein GLP-1 infusion ($p=0.0582$; figure 1B).

With increasing doses of GLP-1, plasma insulin concentrations rose significantly during both portal (282±33 pM to 577±71 pM) and jugular vein (318±29 pM to 1178±235 pM) infusion ($p<0.0001$). Infusion of GLP-1 into the portal vein caused significantly lower insulin levels than GLP-1 infusion into the jugular vein ($p=0.0207$). Post-test analyses revealed a significantly lower insulin concentration during infusion of GLP-1 at a dose of 5 µg/kg/h into the portal vein when compared to infusion of the same amount into the jugular vein ($p<0.05$) (figure 1C). Plasma insulin levels during the clamps are summarized in table 2.

DPP4 inhibition partially protects the insulinotropic potency of portal GLP-1

In the experiments with a preclamp dose of vildagliptin, hyperglycemia was significantly altered by GLP-1 dose ($p<0.0001$) but not by infusion site ($p=0.9257$). With both routes of GLP-1 infusion there was a similar reduction of glycemia with the 2.5 µg/kg/h GLP-1 infusion (pv 213.3±2.6 mg/dl to 192.0±4.3 mg/dl; jv 208.5±3.4 mg/dl to 195.8±3.3 mg/dl), but increased glycemia towards the end of the clamp with the highest dose of GLP-1 (pv 210.9±3.8 mg/dl; jv 214.8±2.4 mg/dl) (figure 2A). GIR increased significantly with higher doses of GLP-1 ($p<0.0001$) with no difference between sites of infusion ($p=0.2680$). The GIR increased steadily with each dose of GLP-1 from 36.8±2.1 mg/kg/min to 51.3±3.1 mg/kg/min during portal infusion of GLP-1 and from 40.0±1.9 mg/kg/min to 54.2±4.2 mg/kg/min during jugular GLP-1 infusion figure 2B).

After DPP4 inhibition plasma insulin concentrations increased significantly with higher doses of GLP-1 ($p<0.0001$) but unlike the previous experiments without the DPP4 inhibitor there was no significant difference between portal and jugular vein GLP-1 infusion ($p=0.2799$). With infusion of GLP-1 into the portal vein, plasma insulin levels increased stepwise from 543±59 pM during hyperglycemia only, to 932±168 pM with 1.5 µg/kg/h GLP-1, to 1535±366 pM with 2.5 µg/kg/h GLP-1 and ultimately 1822±300 pM with 5 µg/kg/h of GLP-1. Similarly, with jugular vein administration, insulin concentrations increased from 672±135 pM (no GLP-1) to a maximum of 2310±340 pM with the second dose of GLP-1 (2.5 µg/kg/h), but declined to 1788±425 pM with the highest dose of jugular vein infusion of GLP-1 (5 µg/kg/h) (figure 2C, table 2).

Injection of the DPP4 inhibitor vildagliptin before the clamp increased plasma insulin levels in response to the GLP-1 infusion into either site significantly (2 way ANOVA pv vs. pv + vilda: dose $p<0.0001$, vilda $p=0.0007$; jv vs. jv + vilda: dose $p<0.0001$, vilda $p=0.0034$). Bonferroni post-test demonstrated a significant effect of vilda on insulin levels at all GLP-1 doses infused into the portal vein ($p<0.05$). Infusion of 1.5 µg/kg/h and 2.5 µg/kg/h GLP-1 into the jugular vein resulted in significantly higher plasma insulin levels after addition of vilda ($p<0.01$) but not at a rate of 5 µg/kg/h (figure 3).

Plasma levels of GLP-1(7-36) during portal or jugular vein infusion

In a separate cohort of animals, plasma concentrations of active GLP-1(7-36) were measured in arterial blood under all four conditions (pv vs. jv infusion ± DPP4 inhibition). Without vildagliptin basal GLP-1 was 2.6±0.4 pM (pv experiments) and 3.3±0.9 pM (jv). Both dose ($p<0.0001$) and infusion site ($p=0.0001$) had significant impact on the measurement of plasma GLP-1 concentration. Post-test analysis revealed that jugular vein infusion resulted in significantly higher arterial plasma GLP-1 levels than portal vein infusion both at a rate of 2.5 µg/kg/h ($p<0.001$) and at a rate of 5 µg/kg/h ($p<0.001$).

