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The insulinotropic effect of the enteral hormone glucagon-like peptide-1 in the portal and systemic circulation of rats

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List of abbreviations

ANOVA	Analysis of variance
CD-26	Cluster of differentiation 26
cDNA	Complimentary deoxyribonucleic acid
CNS	Central nervous system
DIRKO	Dual incretin receptor knockout mouse
DPP-IV	Dipeptidylpeptidase-IV
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Gcgr	Glucagon receptor
GIP	Glucose-dependent insulinotropic polypeptide
GIR	Glucose infusion rate
GLP-1	Glucagon-like peptide-1
GLP-1r	GLP-1 receptor
GLP-2	Glucagon-like peptide-2
GRPP	Glicentin related pancreatic polypeptide
HbA1c	Glycated hemoglobin A1c
IP1	Intervening peptide 1
IP2	Intervening peptide 2
JV	Jugular vein
MPGF	Major proglucagon fragment
mRNA	Messenger ribonucleic acid
OXM	Oxyntomodulin
PC 1/3	Prohormone convertase 1/3
PC 2	Prohormone convertase 2
PE	Polyethilene
PV	Portal vein
RBC	Red blood cells
RIA	Radioimmunoassay
RM 2-way ANOVA	Repeated measures 2-way ANOVA
sDPP-IV	Soluble DPP-IV
SEM	Standard error of the mean
T2D	Type 2 Diabetes
US	United States

1 Introduction

1.1 The incretin effect and the discovery of GLP-1

Studies concerning an endocrine activity of the gastrointestinal tract started around the beginning of the 20th century with the discovery of gut hormones such as secretin by Bayliss and Starling [1]. Glucose-lowering effects of duodenal mucosa extracts was shown as early as 1906 by Moore et al. [2]. The term “incretin” for gut derived substances that lower blood glucose was first introduced by La Barre in 1932 [3]. The development of a radioimmunoassay for insulin allowed the demonstration of the so-called “incretin effect” by showing that glucose administered orally stimulated a greater insulin response than if administered intravenously [4, 5].

In healthy individuals the incretin effect accounts for 50-70% of postprandial insulin secretion [6], whereas this effect is reduced in patients with type 2 diabetes (T2D) and subjects at risk for developing diabetes [7, 8]. Two hormones have been identified that mediate the majority of the incretin effect: Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), hence called incretins or incretin hormones.

GIP was extracted from duodenal mucosa of pigs in the early 1970s by the group of John Brown in Vancouver [9, 10]. Initially it was termed gastric inhibitory polypeptide, because it reduced gastric acid secretion in dogs. However, this occurs only at pharmacological doses, whereas purified preparations of GIP were shown to potently augment glucose-stimulated insulin secretion in humans [11] and rats [12]. It was later revealed that GIP is secreted from enteroendocrine K-cells within the mucosa of the stomach and duodenum in response to luminal nutrients and has a variety of biological actions beyond its action as an incretin. Among these GIP is known to induce glucagon secretion, inhibit apoptosis in the pancreatic β-cells and promote their proliferation, as well as to induce fat deposition in adipose tissue. Furthermore, GIP is involved in bone metabolism [6]. Although GIP was soon recognized as a mediator of the incretin effect it became quickly evident that GIP was not the only incretin [13].

In the 1980s GLP-1 was discovered by cDNA sequencing of proglucagon mRNA [14]. Glucagon is a peptide with 29 amino acids that is spliced from a much larger

proglucagon protein with 158 amino acids (figure 1). Next to glucagon other peptide fragments, namely glicentin related pancreatic polypeptide (GRPP), intervening peptide 1 (IP1), and major proglucagon fragment (MPGF) are included in the proglucagon transcript of the pancreas. Within the MPGF two glucagon-like sequences were found and named GLP-1 and glucagon-like peptide-2 (GLP-2), respectively. Peptide sequencing suggested that GLP-1 would be cleaved in position 72 of proglucagon, where two consecutive basic amino acids are encoded and thus consists of 37 amino acids (GLP-1(1-37)) (figure 1) [14]. Further research revealed that only the truncated form of the peptide cleaved at another basic amino acid in position 77, followed by a histidine in position 78, called GLP-1(7-37), is bioactive and stimulates cAMP production and insulin release in rat islet cells [15]. Moreover, the c-terminal peptide sequence predicts a prohormone convertase–directed cleavage site followed by an amidation of the penultimate arginine resulting in GLP-1(1-36)amide and GLP-1(7-36)amide, respectively. Until today no bioactivity has been shown for the non-truncated forms GLP-1(1-37) and GLP-1(1-36)amide [14].

It had been known for some time that a so-called “gut glucagon” existed [16], and it was suspected that it contributed to hyperinsulinemic reactive hypoglycemia seen after gastric surgery [17]. However, it was found that the immunoreactivity for glucagon in the gut came from a larger peptide that included the sequence of glucagon, called glicentin (figure 1) [18]. Because all the components for glicentin and glucagon were both present in the pancreas and the intestine, it was suspected that differential processing in the various tissues is responsible for the different peptide products [14]. Because neither glicentin nor the other peptide within the glicentin fragment, oxyntomodulin (OXM), has significant insulinotropic potency, the focus shifted towards the MPGF and the two glucagon-like peptides expressed within this fragment.

The discovery that GLP-1(7-37) produced in the gut of various mammals is a potent stimulus for insulin secretion in rats [19], pigs [20], and humans [21] was a major breakthrough in 1987. Henceforth, GLP-1 was recognized as the second incretin along with GIP, and became the topic of intensive research about its physiological and pharmacological actions [14].

We know today, that the preproglucagon gene is expressed in the pancreatic α-cells, enteroendocrine L-cells of the gut and the central nervous system (CNS), namely neurons within the nucleus of the solitary tract [22]. Differential splicing by enzymes called prohormone convertase 1/3 (PC1/3; gut, brain) and prohormone convertase 2 (PC2; α-cell) results in tissue specific posttranslational processing. The α-cell produces predominantly glucagon, along with GRPP, IP1 and MPGF. In the L-cells and the brain cleavage of proglucagon by PC1/3 yields the peptides GLP-1, GLP-2, OXM, intervening peptide 2 (IP2), and glicentin (figure 1). The latter two products have no known biological function [22]. GLP-2 has potent intestinotrophic properties and has been developed into a treatment for short-bowel syndrome [14]. OXM has weak agonistic activity at the glucagon (Gcgr) and GLP-1 receptors (GLP-1r) without a known receptor of its own and its biological importance is unclear. More recently it has been suggested to be used as a co-agonist with GLP-1 to treat diabetes and obesity [23]. Newer studies

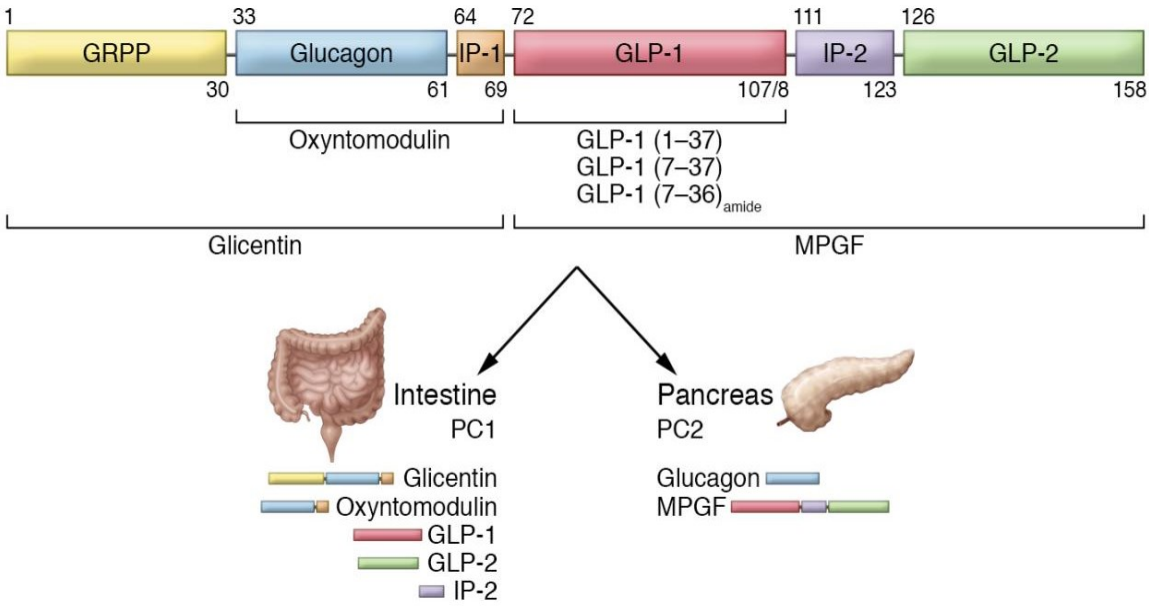

Figure 1: Proglucagon and its cleavage products by tissue specific differential splicing (from Drucker et al. 2017 [14]). In the intestine (and brain) activity of prohormone convertase 1/3 (PC1) results in production of glicentin, oxyntomodulin, GLP-1, GLP-2, and intervening peptide-2 (IP-2). In the pancreas proglucagon is processed into glucagon, glicentin related pancreatic polypeptide (GRPP), intervening peptide-1 (IP-1), and major proglucagon fragment (MPGF) by prohormone convertase 2 (PC2).

suggest that splicing of proglucagon in the pancreas does not exclusively result in glucagon production but small amounts of PC2 are also present within the α-cell [22].

Enteroendocrine L-cells are present in the small and large intestine, where nutrients within the lumen of the gut stimulate rapid secretion of GLP-1 into the circulation. Within the intestine, the density of glucagon gene expression and proglucagon synthesis increases from the proximal to distal gut and expression is highest in the colon. Besides luminal contact of nutrients with the L-cells, also neural, endocrine, and paracrine mechanisms are suggested to be involved in GLP-1 secretion [22]. Both, GLP-1(7-37) and GLP-1(7-36)amide, are secreted from the intestine with GLP-1(7-36)amide representing the major form of active GLP-1 in human plasma [24]. There is no evidence towards a differential effect or potency of the amidated and non-amidated form of GLP-1 [14]. GLP-2 is secreted along with GLP-1 in equimolar amounts from intestinal L-cells.

1.2 Biological actions of GLP-1

Because GLP-1 was expected to act as an incretin hormone, its glucose-dependent insulinotropic properties were revealed as soon as the functional peptide was discovered. However, numerous biological actions of GLP-1 beyond insulin secretion have since been identified. GLP-1 mediates its effects via a specific receptor expressed in a variety of tissues.

The GLP-1r is a 7-transmembrane-spanning, heterotrimeric G-protein-coupled receptor from the same family as the receptors for glucagon, GLP-2, and GIP. Its expression was confirmed in various tissues including the pancreatic α, β, and δ-cells, lung, heart, kidney, intestine, and parts of the central and peripheral nervous system [6]. Mice with a deleted GLP-1r gene (GLP-1r -/-) or a lack of the preproglucagon gene (Gcg -/-) have been particularly helpful in unravelling the physiology of GLP-1. Similarly, the use of a specific GLP-1r antagonist exendin(9-39), the truncated form of the naturally occurring GLP-1r agonist exendin-4, in humans has contributed significantly to understanding the importance of GLP-1 in health and disease [25].

A summary of the most prominent biological actions of GLP-1 is outlined in figure 2 [24]. Most is known about the physiological role and molecular mechanisms of the GLP-1r in the pancreatic β-cell, from where the receptor was initially cloned. In the islets of Langerhans, GLP-1 also reduces glucagon secretion from α-cells, stimulates insulin biosynthesis and enlarges β-cell mass in

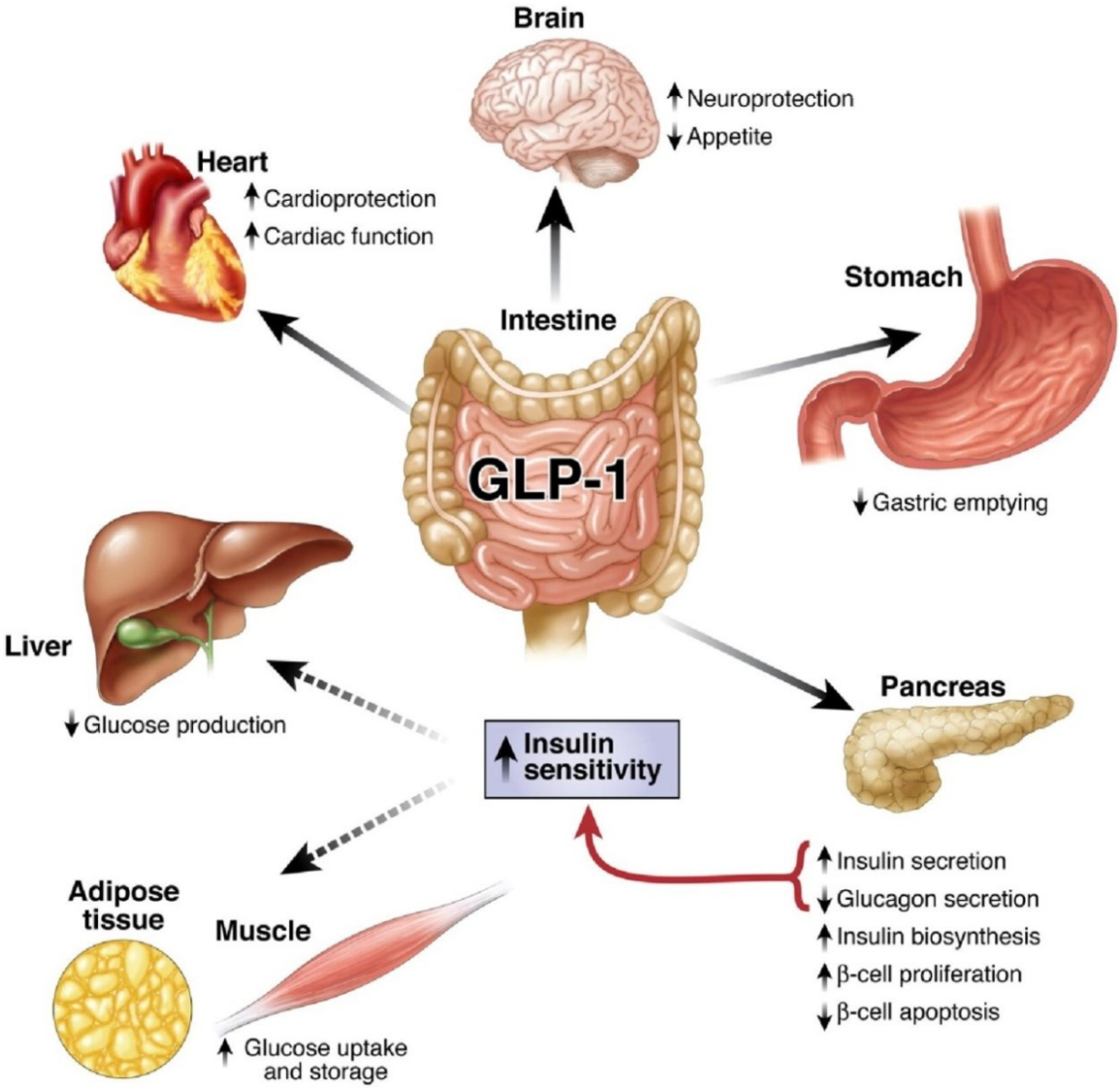

Figure 2: Direct and indirect effects of GLP-1 action in different target tissues (from Baggio and Drucker 2007 [6]). GLP-1r signaling has direct effects in different target tissues like the pancreatic islets, stomach and intestine, central nervous system and the heart. Other effects like glucose lowering actions in the liver, muscle and adipose tissues seem to be mediated indirectly through neural pathways.

rodents by increased proliferation and downregulation of apoptosis. However, the latter effects are only seen in preclinical studies and experiments in humans have failed to show an enlargement of β-cell mass or persistent improvement in β-cell function in diabetic patients after treatment with GLP-1-based pharmacotherapies [26].

Other glucoregulatory actions of GLP-1 include enhancement of hepatoportal glucose sensing, reduction of hepatic glucose production and increased peripheral insulin sensitivity. Because liver, adipose tissue, and muscle do not conclusively express the GLP-1r, these actions seem to be mediated indirectly through neural mechanisms [26].

GLP-1r -/- mice show impairment of glucose tolerance and reduced glucose-stimulated insulin secretion [27]. Interestingly, these mice also have increased fasting glycemia, proving that the glucoregulatory actions of GLP-1 are not limited to the postprandial phase. In humans, acute antagonism of the GLP-1r with exendin-(9-39) results in impaired glucose tolerance and reduced insulin secretion along with an increase of glucagon [28, 29]. Thus, GLP-1 is a physiological regulator of glucose metabolism in rodents and humans.

Other prominent actions of GLP-1r signaling include inhibition of gastric emptying, increased satiety with reduction of food intake and weight loss, as well as cardio- and neuroprotective effects [6]. However, these effects are much more prominently seen during pharmacological interventions using supraphysiologic doses of native GLP-1 or GLP-1r agonists [30]. Particularly the effects on body weight and cardiovascular function have not consistently been shown to be physiologically regulated by GLP-1 in humans. On the other hand, these actions explain very well the prominent side-effects of GLP-1r agonists in the treatment of diabetes. While some of these effects are favorable (weight loss, cardiovascular benefits) others limit the clinical use in some patients (nausea, vomiting) [26].

GLP-1 actions in the heart are complex and the GLP-1r seems to be expressed in different cell types of the rodent and human heart [31]. In addition, cardiac effects of GLP-1 have been shown in GLP-1r -/- mice and with GLP-1r blockade suggesting some receptor independent effects. At least in rodents GLP-1 seems to improve ventricular function and plays a potentially physiologic role [31].

However, in humans there was no clinical benefit of chronic GLP-1r activation in patients with congestive heart failure [32]. The cardiovascular biology of GLP-1 has been extensively reviewed by Ussher and Drucker in 2012 [31] and Drucker in 2016 [33]. More recently, the reduced rate of cardiovascular events and mortality in diabetic patients treated with the GLP-1r agonists liraglutide [34] and semaglutide [35] has further triggered interest in the cardioprotective properties of GLP-1 [32].

Although GLP-1r expression is abundant in the lung the precise function of GLP-1 in the respiratory system and its relevance to normal pulmonary physiology are not known [6]. While the GLP-1r mRNA transcripts are found in the kidney the precise cellular localization is not yet revealed. Infusion of GLP-1 into rodents and humans evokes a natriuretic and diuretic response. Nevertheless, there is little compelling evidence implicating a protective role for GLP-1r agonists in the kidney [30]. Newer research suggests a role of GLP-1 in the immune system and inflammation. The GLP-1r is also expressed in intraepithelial lymphocytes and there seems to be interaction with the gut microbiome. However, a physiological or pharmacological relevance of these findings in humans has not yet been established [26].

1.3 Rapid degradation of GLP-1 (and GIP) by Dipeptidylpeptidase-IV (DPP-IV)

The glucose-lowering and anorectic effects of GLP-1 have soon generated great interest into developing the incretins into a pharmacotherapy for diabetes and obesity. However, native GLP-1 has a dramatically short half-life of 60-90 seconds *in vivo*. This is due to rapid cleavage of the first two amino acids histidine-7 and alanine-8 of the peptide by the ubiquitous enzyme DPP-IV [36]. Thus, the vast majority of circulating GLP-1 is comprised of the non-insulinotropic cleavage product GLP-1(9-36) [37]. Until today, there is an ongoing debate whether GLP-1(9-36) has important biological actions. However, it is generally accepted that DPP-IV inactivated GLP-1 does not act as an incretin hormone [38]. Similarly GIP is cleaved after the first two n-terminal amino acids and inactivated by DPP-IV with a slightly longer circulating half-life of 5-7 minutes [39].

DPP-IV, also known as CD-26, is ubiquitously expressed in a variety of tissues. It exists in two different forms, a membrane bound receptor protein stimulating intracellular signaling pathways, and as soluble DPP-IV (sDPP-IV) found in the circulation of various mammals, including humans and rodents. Both forms of DPP-IV retain their enzymatic activity [24]. CD-26 seems to have an important role as signaling pathway of the immune system and is best characterized in T-cells. Despite this, the immunological phenotype of DPP-IV -/- mice is astonishingly benign without any major defect in immune function [40, 41].

On the other hand, the role of DPP-IV activity in regard to glucose homeostasis has been extensively studied and DPP-IV loss-of-function models helped greatly to understand the physiology of the enzymatic component. DPP-IV -/- mice as well as a naturally occurring strain of Fisher 344 rats with absent or reduced activity of DPP-IV show improved glycemic control, elevated insulin levels as well as reduced degradation of GIP and GLP-1 [40, 42]. Furthermore, DPP-IV null mice are protected from obesity and insulin resistance induced by a high-fat diet [43].

Pharmacological inhibition of DPP-IV activity also results in prolonged half-life of the incretins GLP-1 and GIP and a reduction of hyperglycemia with an enhancement of glucose-stimulated insulin release [6]. Furthermore, the metabolic actions of DPP-IV inhibitors seem to be exclusively mediated by GLP-1 and GIP as the disruption of both their signaling in a dual incretin receptor knockout mouse (DIRKO) abolishes the metabolic actions of DPP-IV inhibitors like vildagliptin [44].

In addition to DPP-IV various other peptidases and renal clearance play a role in the clearance of GLP-1 and GIP but to a much lesser extent.

1.4 Pharmacotherapies based on the incretin system

The potential of GLP-1 as diabetes therapy was soon recognized and generated great interest in how to exploit the incretin system for pharmaceutical purposes. The combination of the glucose-lowering actions of GLP-1 with little risk of hypoglycemia and concomitant weight-loss promised superiority to any previously established pharmacotherapy of diabetes.

A fundamental observation driving much of the research into GLP-1 action was the blunted incretin effect in patients with T2D [7]. While the responsiveness of β-cells to GIP is severely attenuated in diabetic patients, GLP-1 retains its insulinotropic actions and lowers glycemia [45]. Hence forth the majority of studies exploring the incretin system as a therapy for diabetes have focused on GLP-1 [25]. In a proof-of-concept study, continuous subcutaneous infusion of GLP-1 in diabetic patients over 6 weeks resulted in a reduction of HbA1c of 1.3% and a weight loss of 1.9 kg [46]. However, the rapid degradation of native GLP-1 by DPP-IV and the parenteral administration route limited GLP-1 as a feasible long-term therapy for diabetic patients.

John Eng explored the biological actions of peptides extracted from the venomous saliva of the lizard *Heloderma suspectum* in his laboratory. The extracted peptide exendin-4 shows 50% homology with the amino acid sequence of mammalian GLP-1 and acts as an agonist on the GLP-1r [47, 48]. Because it does not share the alanine in position two of the N-terminus, exendin-4 is resistant to degradation by DPP-IV. It was proven useful in the treatment of T2D as a subcutaneous injection and approved by the US Food and Drug Administration in 2005 under the name exenatide [25]. Other specifically designed GLP-1r agonists with long-term stability and resistance to DPP-IV degradation have followed since and proven helpful pharmacologic agents in T2D. Particularly the reduction of cardiovascular events and mortality by some agents in this drug class has underlined the potential of the GLP-1r agonists for the treatment of T2D. To date there are only two established drug classes in diabetes therapy with proven cardiovascular benefits [32]. In 2014 the GLP-1r agonist liraglutide has been approved for the treatment of obesity in non-diabetic patients [49]. Currently dual agonists of the GLP-1 and GIP receptors are developed into novel diabetes and obesity therapeutics and show superior efficacy compared to agonists of the GLP-1r alone [50-52].