With prior administration of i.p. vildagliptin basal plasma levels of active GLP-1 were similarly elevated to 7.0±2.5 pM (pv) and 7.7±2.2 pM (jv) in both cohorts. There were significant effects of both dose ($p<0.0001$) and infusion site ($p=0.0081$ for pv vs. jv) on active plasma GLP-1 during the experiments with DPP4 inhibition (table 3), with jugular vein administration giving consistently higher concentrations than portal vein infusion.

Discussion

While classically considered an incretin, and by definition a hormone, there is emerging evidence against an endocrine mechanism of GLP-1 action (16). Much of this evidence is related to the rapid rate of GLP-1 inactivation by DPP4, and the implausibility that much active peptide reaches target organs like the pancreatic islet through the circulation. Our group and others have suggested that a component of GLP-1 effects is mediated through a neuro-humoral circuit initiated in the portal vein (1, 17, 28, 37). Since insulin secretion is a primary action of GLP-1, we hypothesized that an infusion of synthetic GLP-1 into the portal vein would elicit a larger insulin response than central venous administration. Contrary to this prediction, we observed that GLP-1 given into the jugular vein caused greater insulin secretion than an equimolar portal vein infusion. Consistent with the augmented effect on β-cell secretion, arterial concentrations of active GLP-1 were higher after jugular compared to portal infusion, and the differential levels of circulating peptide were not mitigated by a pharmacologic dose of the DPP4 inhibitor vildagliptin. These findings do not support significant portal mediation of insulinotropic GLP-1 activity, and raise the possibility that metabolism of GLP-1 occurs in the hepato-portal bed independent of DPP4.

For this study we chose rats as the experimental model since we had earlier demonstrated specific portal vein neural GLP-1 sensors in this model, and were able to induce glucose intolerance in rats with infusion of a GLP-1r antagonist specifically into this vascular system (37). While maintaining intact vascular cannulae in the carotid artery and jugular and portal veins is challenging we were able to generate adequate numbers of animals to perform experiments of moderate statistical power. We used graded infusions of GLP-1 to test a range of plasma concentrations that varied from physiologic to pharmacologic levels across both experiments. The hyperglycemic clamp provided generally stable levels of glycemia from group to group and between the two experiments, allowing the effects of GLP-1 dose and site of infusion to be examined in isolation. Finally, we chose a dose of vildagliptin previously demonstrated to cause pharmacologic effects in rodents (20).

The major finding in this study was that portal venous administration of GLP-1 resulted in significantly lower arterial concentrations of active GLP-1, and lesser insulin responses than peptide infused into the jugular vein. These findings indicate that the liver or portal venous circulation has substantial capacity to metabolize GLP-1 accounting for differential concentrations of intact peptide in arterial blood. The increase in hepato-portal GLP-1 clearance was due to either amounts of DPP4 that could not be fully inhibited by the dose of vildagliptin used, or another system of peptide removal not susceptible to DPP4 inhibition, compatible with previous result from studies in swine (31). However, we assume that the concentrations of active GLP-1 in the portal vein, immediately downstream of the infusion catheter, were comparable to those in the jugular vein. Thus, the results of this experiment do not support specific signaling by GLP-1 through sensors located in the portal vein across a broad range of concentrations. In both experiments, there was a general correlation of plasma insulin with arterial GLP-1, suggesting that stimulation of insulin release by GLP-1 was a direct action on β-cells. Moreover, plasma insulin was similar in the portal group at the 5 µg/kg/h dose and the jugular vein group at the 2.5 µg/kg/h dose (table 2), treatments that caused comparable arterial GLP-1 concentrations (table 3). Finally, there was very little stimulation of insulin secretion in either group at the lowest dose of GLP-1, a condition we predicted to be useful for distinguishing selective sensing for the peptide in the portal vein. Taken together our results are not compatible with an important insulinotropic action of GLP-1 mediated specifically in the portal vein of Long-Evans rats, a strain where there is evidence for hepato-portal GLP-1 sensing (37).