Another mechanism to exploit the incretin system as a diabetes therapy is preventing the degradation of endogenous GLP-1 and GIP by inhibition of DPP-IV. As can be expected circulating concentrations of non-degraded active forms of both incretins are increased with pharmacologic inhibition of DPP-IV [53]. This results in a reduction of fasting and postprandial hyperglycemia in patients with

T2D by enhanced glucose-stimulated insulin secretion from β-cells and suppression of glucagon [25]. The first DPP-IV inhibitor approved for the treatment of T2D by the US Food and Drug Administration was sitagliptin in 2006. Multiple other substances in this drug-class have since been approved and are available for treatment of diabetes [14].

Unlike GLP-1r agonists, treatment with DPP-IV inhibitors has a neutral effect on body weight and does not impact clinically significant gastric emptying. A great advantage of DPP-IV inhibitors in the treatment of diabetes is the convenient oral administration of the small-molecule enzyme inhibitors and the lack of clinically relevant adverse events in the majority of patients (except for mild gastrointestinal side effects). Because the insulinotropic and glucagonostatic effects are mediated through the incretins in a glucose-dependent manner, there is practically no risk of hypoglycemia with these agents [25]. However, the relative potency to lower glycemia measured by HbA1c is somewhat weaker than that seen with GLP-1r agonists and the weight neutrality of this drug-class makes it less favorable for obese or overweight diabetic patients [54]. Furthermore, none of the many DPP-IV inhibitors licensed for therapy have proven cardiovascular benefits compared to standard treatment of diabetes [32].

GLP-1r agonists and DPP-IV inhibitors show distinct differences in their therapeutic actions. While DPP-IV inhibition results in significant but small increase of active GLP-1 (and GIP) plasma concentrations with a reduction of total circulating GLP-1 (mediated through a putative feed-back inhibition on L-cells), injection of GLP-1r agonists results in potent and supraphysiologic stimulation of GLP-1r in all compartments and tissues expressing the receptor. This underlines the importance to differentiate between physiologic and pharmacologic GLP-1 actions [54].

1.5 Evidence of a non-humoral mechanism of action of GLP-1 to release insulin secretion

In healthy humans glycemia is very tightly regulated despite major challenges through nutrient and carbohydrate ingestion and phases of intermittent or prolonged fasting. Increasing meal-sizes lead to a larger insulin release despite very similar glycemic excursions and this feed-forward mechanism is thought to

be regulated by the incretins. The classical model of incretin action is that GLP-1 and GIP get secreted from intestinal endocrine cells into the circulation in response to nutrients. From there the incretin hormones reach the pancreatic β-cell with its specific receptors and stimulate insulin release in a glucose-dependent manner [55].

To maintain tight glycemic control plasma insulin concentrations underlie a wide dynamic range with more than 10-fold differences between the fasting and postprandial state. Similarly, plasma levels of GIP have a similar broad physiological range, with levels increasing 5-10 fold over fasting after meals. On the other hand, GLP-1 concentrations in the circulation are very low and barely detectable in the fasting state and increase about two- to maximally threefold in the postprandial state. Furthermore, it is estimated that only about 10% of secreted GLP-1 remains active by the time it reaches the central circulation due to the abundant degradation by DPP-IV (figure 3) [56]. 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 results in an almost exponential insulinotropic effect [57]. In addition there is experimental data suggesting that infusion of GLP-1 at a dose mimicking physiological postprandial levels is not able to stimulate insulin secretion in a canine model [58]. Whether a systemic

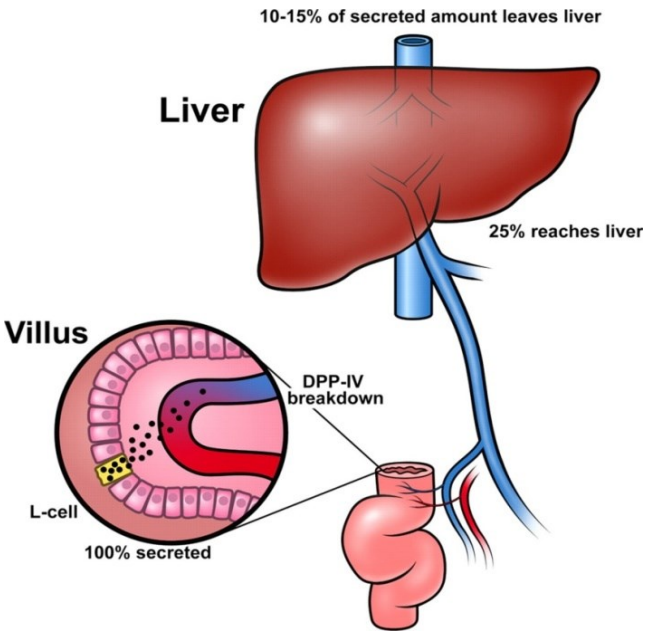

Figure 3: Rapid degradation of GLP-1 after secretion from L-cells (from Holst 2007 [56]). DPP-IV activity is present in the tissue surrounding the L-cell and the endothelium. It can be estimated that only 25% of active GLP-1 reaches the liver and further degradation leave only about 10-15% uncleaved by the time it reaches the circulation.

infusion of GLP-1 into humans in an allegedly physiological range is insulinotropic, is under debate [59]. In addition, the clinical effectiveness of DPP-IV inhibitors despite the only moderate increase in circulating active levels of GLP-1 has further challenged the classical model of a humoral endocrine mechanism of action of GLP-1 [60].

Hence, alternative models of GLP-1 action have been proposed and there is compelling evidence that GLP-1 might mediate its incretin effect in a neuroendocrine fashion (figure 4). Nakabayashi et al. have shown that GLP-1r signaling in the portal vein of rats augments the firing of hepatic vagal afferents and pancreatic vagal efferents, suggesting a neuroendocrine signal originating in the portal vein to stimulate insulin secretion from pancreatic β-cells [61]. In another study in rats it was demonstrated that a bolus of GLP-1 and glucose into the portal vein induces a robust insulinotropic effect that was almost abolished by administration of the ganglionic blocker chlorisondamine [62]. The insulinotropic action of a bolus of GLP-1 into the jugular vein was not affected by chlorisondamine treatment and led to a similar insulin release as portal GLP-1. It was concluded that GLP-1 can mediate insulin release both directly in the pancreatic islet as well as via a nervous mechanism triggered in the hepatoportal system [62]. A study by Burcelin et al. showed in mice that antagonism of the GLP-1r with exendin(9-39) in the portal vein but not the femoral vein reduced the potency of portal glucose to stimulate its own clearance (hepatoportal glucose sensor), while GLP-1 infusion had no such effect. It was suggested that there is a constitutive activation of the GLP-1r in the portal vein in the fasting state that is necessary for glucose sensing. Interestingly insulin levels increased with the portal GLP-1 infusion but the differences in glycemia did not allow estimating its contribution to the incretin effect [63]. In addition various canine studies with portal vein glucose and GLP-1 infusion showed consistent effects on peripheral glucose uptake. This effect seems to be dependent on portal but not systemic co-infusion of glucose and in this setting GLP-1 does not change pancreatic hormone release [64-67].

Previous studies in our laboratory showed that the GLP-1r is expressed in vagal afferent neurons and immunohistochemistry revealed co-localization of synaptophysin and GLP-1r in the portal vein of rats. Furthermore, a low-dose

infusion of the GLP-1r antagonist [desHis[1], Glu[9]]exendin-4 into the portal vein but not the jugular vein impaired tolerance to oral glucose in rats. Thus, it was concluded that portal vein GLP-1r signaling is required for normal glucose tolerance (figure 4). Because glycemia was different between the two experimental conditions, the effect of portal GLP-1 antagonism on insulin release was not further interpretable [68].

1.6 Hypothesis: GLP-1 released from the gut into the portal vein mediates insulin secretion from pancreatic β-cells through a neuroendocrine mechanism via afferents in the hepatoportal bed

While most of these studies show that neuroendocrine GLP-1r signaling in the portal vein is essential for glucose tolerance and interfering with it by local antagonism or vagal denervation interferes with glucose disposal, none have proven that the most prominent action of GLP-1, namely mediating the incretin effect, depends on this mechanism. Particularly there is no study that compares the effect of GLP-1 infusion into the portal vein with infusion into the jugular vein

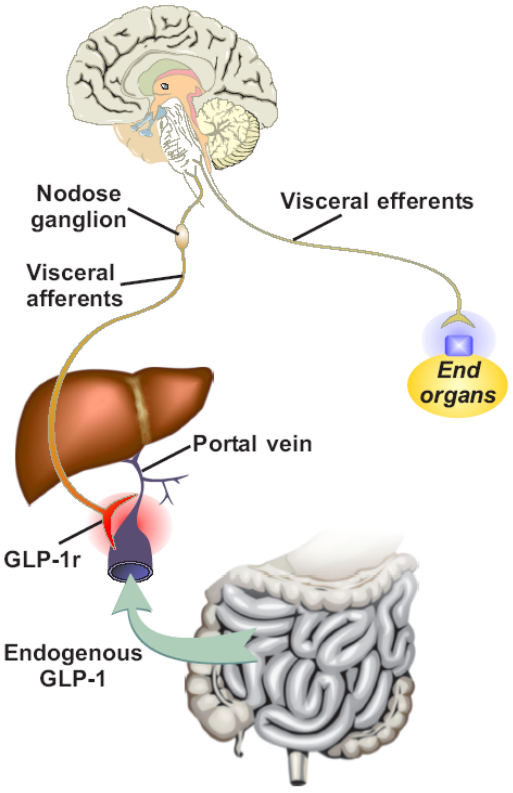

Figure 4: Alternative model of GLP-1 action. Endogenous GLP-1 gets secreted from intestinal L-cells and acts on GLP-1r in the hepatic portal vein. Vagal afferents transmit the GLP-1 signaling to neurons in the hindbrain from where visceral efferents mediate insulin secretion from pancreatic β-cells.

during isoglycemic conditions that allows comparing insulin secretion in response to this intervention.

Therefore, we hypothesized that site specific infusion of GLP-1 into the portal vein would result in higher plasma insulin concentrations during a hyperglycemic clamp than jugular infusion of the same dose of GLP-1.

1.7 Hypothesis II: Pharmacological DPP-IV inhibition will result in a similar insulinotropic potency of portal and jugular GLP-1

As a secondary hypothesis we tested an alternative model whereby portal GLP-1 is more susceptible to degradation by DPP-IV in the hepatoportal bed where the enzyme is highly expressed. In this model the insulinotropic effect of portal GLP-1 would be weaker than peripherally administered peptide, and pharmacological inhibition of DPP-IV would result in similar GLP-1 plasma concentrations in the arterial circulation with equal insulinotropic potency of portal and jugular GLP-1.

2 Material and methods

2.1 Animals

Experiments were performed on male Long Evans rats with a mean body weight of 270-300 g, delivered from Harlan Laboratories Inc. (Indianapolis, IN – USA) directly to the animal facility at the Genome Research Institute of the University of Cincinnati. 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 from 6:00 to 18:00 daily.

Starting on the day of arrival rats had immediate contact with the researchers involved in the handling of the animals and the experiments. Rats were weighed and handled daily, usually in the morning.

Beginning one week after arrival at our facility rats underwent surgery (section 2.3). During post-surgical recovery the rats were continuously handled and weighed daily and their body weight was monitored until they reached their pre-surgical body weight. Rats suffering consistent weight loss after surgery were fed wet chow, mostly resulting in increased food intake and facilitating recovery. If animals had signs of dehydration they were given a subcutaneous injection of a balanced electrolyte solution (Normosol®, Pfizer Inc., New York City, NY – USA) to improve well-being. Experiments were performed only on fully recovered, healthy rats. Health was assessed by monitoring of behavior, food and water intake, and general appearance (e.g. rough fur).

Rats were fasted approximately 15 hours before the experiments having free access to water. On the day of the clamp experiment rats were weighed and subsequently brought to the room where the experiments took place. There they were able to adjust to the new environment for 20-40 minutes. During the experiments the animals were conscious and freely moving at all times.

After the clamp rats were fed and brought back to their housing facility. On the following day the animals were examined and weighed to ensure full recovery from the experiment.

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.

2.2 Materials

All catheters were prepared manually in our laboratory as described below before the surgeries.

2.2.1 Carotid catheter

For the carotid artery polyethylene (PE) catheters were used as PE is a stronger and more resistant material compared to silicone tubing. The PE-50 was produced by Instech Solomon (Plymouth Meeting, PA – USA) and had an inner diameter of 0.058 cm and an outer diameter of 0.096 cm.

Catheters were all cut 20 cm long with a scalpel and 4 cm from the tip a small bubble was placed by heating up the PE. To avoid inner collapsing a steel wire was inserted into the catheter before. The bubble would help to secure the catheter into the carotid artery. The catheters were sterilized by gas or alcohol and before their use they were flushed with sterile 0.9% saline (Baxter, Deerfield, IL – USA).

The length of the catheter part inserted into the carotid artery would vary based on the individual rat's body weight: 2.7 cm /300 g and additional 0.1 cm per 20 g of body weight.

2.2.2 Jugular catheter

For the jugular vein catheter silicone tubing from Braintree Scientific Inc. (Braintree, MA – USA) was used. The catheters had an inner diameter of 0.063 cm and an outer diameter of 0.119 cm and were 20 cm long. With liquid silicone a small bubble was made 4.2 cm from the tip to secure the catheter internally to the jugular vein.

2.2.3 Portal catheter

Silicone tubing (Braintree Scientific Inc.) was also used for the portal vein catheter as it is a softer and gentler material. The catheters had an inner diameter of 0.050 cm and an outer diameter of 0.093 cm and were 28 cm long. The inner tip was beveled to facilitate insertion in the quickly collapsing portal vein. With liquid silicone a small bubble was placed 1 cm from the tip to help secure the catheter to the portal vein. 5 cm from the inner tip a Dacron mesh (Braintree Scientific Inc.) with 0.5 cm diameter was attached around the catheter with liquid silicone. This mesh was sutured to the abdominal wall to further secure a correct placement of the portal catheter.

2.3 Surgery

2.3.1 General preparation

A sterile table cloth was placed on the operating table, all sterile instruments and materials were then placed on it. Under the sterile table cloth a heating pad was placed covered by two layers of gauze to avoid over-heating and burning.

The rats received general anesthesia through a standard Isoflurane inhalation (99%Iso/ml, Abbott Laboratories, North Chicago, IL – USA). Rats were placed in a transparent box with an oxygen and isoflurane supply. After approximately 5 minutes they were taken out and shaved on the neck, abdomen and back with a commercial razor.

The shaved spots were then disinfected with povidone-iodine antiseptic (Betadine®, Purdue Pharma L.P., Stamford, CT – USA). The rats were placed on the operating table with their head towards the surgeon for insertion of the carotid and jugular catheter and opposite to the surgeon for the portal catheter. The nasal mask for anesthesia was moved during surgeries according to the rat's position.

Rats were injected with 160 µl of bupivacaine 0.25% (Hospira, Lake Forest, IL – USA) on the neck where the catheters exited the skin to minimize foreign body sensation. Additionally the animals were injected subcutaneously with 300 µl buprenorphine (Burpenex®, 0.3 mg/ml, Hospira) and intraperitoneally with 210 µl

enrofloxacin 2.27% (Baytril®, Bayer Healthcare, Shawnee Mission, KS – USA) as a perioperative antibiotic prophylaxis.

The rat's eyes were protected from ulcera by placing petrolatum ophthalmic ointment (Puralube® Vet Ointment, Pharmaderm, Melville, NY – USA) between the eyelids with a Q-tip.

2.3.2 Placement of carotid catheter

Access to the neck was through a midline incision. After careful, blunt dissection of the sternohyoid and the sternocleidomastoid muscles the carotid artery and its common path with the vagal nerve was identified. With curved forceps the carotid artery was dissected and separated from the vagal nerve and the curved forceps were placed underneath the carotid artery. The carotid artery was tied off cranially with a non-absorbable 4-0 silk-suture (Tyco Health Care, Norwalk, CT – USA) and 2 further open sutures underneath the vessel were placed. The vessel was perforated with a 21 gauge needle (BD, Franklin Lakes, NJ – USA) and the catheter was inserted with the help of a plastic introducer (BD). The catheter was further introduced with the curved forceps until the bubble of the catheter was reached. On the opposite, external side of the catheter was a 3 ml syringe (BD) filled with 0.9% saline (Baxter) to immediately flush the line. The catheter was then clamped off to avoid retrograde filling of the catheter with blood. Subsequently the 2 sutures lying underneath the vessel around the artery were tied around the catheter to secure it. The distal suture that tied off the upper part of the artery was used to additionally secure the catheter.

Finally, a 16 gauge tunneling needle (Roboz Surgical Instrument Co., Gaithersburg, MD – USA) was used to tunnel the catheter underneath the rat's skin and to perforate it on the back between the shoulder blades, where the animal was previously shaved.

2.3.3 Placement of jugular catheter

Using the same access as for the carotid artery catheter the tissue lateral of the sternocleidomastoid muscle was dissected until the jugular vein was visible. The jugular vein was elevated by dissecting the tissue underneath with the curved forceps and then clamped distally with help of a mosquito hemostat. The vein was perforated with a curved 20 gauge needle (BD) and the lumen of the vein

was held open with sharp jeweler's-forceps while simultaneously inserting the catheter holding it with the curved forceps and making it advance up to the bubble. After confirming that the catheter was patent by drawing blood and flushing it with saline the vessel was ligated around the catheter both proximal and distal of the bubble with non-absorbable 4-0 silk (Tyco Health Care). The hemostat was then removed. The catheter was closed with a 1 cm long steel wire and tunneled subcutaneously to the back with the help of the 16 gauge needle.

Finally the skin was sutured with antibacterial 4-0 Vicryl Plus (Ethicon, Somerville, NJ – USA) by single interrupted stitches.

2.3.4 Placement of portal catheter

A 5 cm long midline abdominal incision was performed starting at the xiphoidal process. The abdominal wall as opened by lifting it with the rat-tooth forceps to avoid injury to the inner organs and cutting along the *linea alba*. A warm, wet, sterile 4x4 gauze was inserted on the right side of the abdomen to keep a good access to the abdominal cavity because no retractors were used. With sterile, wet Q-tips the bowel was mobilized and laid into warm wet sterile gauze to the left side of the animal.

After identifying the portal vein a wet piece of 2x2 gauze was placed under the liver to gently keep it lifted. After careful preparation and cleaning of connective tissue around the portal vein a small hole was made with a 25 gauge needle (BD). Subsequently the tip of the catheter was inserted by holding it with forceps and pushing it into the vein up to the bubble. The catheter was secured using 7-0 non-absorbable suture (Ethicon) to the omentum. With a mosquito hemostat the abdominal wall on the right side of the animal was perforated and the distal part of the catheter was pulled through it until the second bubble and the mesh were in place on the abdominal wall. The catheter was then tunneled to the back of the animal with help of the 16 gauge needle and closed with a 1 cm long steel wire. The intestine was carefully replaced and the muscular abdominal wall was sutured with single interrupted stitches with antibacterial 4-0 Vicryl Plus. The skin incision was closed by also suturing with 4-0 Vicryl and single interrupted stitches and by additionally stapling with Michael-clips (Roboz Surgical Instrument Co.).

All catheters were pulled through a larger, thicker, 5 mm long piece of silicone tubing (Braintree Scientific Inc.) holding them together and were flagged and color-coded with a small piece of tape. The tape would simultaneously prevent the lines from slipping back under the skin. The lines were then all flushed with heparinized saline (100 U/ml heparin in 0.9% saline (both Baxter).

2.4 Hyperglycemic clamps with site-specific GLP-1 infusion

2.4.1 General Set-Up

Before the experiment the clamp room had to be prepared. On a regular table the infusion pumps (11 Plus, Harvard Apparatus, Holliston, MA – USA) for glucose and GLP-1, 1 ml Syringes for blood sampling, 4x4 non-sterile gauze, glucometers and test strips, a mini centrifuge, labeled Eppendorf tubes, and an ice bucket were placed. A second table with lower height (2 x the height of the shoebox container) was placed next to the experimental table and the rat was placed on top of it in its container. The general clamp setup is depicted in figure 5.

Catheter adapters were prepared with PE-60 tubing (Instech Solomon) with an inner diameter of 0.0762 cm and an outer diameter of 0.1220 cm. To check the patency of all catheters a 20 cm piece of PE tubing was attached to each catheter on the back of the animal. To connect the tubing with the catheters a metal connector was used for the jugular and portal vein (Braintree Scientific Inc.) and silicone tubing for the carotid artery catheter (Braintree Scientific Inc., inner diameter 0.0762 cm and outer diameter 0.1650 cm). On the other side of the tubing a syringe with a 21 gauge needle was placed and heparinized saline (0.9% with 100 U/ml heparin) was used to flush the catheter. Animals were used for the clamp experiment if all 3 catheters would allow both injection and drawing blood from them.

If all catheters were patent they were connected to the prepared infusion catheters made of PE-60. The jugular and the portal catheter were connected to a 2-channel swivel (Instech Solomon) placed on the top of the cage. This would allow the rat to move freely within the cage during the clamp experiment (figure 6). The other side of the swivel was connected to the respective syringe pumps with the same tubing. The carotid catheter was connected separately and had to be untwisted manually when the rat moved.

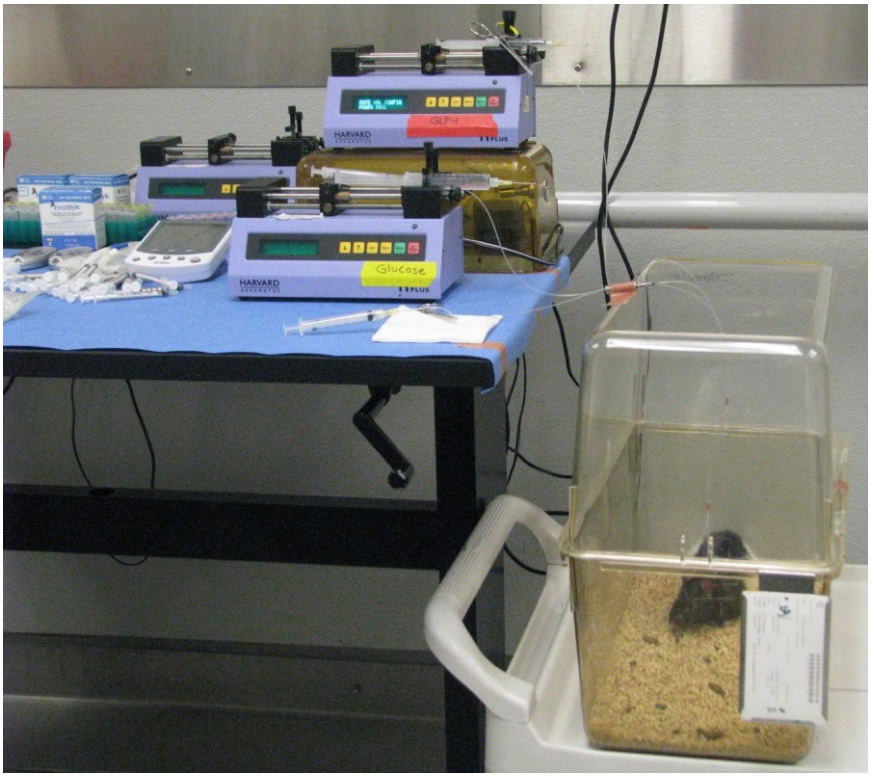

Figure 5: General clamp setup. Freely moving rat in a shoebox container. Infusion pumps are set up on the experimental table and plastic tubing is connected with the indwelling catheters externalized on the rat's back. 1 ml syringes and glucometers are prepared for drawing blood and measuring blood glucose.