Administration of vildagliptin, a potent DPP4 inhibitor, increased arterial GLP-1 levels and insulin secretion in animals that received GLP-1 through both the portal, and jugular, veins. However, despite using doses of vildagliptin previously demonstrated to be on the maximal portion of the dose-response curve in rats (6) or to protect intact GLP-1 comparably to DPP4 gene deletion in mice (20), we were not able to equalize the concentrations of active GLP-1 in the arterial circulation of the jugular and portal vein infusion groups. This suggests that passage of GLP-1 through the liver causes significant inactivation of GLP-1 beyond what occurs in the general circulation. The ELISA assay that we used to measure active GLP-1 is blind to the site and mechanism of GLP-1 metabolism such that the plasma levels obtained in this study do not

necessarily reflect peptide cleavage by DPP4. A recent study has demonstrated substantial metabolism of GLP-1 peptides by neutral endopeptidases in mice (41) and humans (40) suggesting a potential mechanism to account for the significant removal of GLP-1 across the hepato-portal bed. Regardless, the results here support hepatic metabolism or clearance independent of DPP4, consistent with previous work indicating ~ 95% first-pass clearance in the liver (15). This finding has physiologic implications since intestinally released GLP-1 must traverse the hepatic circulation before reaching extra-splanchnic target organs. Furthermore, the substantial degradation of GLP-1 in the hepato-portal bed in the presence of vildagliptin suggests that a mechanism other than endocrine action accounts for the glucose lowering of DPP4 inhibition. In light of the broad use of this class of drugs for the treatment of type 2 diabetes, a better understanding of its pharmacological mechanism could improve patient care and allows individualized treatment concepts.

The results of our study differ from the conclusions of several other groups who have studied mediation of GLP-1-stimulated insulin secretion through a portal neural reflex (1, 4, 28). The study by Nishizawa et al. is most similar to the results reported herein, because they also tested the presence of hepato-portally mediated insulin secretion by direct infusions of GLP-1 into the portal and jugular veins of rats (28). Their primary finding was that a brief, low dose portal vein infusion of GLP-1 together with portal vein glucose caused higher insulin release than infusion of glucose alone, and that this effect was abolished by vagotomy. These investigators also noted that insulin concentrations were about 2-fold higher when GLP-1 was given through the jugular compared to portal vein at the same dose but did not measure plasma GLP-1 in these experiments. They concluded that in the setting of portal glucose and low dose GLP-1, mimicking the prandial state, GLP-1 mediates insulin release mainly through a vagal signal originating in the portal vein, whereas higher doses, or administration into the jugular vein, act directly on pancreatic β-cells (28). The low dose of GLP-1 used by Nishizawa was ~ 7-fold less than the smallest dose infused in our study, and they infused lesser amounts of glucose with ~ 5-fold lower glucose stimulated insulin secretion than the baseline we observed. These features may have increased the sensitivity of their experiments to detect an effect of portal GLP-1 sensing on insulin secretion. On the other hand, the amount of insulin stimulated through the portal neural pathway was small and did not

affect glucose clearance, and is at odds with the notion that GLP-1 is the major mediator of to the incretin effect and postprandial glucose clearance in rodents (22, 33) and humans (14, 32). Hence, it seems unlikely that the vagally mediated insulinotropic effect seen in the study by Nishizawa et al. is the primary mechanism by which GLP-1 mediates its insulinotropic actions and may explain why our less physiologic but more rigorous clamp design did not produce similar results. It is notable that Nishizawa et al. reported a differential effect of jugular and portal GLP-1 infusion, similar to what we observed, supporting the liver as a site of substantial clearance of GLP-1.