2.4.2 Glucose infusion, peptide preparation and blood samples

For the variable glucose infusion of the hyperglycemic clamp a commercial Dextrose-50% solution (Baxter) was diluted 1:1 with saline 0.9% (Baxter) to receive D-25% in a 10 ml syringe.

In all experiments an additional constant amount of glucose (D-20%, Baxter) was infused into the portal vein at a rate of 4 mg/kg/min. This was done to rule out that the portal GLP-1r acts only in a glucose-dependent manner. Physiologically GLP-1 and glucose as well as other nutrients appear almost simultaneously in the portal vein after a meal.

After putting the rat back into the cage allowing it another 10 minutes to adapt to the environment, a baseline blood sample was drawn.

The syringe for the peptide infusion was prepared from a stock of frozen GLP-1 aliquots with 2.5 µg/ml (Bachem, Torrence, CA – USA) stored at -20°C. The peptide was gently thawed on ice before the experiment. To avoid that the peptide sticks to the large plastic surface (syringe, tubing, catheters) 200 µl of blood from the rat was taken together with the first baseline sample and mixed with the peptide in the final 5 ml infusion syringe (protein coating).

Now the infusion catheters were primed up to where the catheters entered the rat's skin so that the glucose and peptide infusions would reach the circulation immediately after the syringe pumps were started.

Blood samples were taken from the carotid artery catheter that was connected to PE-60 tubing with a needle on the other end. For every blood sample first the saline for flushing the tubing was removed with a 1 ml syringe until the catheter was completely filled with blood. To avoid diluted samples 0.5 ml blood was drawn into a separate 1 ml syringe but not discarded. Then the actual samples were drawn into another 1 ml syringe. For measuring glucose only a few drops of blood were drawn; for measuring insulin 0.3 ml of whole blood was taken.

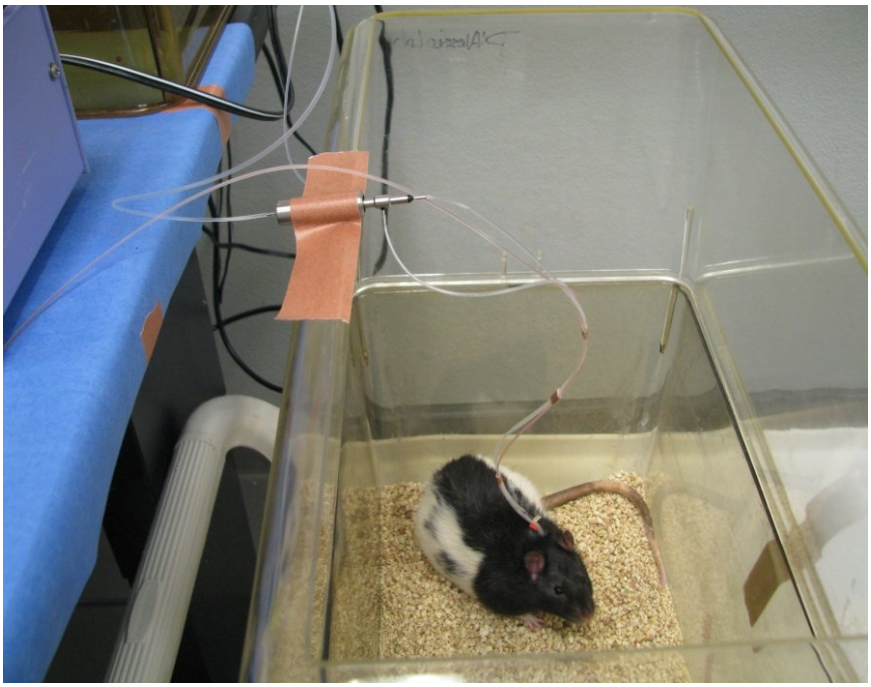

Figure 6: Conscious and freely moving rat during clamp experiment. The catheters are connected through a swivel that would allow untwisting the infusion lines multiple infusion syringes are inserted into the pumps without interruption.

Afterwards 0.5 ml of blood previously removed was reconstituted in saline and infused into the carotid artery to avoid hypovolemia and acute anemia and the catheter was flushed with 0.7 ml of heparinized saline (20 U/ml).

Insulin samples were put into sterilized, heparinized (1000 U/ml) Eppendorf tubes. Samples were immediately centrifuged for 2 minutes at 6000 rpm (2000 g) in a mini centrifuge (Research Products International Corporation, Mount Prospect, IL, USA) and approximately 150 µl of plasma was pipetted into a pre-labeled empty Eppendorf tube 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.

After all preparations were finished another baseline sample was drawn immediately before the glucose infusions were started (time point 0). The glucose infusion for the hyperglycemic clamp was started with an initial dose of 90 mg/kg/min for the first 2 minutes, followed by 25 mg/kg/min for another 3 minutes and 20 mg/kg/min thereafter given through the jugular catheter. This dose was the result of previous dose-finding studies performed at our laboratory to induce a square-wave of hyperglycemia (data not shown).

Blood glucose was measured in 5-minute intervals from 0-120 minutes and the glucose infusion rate (GIR) adjusted accordingly by an ad-hoc algorithm to maintain constant hyperglycemia. Glucose was measured using a standard bedside glucometer (Freestyle Flash, Abbott Diabetes Care, Alameda, CA – USA) in duplicate (with 2 glucometers) for each time point and the mean of both measurements was calculated.

Blood draws for insulin samples were taken at 0, 10, 30, 40, 50, 55, 60 minutes during the first hour of the clamp before the GLP-1 infusion was started.

2.4.3 GLP-1 infusion

After 60 minutes of constant hyperglycemia the graded GLP-1 infusion was started to go either into the portal or jugular vein. Hyperglycemia was maintained for the remainder of the experiment (until 120 min) with the variable glucose infusion. From 60-80 minutes GLP-1 was infused at a rate of 1.5 µg/kg/h. From 80-100 minutes the rate was increased to 2.5 µg/kg/h, followed by 5 µg/kg/h from

100-120 minutes. The doses were chosen based on previous experiments conducted in our laboratory (data not shown).

During the infusion of GLP-1, samples for plasma insulin measurements were taken at 70, 75, 80, 90, 95, 100, 110, 115, and 120 minutes (3 samples for each GLP-1 dose).

At the end of the hyperglycemic clamp all catheters were clamped with hemostats between the rat and the swivel and cut off distally of the hemostat. The rats were then taken out of the cage and the tubing was removed where the connectors were. After flushing the catheters with heparinized saline (100 U/ml) they were closed with the steel wires. Rats were then fed.

Plasma samples for insulin measurements were immediately taken to the laboratory, put in boxes and frozen at -20 C until they were assayed within 1-4 weeks.

2.5 Hyperglycemic clamps with DPP-IV inhibition

To test the hypothesis whether GLP-1 infused into the portal vein could be protected from rapid degradation by pharmacological DPP-IV inhibition, we performed the same experiments after administration of vildagliptin.

For this experiment rats were injected with 10 mg vildagliptin (suspended in 1 ml of saline) intraperitoneally. Vildagliptin was kindly provided by Dr. Bryan Burkey of Novartis (Cambridge, MA – USA). The injections were performed with a 23 gauge needle (BD) on conscious animals in their housing room 30 minutes before the hyperglycemic clamp. Afterwards the animals were moved to the room where the experiments took place. The experimental setup was then identical to that of hyperglycemic clamps without DPP-IV inhibition as described above.

2.6 Arterial plasma concentrations of active GLP-1(7-36) after site specific infusion into the portal or jugular vein

Because of the limited amount of blood that could be taken during the hyperglycemic clamps without severe hemodilution no plasma samples for measuring GLP-1 concentrations were taken during these experiments. Instead

GLP-1 infusion into either the portal or jugular vein with sampling from the carotid artery was performed in a separate cohort of rats.

In general the setup was similar to the hyperglycemic clamps but there was no infusion of glucose. An infusion of GLP-1 was prepared as described in detail above. The GLP-1 syringe was put into the syringe pump and attached to one arm of the swivel. The GLP-1 infusion was given either into the portal or jugular vein. A baseline sample of 1 ml was taken from the carotid artery catheter immediately before the GLP-1 infusion was started (time point 0). From 0-20 min GLP-1 was infused at a rate of 2.5 µg/kg/h and from 20-40 min at a rate of 5 µg/kg/h. 1 ml samples were taken at the end of each infusion step (time points 20 and 40 minutes).

All animals received an intraperitoneal injection of either saline or the DPP-IV inhibitor vildagliptin 30 minutes prior to start of the GPL-1 infusion (1 ml).

Whole blood samples for GLP-1 measurements were 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 brought immediately to the laboratory. There they were spun at 10,000 rpm at 4°C for 10 minutes in a tabletop centrifuge (Fisher Thermo Scientific, Waltham, MA – USA). Plasma samples were pipetted into pre-labeled Eppendorf tubes, put in a box and stored at -80°C until they were assayed.

2.7 Insulin RIA

Insulin assays were performed using a commercially available RIA-kit from Millipore (Millipore Corporation, Billerica, MA – USA) following the manufacturer's instructions except of the use of a specific rabbit insulin antibody as previously described [69].

2.8 GLP-1 ELISA

GLP-1(7-36) plasma concentrations were measured using a commercially available ELISA for active GLP-1 (Millipore Corporation) according to the manufacturer's instructions.

2.9 Statistical Analysis

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 (tables 2-4). A p-value of <0.05 was considered statistically significant. The results are expressed as mean ± standard error of the mean (SEM) for the different cohorts. Analysis and graph plotting was done using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA – USA).

3 Results

3.1 Surgery

A total of approximately 240 rats was purchased for surgeries and experiments. Almost all of these animals underwent the surgical placement of catheters. Of these approximately 50 animals underwent clamp procedures and about 20 animals were used for measurement of arterial GLP-1 plasma levels. The majority of the latter animals were already used for a previous clamp procedure, after which they were allowed to recover for at least one week.

There are multiple reasons for the relatively large number of operated animals compared to actual clamp experiments. It should be noted that both, the placement of three catheters including the portal vein in one surgical procedure, as well as the hyperglycemic clamps, had to be established newly at the laboratory. Naturally there was a learning curve, particularly regarding the surgery with decreasing rates of casualties and drop outs over time. Few rats died from apnea during anesthesia. In some cases the placement of the catheters led to dissection of the vessels and consequently fatal hemorrhage. Particularly the placement of the portal vein catheter was challenging and rats were at risk of bleeding out quickly. The portal vein was not clamped during the procedure to avoid manipulation of the vessel and the surrounding vagal afferents.

As described above, clamps were only performed in healthy rats that fully regained their pre-surgical body weight. Some rats presented with neurological symptoms during the recovery period indicating a stroke and therefore had to be euthanized. Some animals scratched or bit holes in their catheters during the recovery period despite the careful externalization of the catheters between the scapulae and individual housing.

Rats could not be used for the clamp procedures unless all three catheters were fully patent (easy flushing and aspiration of blood). Despite the heparin block of the catheters, clotting was a recurring problem, particularly with the portal vein catheter that had the smallest inner diameter.

A few hyperglycemic clamps had to be terminated because a rat had irreversibly twisted the infusion catheters preventing a continuous and stable infusion of glucose or GLP-1 and thus affecting the hyperglycemic clamp and the reliability of the insulin response to GLP-1. Even fewer animals bit holes into the infusion catheters during the clamp procedure that were not fixable and led to abortion of the experiment or fatal bleeding. Some rats showed focal neurological symptoms during the experiments indicating a stroke which led to immediate termination of the clamp.

3.2 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 very similar in both cohorts with 315.1±5.9 g and 314.8±5.2 g (pv vs. jv, p=0.97). Also the levels of fasting glucose (97.6±4.9 mg/dl vs. 95.9±2.9 mg/dl, p=0.76), average glucose during the hyperglycemic clamp (212.1±3.5 mg/dl vs. 206.3±2.5 mg/dl, p=0.19) and glucose over basal (114.5±6.0 mg/dl vs. 110.4±2.7 mg/dl, p=0.54) did not differ significantly between the cohorts. The coefficient of variation (CV) for the duration of the hyperglycemic clamps were equally low in both groups with 8.7±0.6 % and 8.8±0.5 %, respectively (p=0.96).

After administration of ip vildagliptin 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. Body weight at the day of the clamp was not significantly different with 335.2±5.8 g and 319.7±8.4 g (pv vs. jv, p=0.17). Similar to the experiments without the DPP-IV inhibitor, fasting glucose (99.9±4.8 mg/dl vs. 98.1±2.7 mg/dl, p=0.72), average glucose during the hyperglycemic clamp (201.2±1.4 mg/dl vs. 202.7±1.1 mg/dl, p=0.38) and glucose over basal (101.2±1.4 mg/dl vs. 104.6±2.4 mg/dl, p=0.49) did not differ significantly between the cohorts. The coefficient of variation (CV) was 8.6±0.7 % in the pv-group and 9.4±0.7 % in the jv-group (p=0.38). The results are summarized in table 1.

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 DPP-IV 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 ± SEM 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 statistical significant. None of the parameters differed significantly between portal and jugular vein GLP-1 infusion.

3.3 Effect of portal vs. jugular vein GLP-1 infusion

Glycemia, glucose infusion rates and insulin concentrations during the constant hyperglycemic clamp and concomitant infusion of increasing doses of GLP-1 into the portal or jugular vein are summarized in table 2.

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) towards 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 7a).

Table 2: glucose, glucose infusion rate (GIR) and insulin during clamp

Glucose (mg/dl)			
	Portal Vein GLP-1 (N=10)	**Jugular Vein GLP-1 (N=10)**	**p-Value**
Hyperglycemia	216.2±4.0	212.4±3.2	ns
+ GLP-1 1.5 µg/kg/h	219.3±4.0	216.1±2.9	ns
+ GLP-1 2.5 µg/kg/h	218.9±6.5	201.0±3.4	ns
+ GLP-1 5.0 µg/kg/h	201.4±7.4	198.8±3.3	ns

RM 2-way ANOVA: Dose ($p<0.0001$) of GLP-1 but not infusion site ($p=0.1568$) had a significant impact on plasma glucose levels.

Glucose infusion rate (mg/kg/h)			
	Portal Vein GLP-1 (N=10)	**Jugular Vein GLP-1 (N=10)**	**p-Value**
Hyperglycemia	27.7±3.4	31.2±2.6	ns
+ GLP-1 1.5 µg/kg/h	28.0±3.3	30.3±2.5	ns
+ GLP-1 2.5 µg/kg/h	28.5±3.3	35.1±2.6	ns
+ GLP-1 5.0 µg/kg/h	34.5±3.8	54.8±3.6	ns

RM 2-way ANOVA: Dose ($p<0.0001$) of GLP-1 but not infusion site ($p=0.0582$) had a significant impact on the glucose infusion rate.

Insulin (pmol/l)			
	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

RM 2-way ANOVA: Dose ($p<0.0001$) of GLP-1 and infusion site ($p=0.0207$) had a significant impact on plasma insulin levels. Bonferroni post-hoc testing revealed 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 $p<0.05$

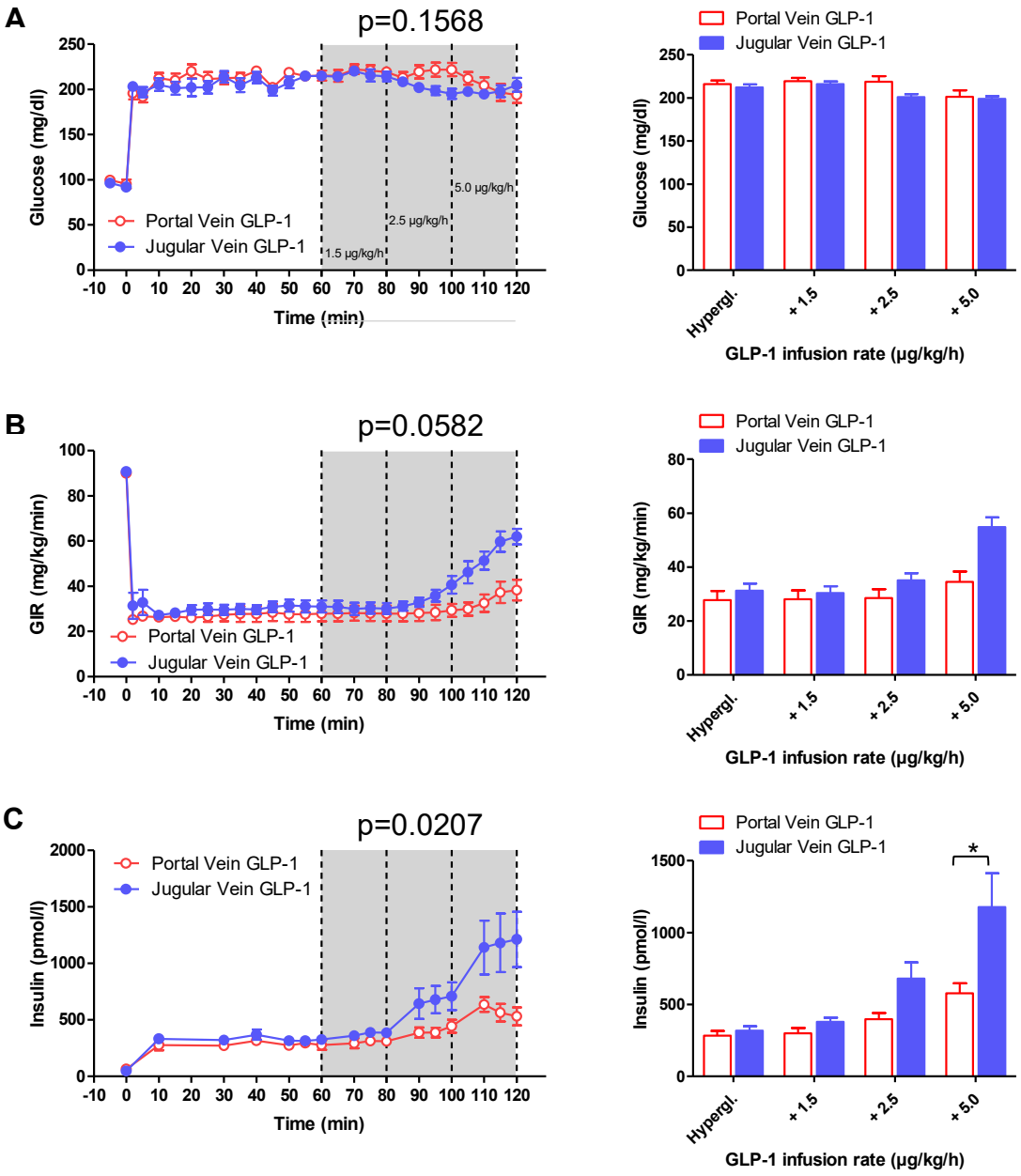

Figure 7: Glucose (A), glucose infusion rate (B), and insulin (C) during hyperglycemic clamp. Line graphs (left) depict infusion of portal (red circles) or jugular (blue dots) GLP-1 infusion starting at time point 60 min, with increasing doses (61-80 min 1.5 µg/kg/h; 81-100 min 2.5 µg/kg/h; 101-120 min 5.0 µg/kg/h). Bar graphs (right) depict average glucose (top), glucose infusion rate (middle), and insulin (bottom) levels during infusion of portal (white with red border) or jugular (blue) infusion of GLP-1. Hypergl.(ycemia) reflects the average values from 50 to 60 min before the GLP-1 infusion was started. GLP-1 infusion in to the jugular vein at the highest dose had a significantly greater effect on insulin plasma concentrations than portal infusion (*$p<0.05$).

Also 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 ($p<0.0001$). Portal vein GLP-1 infusion tended to result in lower GIR than jugular vein GLP-1 infusion ($p=0.0582$) but this difference did not reach statistical significance (figure 7b).

With increasing doses of GLP-1, plasma insulin concentrations raised 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 lead to significantly lower insulin levels than GLP-1 infusion into the jugular vein ($p=0.0207$). Post-hoc 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 7c).

3.4 Effect of DPP-IV inhibition on portal and jugular vein GLP-1 infusion

Glycemia, glucose infusion rates and insulin concentrations during hyperglycemic clamps with prior inhibition of DPP-IV activity by vildagliptin and portal vs. jugular vein infusion of GLP-1 are summarized in table 3.

Hyperglycemia during the clamps was significantly altered by GLP-1 dose ($p<0.0001$) but not by infusion site ($p=0.9257$) with a similar reduction of glycemia under 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 higher glycemia towards the end of the clamp with higher infusion rates of glucose (pv 210.9±3.8 mg/dl; jv 214.8±2.4 mg/dl).

GIR increased significantly with higher doses of GLP-1 ($p<0.0001$) with no difference between pv and jv infusion of the peptide ($p=0.2680$). The GIR increased constantly 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.

Table 3: glucose, glucose infusion rate (GIR) and insulin during clamp after DPP-IV inhibition

Glucose (mg/dl)			
	Portal Vein GLP-1 (N=10)	**Jugular Vein GLP-1 (N=10)**	**p-Value**
Hyperglycemia	213.3±2.6	208.5±3.4	ns
+ GLP-1 1.5 µg/kg/h	205.6±3.9	203.6±3.2	ns
+ GLP-1 2.5 µg/kg/h	192.0±4.3	195.8±3.3	ns
+ GLP-1 5.0 µg/kg/h	210.9±3.8	214.8±2.4	ns

RM 2-way ANOVA: Dose ($p<0.0001$) of GLP-1 but not infusion site ($p=0.9257$) had a significant impact on plasma glucose levels.

Glucose infusion rate (mg/kg/h)			
	Portal Vein GLP-1 (N=10)	**Jugular Vein GLP-1 (N=10)**	**p-Value**
Hyperglycemia	36.8±2.1	40.0±1.9	ns
+ GLP-1 1.5 µg/kg/h	36.9±2.9	41.7±2.3	ns
+ GLP-1 2.5 µg/kg/h	47.5±3.2	53.1±3.6	ns
+ GLP-1 5.0 µg/kg/h	51.3±3.1	54.2±4.2	ns

RM 2-way ANOVA: Dose ($p<0.0001$) of GLP-1 but not infusion site ($p=0.2680$) had a significant impact on the glucose infusion rate.

Insulin (pmol/l)			
	Portal Vein GLP-1 (N=10)	**Jugular Vein GLP-1 (N=10)**	**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

RM 2-way ANOVA: Dose ($p<0.0001$) of GLP-1 but not infusion site ($p=0.2799$) had a significant impact on the insulin concentration.