It has been suggested by several groups that neuroendocrine signaling through the GLP-1r originates proximal to the portal vein within the substance of the intestine (17, 23, 39) where local GLP-1 concentrations are even higher than in the portal vein (9). Sisley and coworkers used genetic deletion of the GLP-1 receptor in nodose neurons in mice and observed only a trend towards glucose intolerance, but without formal evaluation of insulin secretion (34). However, a recent report from Krieger et al. noted that lentiviral knockdown of the GLP-1r in the nodose ganglia of rats increased postmeal hyperglycemia and reduced insulin consistent with mediation of GLP-1 effects by vagal afferent neurons (23). Veedfald et al. used a similar design to ours in pigs to test whether exogenous GLP-1 would mediate insulin secretion via intestinal vagal afferents (38). Similar to the infusion of GLP-1 into the portal vein in our study, site specific infusion of GLP-1 into the mesenteric artery resulted in a lower insulin release than a peripheral intravenous GLP-1 infusion. However, the more proximal infusion of GLP-1 resulted in greater degradation of GLP-1 in the splanchnic and hepato-portal circulation. While these results are compatible with the findings reported herein, it is plausible that the magnitude of stimulation by exogenous infusion of GLP-1 overshadows and obscures any insulinotropic effect via the vagus nerve (38). Future studies of splanchnic/portal GLP-1 would do well to include low as well has high doses of peptide.

An unexpected finding in our study was the drop of plasma insulin concentrations seen with the highest doses of GLP-1 in conjunction with DPP-IV inhibition. The almost exponential increase in arterial plasma concentrations of active GLP-1 with infusion of synthetic peptide into the jugular vein, protected from degradation by vildagliptin, would be expected to increase plasma insulin, but instead reduced insulinemia to a level

comparable to the portal infusion. One explanation for this counterintuitive response is stimulation of the sympathetic nervous system by the massive plasma concentrations of GLP-1. We have previously observed this effect in rats given high doses of GLP-1 peripherally (30) or into the CNS (21), and other groups have reported similar findings with GLP-1 and exendin-4 (29). While we did not measure epinephrine in this study we have demonstrated previously that hyperglycemia and reduced plasma insulin seen in conjunction with very high doses of GLP-1 can be reversed by adrenalectomy (30). Because this unexpected drop in insulin towards the end of the high-dose GLP-1 plus vildagliptin clamp was seen consistently across the whole cohort a random effect or technical problems with the GLP-1 infusion seem unlikely.

In summary, we were not able to show a direct insulinotropic effect through GLP-1r activation in the hepatoportal bed via vagal afferents, as we hypothesized. This finding does not appear to be congruent with our previous demonstration that GLP-1 receptor antagonism limited to the portal vein causes glucose intolerance (37). However, we cannot exclude effects of GLP-1 to initiate non-insulin mediated effects to lower blood glucose based on the study design presented here. A notable finding was the lower arterial GLP-1 concentrations resulting from portal compared to jugular vein administration of peptide. This finding indicates a prominent role for GLP-1 clearance in the hepato-portal bed. Altogether, our findings provide further reason to doubt a primary endocrine mechanism of action of intestinally released GLP-1 and pharmacological DPP4 inhibition.