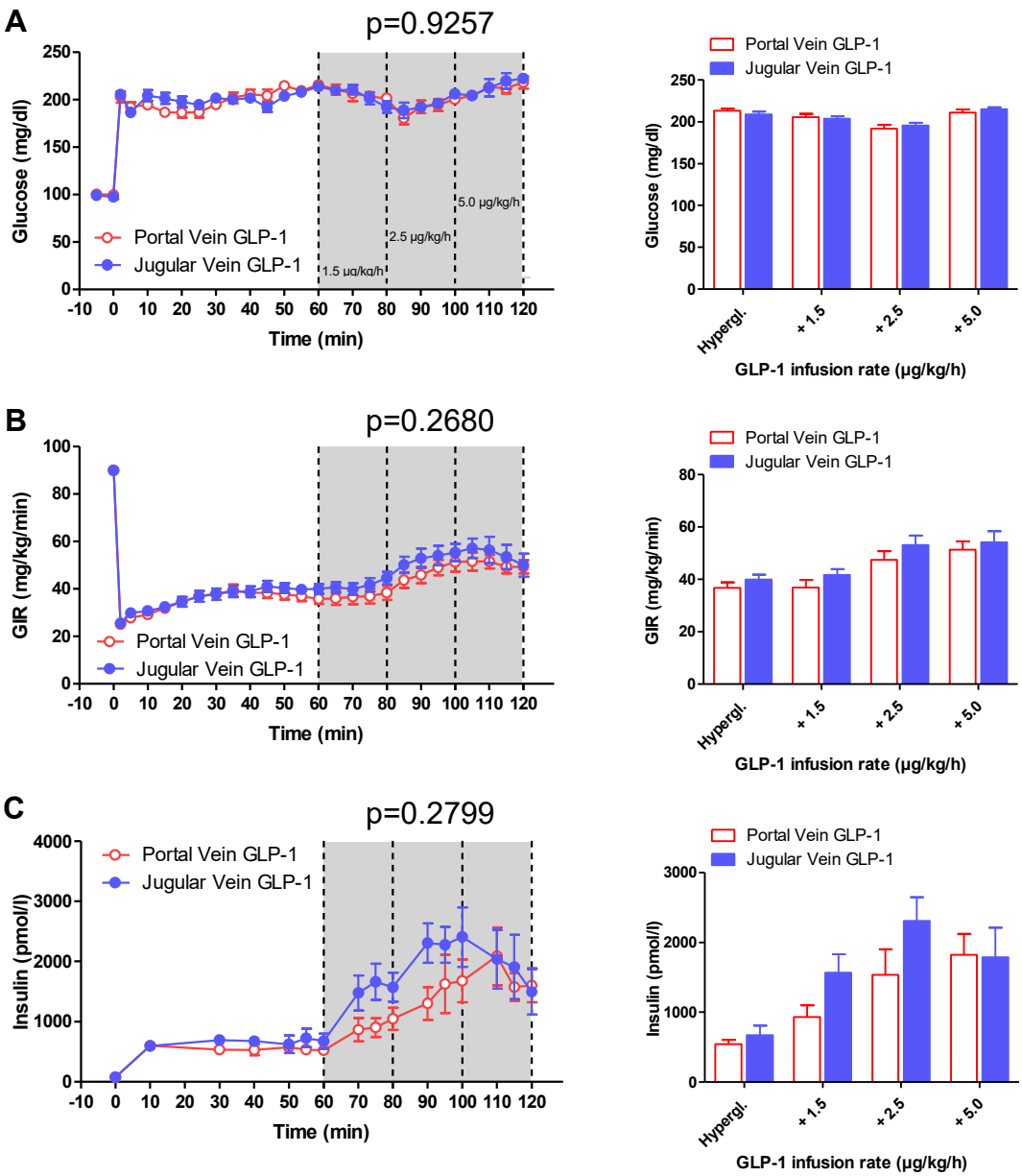

Figure 8: Glucose (A), glucose infusion rate (B), and insulin (C) during hyperglycemic clamp with inhibition of DPP-IV. Line graphs (left) depict infusion of portal (red circles) or jugular (blue dots) GLP-1 infusion starting at time point 60 min, with increasing doses (61-80 min 1.5 µg/kg/h; 81-100 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 insulin (bottom) levels during infusion of portal (white with red border) or jugular (blue) infusion of GLP-1. Hypergl.(ycemia) 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.

After DPP-IV inhibition insulin plasma concentrations increased significantly with higher doses of GLP-1 ($p<0.0001$) but unlike the previous experiments without the DPP-IV inhibitor there was no significant difference between portal and jugular vein GLP-1 infusion ($p=0.2799$). While 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 when infused into the portal vein, insulin plasma 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) in order to fall down to 1788±425 pM with the highest dose of GLP-1 (5 µg/kg/h) when it was infused into the jugular vein. When the 2-way ANOVA for RM was performed until the end of the 2.5 µg/kg/h infusion rate of GLP-1 the jv infusion tended to result in higher insulin than the pv infusion ($p=0.1020$).

Numerically the insulin plasma concentrations were higher in the experiments with the DPP-IV inhibitor as can be expected. Because the experiments with and without vildagliptin were done in separate cohorts and there was no placebo given during the first set of experiments, no direct statistical comparison between those two conditions was performed.

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

In a separate cohort plasma concentrations of active GLP-1(7-36) was measured in the arterial blood stream under all four conditions (pv vs. jv infusion ± DPP-IV inhibition). Without vildagliptin basal GLP-1 was 2.6±0.4 pM (pv) and 3.3±0.9 pM (jv), respectively. Both dose ($p<0.0001$) and infusion site ($p=0.0001$) had significant impact on the measurement of the GLP-1 plasma concentration. Post-hoc analysis revealed that jv infusion resulted in significantly higher GLP-1 plasma levels than pv infusion both at a rate of 2.5 µg/kg/h (jv 43.6±5.0 pM vs. pv 14.3±2.4 pM, $p<0.001$) and at a rate of 5 µg/kg/h (jv 80.9±3.4 pM vs. 36.6±2.8 pM, $p<0.001$).

With previous administration of intraperitoneally 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. 2-way ANOVA revealed again a significant effect of both dose

(p<0.0001) and infusion site (p=0.0081 for pv vs. jv). Bonferroni post-hoc testing showed no significant difference of GLP-1 plasma concentrations at an infusion rate of 2.5 µg/kg/h (pv 47.1±12.3 pM vs. jv 116.7±27.9 pM, ns) but a highly significant difference at an infusion rate of 5 µg/kg/h (pv 184.8±35.7 pM vs. jv 443.2±66.4 pM, p<0.01) (table 4).

Table 4: GLP-1 (7-36) plasma concentration (pmol/l) during portal and jugular vein infusion

without DPP-IV inhibition

	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

RM 2-way ANOVA: Dose (p<0.0001) of GLP-1 and infusion site (p<0.0001) had a significant impact on the GLP-1 plasma concentration. Bonferroni post test revealed 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 DPP-IV inhibition

	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

RM 2-way ANOVA: Dose (p<0.0001) of GLP-1 and infusion site (p<0.0081) had a significant impact on the GLP-1 plasma concentration. Bonferroni post test revealed 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

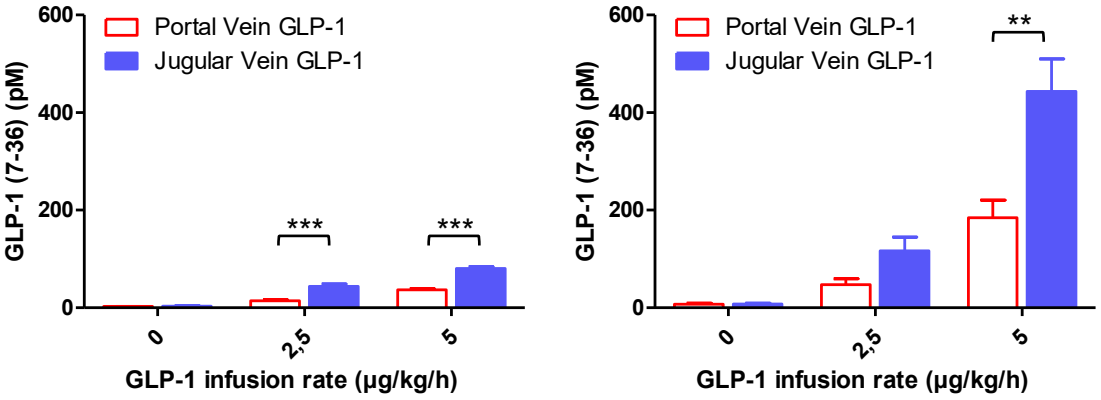

Figure 9: Arterial plasma concentrations active GLP-1 during infusion of GLP-1(7-36)amide into the portal (red/white) or jugular vein (blue). The graph on the left depicts GLP-1 levels between portal or jugular GLP-1 infusion with previous intraperitoneal injection of saline. The graph to the right depicts GLP-1 levels between portal or jugular GLP-1 infusion with previous intraperitoneal injection of vildagliptin. RM 2-way ANOVA shows with and without vildagliptin highly significant effects of both dose and infusion site ($p<0.0001$). Post-hoc tests showed significantly higher GLP-1 levels in the jugular vs. portal vein. *** indicates a $p<0.001$; ** indicates a $p<0.01$

4 Discussion

In recent years evidence against an endocrine mechanism of action of GLP-1 grew [56]. Thus, it was hypothesized that GLP-1 mediated insulin release from pancreatic β-cells is a consequence of a combined neuro-humoral signaling circuit in which GLP-1 released into the portal circulation acts via GLP-1r in nerve endings of vagal afferents in the hepatoportal vein. To test this hypothesis an equimolar amount of synthetic GLP-1 was infused either into the portal or jugular vein during constant hyperglycemia and a larger insulin response to the intraportal GLP-1 infusion was expected. Contrary to the hypothesis, GLP-1 infusion into the jugular vein resulted in a more potent insulin secretion than an equimolar infusion of GLP-1 into the portal vein. This was paralleled by significantly higher arterial plasma concentrations of active GLP-1 after infusion of the peptide into the jugular vs. the portal vein. Because degradation of GLP-1 across the hepatoportal bed after infusion of the active peptide into the portal vein seemed to contribute significantly to this result the experiments were repeated with the potent inhibitor of DPP-IV activity vildagliptin. Also with DPP-IV inhibition the arterial plasma concentrations of GLP-1 were significantly higher with jugular vs. portal vein infusion of the peptide and resulted in numerically higher insulin levels.

There are several possible explanations as to why our hypothesis was not confirmed. First of all, the hyperglycemic clamp and portal infusion of GLP-1 is not a physiological setting. As mentioned above, most studies used a GLP-1r antagonist or vagal denervation in order to show that this results in disturbance of a physiological response to intestinal feeding [67, 68]. Although we co-infused glucose into the portal vein to simulate the physiological appearance of glucose from the gut and GLP-1 in the portal vein, the larger portion of glucose to generate constant systemic hyperglycemia was infused into the jugular vein. Thus there was no negative arterial to portal glucose gradient, which is seen normally after a meal. Previous studies in dogs suggest that this gradient is necessary for glucose and GLP-1 to mediate increased glucose utilization [66]. However, it is unclear to which extend this holds true for rodents and humans and

if this also applies to changes in insulin secretion was not part of the study by Johnson et al.

Another explanation could be that splanchnic afferents that transport the vagal-pancreatic neuroendocrine loop originate proximal from where we placed our catheter. In fact, there is already significant degradation of GLP-1 by the time it reaches the portal vein (figure 3) and afferent GLP-1r carrying vagal fibers have been described also in the intestine surrounding GLP-1 secreting L-cells [56]. This hypothesis could explain why studies inhibiting the neural signal in a physiological oral meal test results in impaired glucose tolerance [70]. Moreover, a study by Hayes et al. showed that specific denervation of the common hepatic branch of the vagus did not alter glucose tolerance and feeding behavior in response to the GLP-1r antagonist exendin-9 while complete subdiaphragmatic vagotomy did [71]. Interestingly, most studies finding positive effects on GLP-1r action used a paradigm antagonizing GLP-1 effects instead of agonism at the receptor. They concluded that vagal afferents mediate the glucose lowering effects of GLP-1 in a paracrine fashion through nerve endings surrounding the L-cells rather than receptors in the portal vein. On the other hand such a hypothesis would be contrary to many positive experiments with infusion of GLP-1 [61, 62] or the antagonist [63, 68] into the portal vein.