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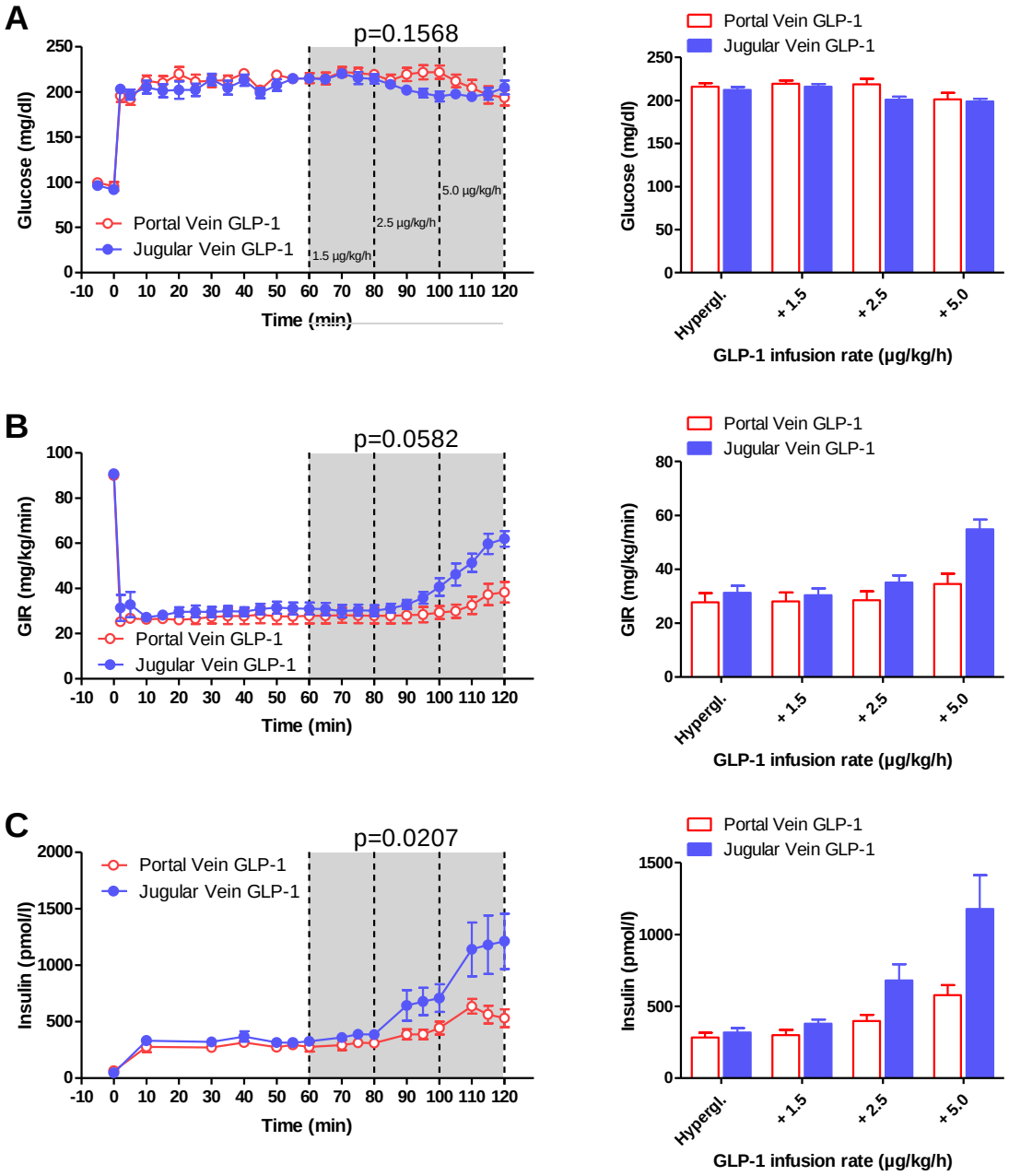
Figures

Figure legends

Figure 1: Glucose (A), glucose infusion rate (B), and arterial plasma insulin (C) during hyperglycemic clamp. Line graphs (left) depict infusion of GLP-1 into the portal (red) or jugular (blue) veins starting at time point 60 min, with increasing doses (61-80 min 1.5 µg/kg/h; 81-100 min 2.5 µg/kg/h; 101-120 min 5.0 µg/kg/h). Bar graphs (right) depict average glucose (top), glucose infusion rate (middle), and arterial plasma insulin levels (bottom) during infusion of portal (red) or jugular (blue) infusion of GLP-1. *Hypergl.* reflects the average values from 50 to 60 min before the GLP-1 infusion was started. GLP-1 infusion into the jugular vein at the highest dose had a significantly greater effect on arterial plasma insulin concentrations than portal infusion (*$p<0.05$). All values are mean ± SE.

Figure 2: Glucose (A), glucose infusion rate (B), and arterial plasma insulin (C) during hyperglycemic clamp with inhibition of DPP4. Line graphs (left) depict infusion of GLP-1 into the portal (red) or jugular (blue) veins starting at time point 60 min, with increasing doses (61-80 min 1.5 µg/kg/h; 81-100 min 2.5 µg/kg/h; 101-120 min 5.0 µg/kg/h), to rats pretreated with vildagliptin. Bar graphs (right) depict average glucose (top), glucose infusion rate (middle), and arterial plasma insulin levels (bottom) during infusion of portal (red) or jugular (blue) infusion of GLP-1. *Hypergl.* reflects the average values from 50 to 60 min before the GLP-1 infusion was started. There was no significant difference between insulin concentrations with portal or jugular GLP-1 infusion. All values are mean ± SE.

Figure 3: Arterial plasma insulin concentrations during the hyperglycemic clamp after portal (left) or jugular (right) infusion of GLP-1(7-36). For both infusion sites arterial plasma insulin concentrations were significantly higher when GLP-1 was infused after DPP4 inhibition by vildagliptin (white) than without vildagliptin (black) (RM 2-way ANOVA $p<0.0001$ for dose and DPP4 inhibition). Bonferroni post-tests showed significantly higher arterial plasma insulin concentrations with vildagliptin compared to native GLP-1 infusions for all concentrations except for infusion of the highest dose into the jugular vein. *** indicates a $p<0.001$; ** indicates a $p<0.01$. All values are mean ± SE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2.5 µg/kg/h
5.0 µg/kg/h
Hypergl.
+1.5
+2.5
+5.0
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GIR (mg/kg/min)
C
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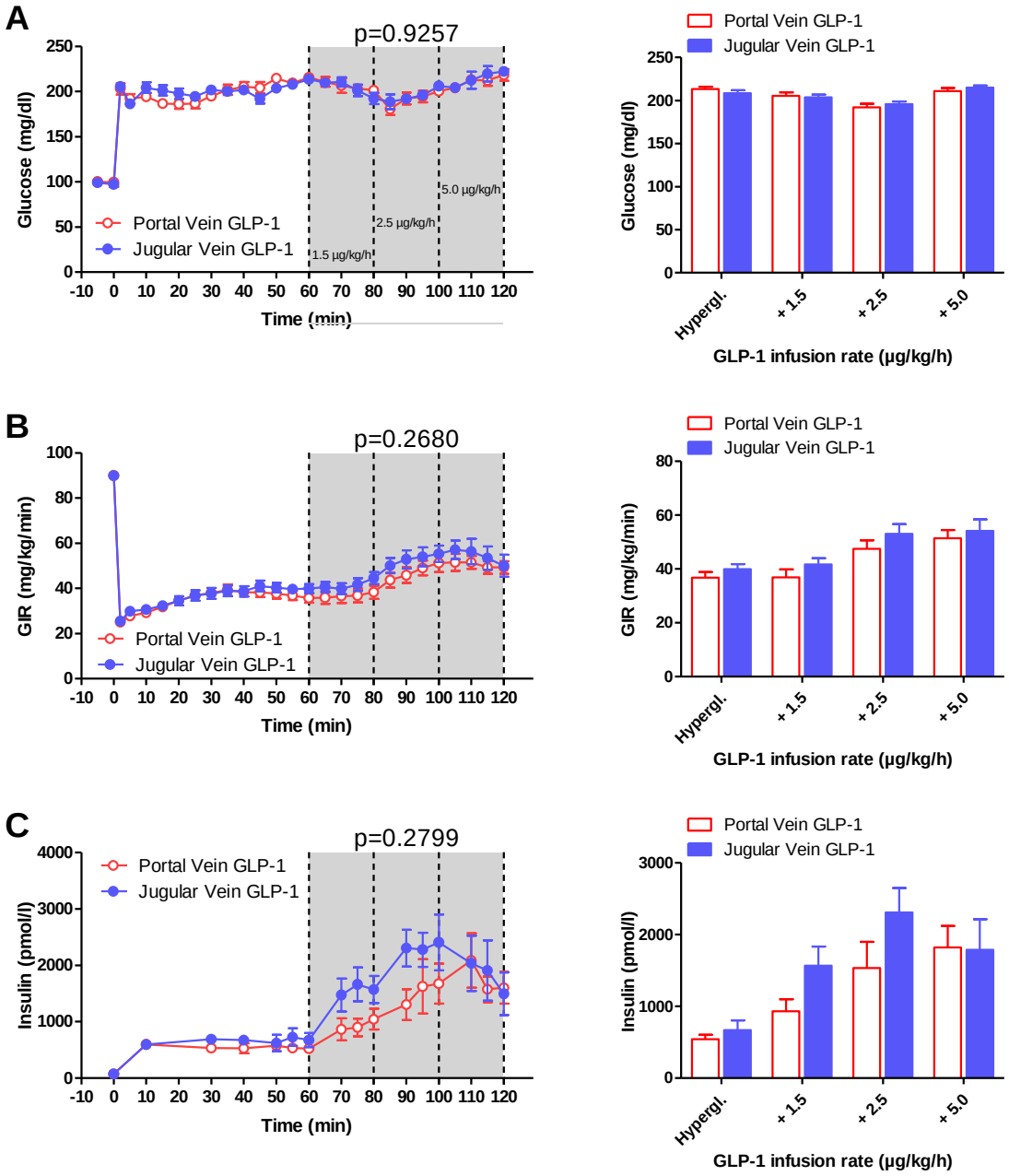
Insulin (pmol/l)


A
p=0.9257
Glucose (mg/dl)
Time (min)
Portal Vein GLP-1
Jugular Vein GLP-1
1.5 µg/kg/h
2.5 µg/kg/h
5.0 µg/kg/h
Hypergl.
+ 1.5
+ 2.5
+ 5.0
GLP-1 infusion rate (µg/kg/h)
B
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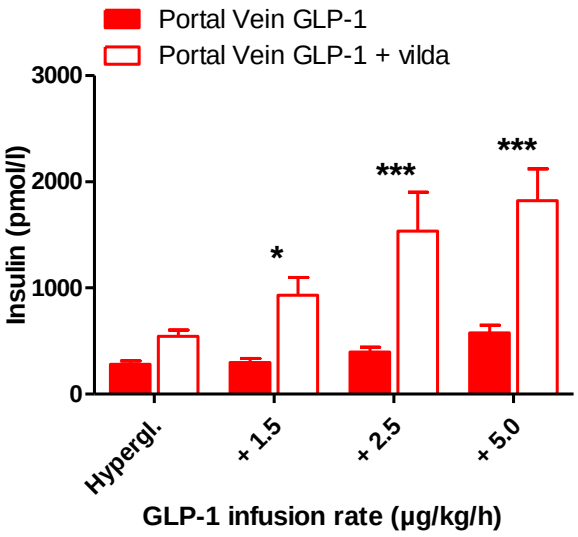
GIR (mg/kg/min)
C
p=0.2799
Insulin (pmol/l)


Portal Vein GLP-1
Portal Vein GLP-1 + vilda
Insulin (pmol/l)
3000
2000
1000
0
*
***
***
Hypergl.
+ 1.5
+ 2.5
+ 5.0
GLP-1 infusion rate (µg/kg/h)

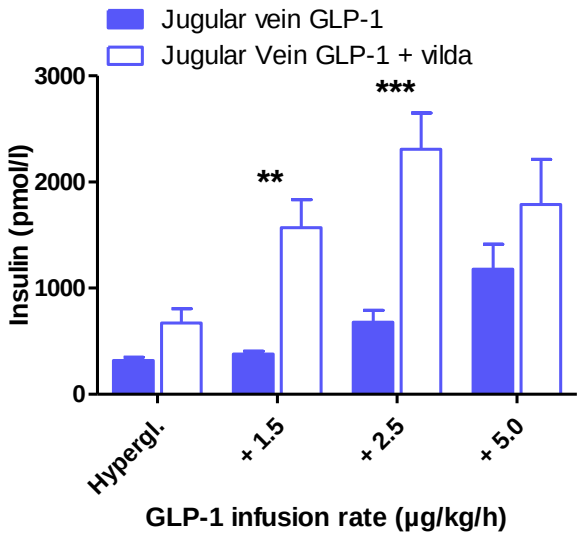

Jugular vein GLP-1
Jugular Vein GLP-1 + vilda
Insulin (pmol/l)
3000
2000
1000
0
**
***
Hypergl.
+ 1.5
+ 2.5
+ 5.0
GLP-1 infusion rate (µg/kg/h)

Table 1: baseline and clamp characteristics

	Portal Vein GLP-1 (N=10)	Jugular Vein GLP-1 (N=10)	p-Value
Body weight (g)	315.1±5.9	314.8±5.2	0.97
Fasting glucose (mg/dl)	97.6±4.9	95.9±2.9	0.76
Clamp glucose (average) (mg/dl)	212.1±3.5	206.3±2.5	0.19
Glucose over basal (mg/dl)	114.5±6.0	110.4±2.7	0.54
CV – Clamp (%)	8.7±0.6	8.8±0.5	0.96

With DPP4 inhibition (vildagliptin)

	Portal Vein GLP-1 (N=9)	Jugular Vein GLP-1 (N=12)	p-Value
Body weight (g)	335.2±5.8	319.7±8.4	0.17
Fasting glucose (mg/dl)	99.9±4.8	98.1±2.7	0.72
Clamp glucose (average) (mg/dl)	201.2±1.4	202.7±1.1	0.38
Glucose over basal (mg/dl)	101.2±1.4	104.6±2.4	0.49
CV – Clamp (%)	8.6±0.7	9.4±0.7	0.38

Mean ± SE for cohorts undergoing the clamp procedure. Differences between the animals receiving portal vs. jugular vein infusion of GLP-1 were compared using a two-sided ttest for unpaired cohorts with equal variances. A $p<0.05$ was considered statistically significant. None of the parameters differed significantly between portal and jugular vein GLP-1 infusion.

Table 2: Arterial plasma insulin levels (pmol/l) during clamp

	Portal Vein GLP-1 (N=10)	Jugular Vein GLP-1 (N=10)	p-Value
Hyperglycemia	282±33	318±29	ns
+ GLP-1 1.5 µg/kg/h	300±36	378±28	ns
+ GLP-1 2.5 µg/kg/h	396±44	679±112	ns
+ GLP-1 5.0 µg/kg/h	577±71	1178±235*	<0.05

Both dose ($p<0.0001$) of GLP-1 and infusion site ($p=0.0207$) had a significant impact on arterial plasma insulin levels when analyzed by RM 2-way ANOVA. Bonferroni post-tests demonstrated significantly higher insulin levels during infusion of GLP-1 into the jugular vs. portal vein at a dose of 5 µg/kg/h.* indicates a $p<0.05$

With DPP4 inhibition (vildagliptin)

	Portal Vein GLP-1 (N=9)	Jugular Vein GLP-1 (N=12)	p-Value
Hyperglycemia	543±59	672±135	ns
+ GLP-1 1.5 µg/kg/h	932±168	1569±264	ns
+ GLP-1 2.5 µg/kg/h	1535±366	2310±340	ns
+ GLP-1 5.0 µg/kg/h	1822±300	1788±425	ns

Dose ($p<0.0001$) of GLP-1 but not infusion site ($p=0.2799$) had a significant impact on arterial plasma insulin levels when analyzed by RM 2-way ANOVA. All values are mean ± SE.

Table 3: Arterial plasma GLP-1 (7-36) concentration (pmol/l) during portal and jugular vein infusion

	Portal Vein GLP-1 (N=5)	Jugular Vein GLP-1 (N=5)	p-Value
Baseline	2.6±0.4	3.3±0.9	ns
2.5 µg/kg/h	14.3±2.4	43.6±5.0***	p<0.001
5.0 µg/kg/h	36.6±2.8	80.9±3.4***	p<0.001

Both dose (p<0.0001) of GLP-1 and infusion site (p<0.0001) had a significant impact on the arterial plasma GLP-1 levels when analyzed by RM 2-way ANOVA. Bonferroni post-tests demonstrated significantly higher plasma GLP-1 levels after infusion of both 2.5 and 5 µg/kg/h GLP-1 into the jugular vs. portal vein (p<0.001 for both doses). *** indicates a p<0.001

With DPP4 inhibition (vildagliptin)

	Portal Vein GLP-1 (N=5)	Jugular Vein GLP-1 (N=6)	p-Value
Baseline	7.0±2.5	7.7±2.2	ns
2.5 µg/kg/h	47.1±12.3	116.7±27.9	ns
5.0 µg/kg/h	184.8±35.7	443.2±66.4**	p<0.01

Both dose (p<0.0001) of GLP-1 and infusion site (p<0.0081) had a significant impact on the arterial plasma GLP-1 levels when analyzed by RM 2-way ANOVA. Bonferroni post-tests demonstrated significantly higher plasma GLP-1 levels after infusion of 5 µg/kg/h GLP-1 into the jugular vs. portal vein (p<0.01). ** indicates a p<0.01. All values are mean ± SE.