A study published by Nishizawa et al. also tested the hypothesis that portal GLP-1 would mediate insulin secretion from the pancreas in a neuroendocrine fashion [72]. However, they used a different study design. First, they measured portal concentrations of GLP-1 and glucose following a meal. Then they showed that a brief low dose infusion of GLP-1 together with a high dose of glucose would result in a higher insulin release than infusion of glucose alone and this effect was abolished by vagotomy. Infusion of a slightly higher GLP-1 dose resulted in an insulinotropic effect that was not completely reversed by vagotomy, suggesting that spill-over of portal GLP-1 was responsible for this effect. Finally infusion of the higher dose into the jugular vein resulted in an insulinotropic effect that was not changed by vagotomy. Although the authors did not directly compare the insulin levels in response to portal and jugular GLP-1 infusion, the levels were about 2-fold higher with a jugular infusion of the same dose. An infusion of the lower dose of GLP-1 was not tested in the jugular vein. The authors concluded

that in a physiological setting (portal glucose infusion, low physiological levels of GLP-1) GLP-1 mediates insulin release mainly through a neuroendocrine signal originating in the portal vein, whereas higher doses or administration into the jugular vein directly act on pancreatic β-cells [72]. There are multiple important differences that may explain the conflicting results with our study. First of all, in the experiments by Nishizawa et al. glucose was completely infused into the portal vein, whereas we used only a low dose glucose infusion in the portal vein and the hyperglycemic clamp was maintained through jugular vein glucose infusion. Second, we used a hyperglycemic clamp of a long duration with a graded GLP-1 infusion rather than a short term infusion with a single dose. Also in the study by Nishizawa et al., the specific effect of portal GLP-1 became only apparent after vagotomy. We refrained from vagotomy because compared to jugular vein infusion there was clearly no prominent insulinotropic effect in the portal vein. Finally, there was no dose-response trial and direct comparison of GLP-1 infusion into either the portal or jugular vein in this previously published study. While there was no direct comparison of the insulinotropic effect between the jugular and portal vein infusion, the graphic result confirm our finding that equimolar doses of GLP-1 infusion into the jugular vein elicits a higher insulin response than portal vein infusion, a consequence of more intensive degradation of GLP-1 in the hepatoportal bed [72]. Furthermore, using a DPP-IV inhibitor to quantify the contribution of GLP-1 inactivation in the hepatoportal bed during pharmacological intervention is unique to our study.

Interestingly the doses of GLP-1 used in the experiments by Nishizawa and ours differed greatly. The dose of 1.5 µg/kg/h corresponds to 7.6 pmol/kg/min and 5 µg/kg/h to about 25 pmol/kg/min. Nishizawa et al. used 1 pmol/kg/min as a low and 3 pmol/kg/min as a high dose [72]. Despite the fact that our lowest dose was >2-fold higher than the high dose of Nishizawa et al. there was no significant effect on insulin secretion either in the portal or jugular vein. Previous studies infusing GLP-1 into the portal vein have employed 5 pmol/kg/min [63] corresponding to about 1 µg/kg/h. In our pilot studies using doses of 1 µg/kg/h and lower there was no insulinotropic effect, so we decided to use a graded infusion ranging from a subthreshold dose to a pharmacologic range (data not shown). Although our dose of 1.5 µg/kg/h is about 8-fold higher than the

1 pmol/kg/min in the study by Nishizawa et al. showing a portal vein specific effect on insulin secretion, this dose was clearly in a subthreshold range in our experiments. Extrapolating the plasma levels of active GLP-1 with infusion of 2.5 µg/kg/h into the portal vein to the lower dose, also suggests that we targeted a physiological range. A possible explanation would be that Nishizawa et al. used a more purified peptide than we did, although both studies used the same peptide producer (Bachem, USA vs. Bachem Switzerland). Nevertheless, the peptide concentrations we used are similar to what has previously been published in dose response studies of GLP-1 [73, 74].

Regarding neurally mediated effects of endogenous GLP-1 or pharmacologic GLP-1r activation even the most recent studies employing state-of-the-art genetic engineering show conflicting results. Using chemogenetics and optogenetics Williams et al. showed that the vast majority of GLP-1r carrying afferent vagal neurons have their nerve endings within the muscle layer, do not participate in nutrient sensing, do not respond to pharmacologic administration of a GLP-1r agonist, and seem to primarily function as gastrointestinal mechanoreceptors [75]. Similarly, Sisley and colleagues showed that neuron specific knockout (both centrally and peripherally) of the GLP-1r did not alter food intake, body weight or glucose tolerance in a physiological setting. The weight reducing effects of the GLP-1r agonist liraglutide however required the brain GLP-1r but not a functional GLP-1r on peripheral neurons [76]. This was confirmed by another study that mapped in great detail the relevant brain regions activated by liraglutide and did also not involve vagal GLP-1r signaling [77]. On the other hand, Krieger et al. showed that lentiviral knockdown of GLP-1r in the nodose ganglia of rats increased postmeal hyperglycemia and reduced insulin but did not alter responses to an oral glucose tolerance test. In this study changes in gastric emptying may have had a significant impact on the findings. Similarly to the previous studies there was no effect on long-term food intake and body weight [78]. However, very conflicting results were published by Iwasaki et al. who showed that the non-caloric sweetener D-allulose stimulated endogenous GLP-1 release, which reduced food intake, body weight, and glycemia through GLP-1r signaling in vagal afferents in genetic mouse models of complete or isolated vagal GLP-1r knockdown [79].

Taken together it remains unclear whether our results are accounted for because GLP-1 does not exert insulinotropic (incretin) effects through a neuroendocrine signal in the portal vein, or if an unphysiological setup prevented us from showing a positive effect. Interestingly, similarly conflicting results have been shown in regards to GLP-1's effects on food intake and satiety. While some studies have found GLP-1 infusion into the portal vein to reduce food intake more potently than systemic infusion [80] others have not [81, 82].

While it was not possible to show that a low dose infusion of GLP-1 into the portal vein exerts an insulinotropic effect in this study the results contribute further to the body of evidence that challenges an endocrine mode of action of GLP-1. In addition these results do not support that enhancement of circulating GLP-1(7-36)amide is responsible for the insulinotropic effects of DPP-IV inhibition. Infusion of GLP-1 at a dose of 1.5 µg/kg/h into the portal vein was clearly not insulinotropic and at 2.5 µg/kg/h resulted in a non-significant 40% increase in insulin plasma concentrations (one-way ANOVA for portal GLP-1 infusion in response to dose). The GLP-1 plasma concentration measured in another cohort but corresponding to the dose of 2.5 µg/kg/h in the portal vein, was 14.3±2.4 pmol/l and thus represented a 6.0±1.2-fold increase over basal (2.6±0.4 pmol/l). Although we did not measure postprandial arterial plasma concentrations of active GLP-1 in this cohort, this relative increment is more pronounced than what is regularly seen after a meal. On the other hand DPP-IV inhibition with vildagliptin increased the plasma concentrations of GLP-1 to 7.0±2.4 pmol/l in the fasting state, which corresponds to about a 3-fold increase vs. no vildagliptin. Similarly to what is seen in human studies [83] DPP-IV inhibition resulted in a 2-fold increase of plasma insulin during the hyperglycemic clamp before the GLP-1 infusion was started (543±59 pmol/l vs. 282±33 pmol/l, unpaired t-test $p<0.001$). Looking at the relative potency to stimulate insulin secretion and the corresponding GLP-1 plasma concentrations seen after infusion of synthetic GLP-1 vs. enhancing endogenous GLP-1 action it becomes apparent that circulating GLP-1 may neither be mediating the incretin effect nor the glucose lowering actions of DPP-IV inhibition.

Despite of the use of the potent pharmacological DPP-IV inhibitor vildagliptin, there remained a significant difference of active GLP-1 reaching the arterial

circulation between portal and jugular infusion of the peptide (table 4, figure 9). As mentioned above, insulin concentrations were about twice as high during constant hyperglycemia with vildagliptin and further amplified by a factor of 3-4 when GLP-1 was infused in either site. With exception to the unexpected drop in insulin levels during the highest dose of GLP-1 infusion into the jugular vein, there was a clear left shift in the dose response curve of both portal and jugular vein infusion of GLP-1 with DPP-IV inhibition. So the gap between plasma insulin levels in response to either portal or jugular vein infusion of GLP-1 remained present (with the exception to the highest dose), even though this was not statistically significant.

Together this supports the role of the hepatoportal bed as a major site of GLP-1 degradation as previously described by Hansen et al. in pigs [84, 85]. It also demonstrates that even with pharmacological DPP-IV inhibition there remains significant DPP-IV action to degrade active GLP-1. Previous studies showed a >80%-reduction of plasma DPP-IV activity with vildagliptin and good protection from degradation of intraperitoneally injected GLP-1 [86, 87]. Hence, membrane bound DPP-IV (i.e. on the endothelium) seems to remain a potent factor of incretin degradation in the hepatoportal circulation under pharmacological DPP-IV inhibition. Again, this observation of intensive GLP-1 degradation in the liver despite pharmacological DPP-IV inhibition makes it unlikely that this drug class acts primarily through enhancement of circulating GLP-1(7-36) amide released from the gut.

Very elegant studies with genetic models of GLP-1r activity have further shed light on the possible insulinotropic mechanism of GLP-1. Lamont et al. created a transgenic model, where the human GLP-1r was restored in the pancreatic ductal and β-cells on the background of a GLP-1r -/- mouse (Pdx1-hGLP1R:*Glp1r -/-* mice). Thus, there was only a functional GLP-1r present in the pancreas but not in the nervous system or other tissues. Interestingly, the pancreatic GLP-1r was sufficient to reverse the glucose intolerance seen in GLP-1r -/- mice and normalize insulin secretion. This suggests that GLP-1 is acting directly on the pancreatic β-cells and does not require additional receptor activation, like a GLP-1r in the portal vein [88]. Unlike glucose tolerance, effects of the GLP-1r agonist exendin-4 on food-intake, hindbrain activation, or gastric emptying were not

restored by isolated pancreatic expression of the GLP-1r. These results however are at odds with another transgenic model, where the GLP-1r has been specifically knocked out in the β-cells of the pancreas with intact GLP-1r signaling in other tissues [89]. Here the results were more complex with differential effects of β-cell GLP-1r knockdown during oral and parenteral glucose administration. While the β-cell GLP-1r was not necessary to allow normal oral glucose tolerance and glucose lowering of DPP-IV inhibition, the insulinotropic effect of intraperitoneal or intravenous GLP-1 was severely blunted. Another recent study in transgenic mice helps to bring together the results of Lamont et al. [88] with the well-founded skepticism towards an endocrine mode of action of endogenous GLP-1. More and more evidence points towards a significant and physiological relevant production of GLP-1 in pancreatic α-cells [55]. In this context Chambers et al. silenced proglucagon expression in mice (thus preventing GLP-1 production) but were able to restore expression specifically in the gut or pancreas [90]. While recovery of proglucagon expression in the intestine was able to almost completely restore circulating GLP-1 levels it did not have an impact on the metabolic phenotype. More importantly, using the GLP-1r antagonist exendin-9 did not impair glucose tolerance with functional GLP-1r and intestinally secreted GLP-1. However, salvage of proglucagon expression only in pancreatic α-cells resulted in a clear effect of GLP-1r blockade with exendin-9 towards decreased glucose tolerance. The authors drew the legitimate conclusion that not intestinal GLP-1 stimulates insulin secretion through β-cell GLP-1r, but activation happens in a paracrine fashion by locally produced GLP-1 from the α-cells.

Of course, there are limitations with transgenic mouse models and it is unclear if the same mechanisms hold true for rats or even humans. Despite the highly conserved amino acid sequence of GLP-1 across different species and even more of the identical GLP-1r in many mammals including rodents, dogs, pigs, non-human primates, and humans, there seem to be some species specific differences of the GLP-1r signaling [91, 92]. In addition, knockout models tend to develop mechanisms compensating for loss-of-function, as for example seen with the single incretin receptor knockout mice [44]. Furthermore knockdown of GLP-1 expression cannot be separated from disturbing the signaling of all proglucagon derived peptides including glucagon, which is a potent mediator of metabolic

effects. Nevertheless, all those studies support our findings that circulating GLP-1 plasma concentrations in a physiological range do not correlate well with its insulinotropic effect and that other mechanisms than portal GLP-1r activation may mediate insulin secretion.

Another unexpected finding was the drop of plasma insulin concentrations seen with the highest dose 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 while protecting its degradation with vildagliptin would have suggested a parallel increase in plasma insulin with a continuous left shift of the dose response curve between portal vs. jugular vein infusion. A possible explanation is that maximal stimulation of insulin release was already achieved with the second highest dose of GLP-1 corresponding to a drastically supraphysiologic plasma concentration of about 120 pmol/l. In fact some studies have shown that acute administration of high doses of the GLP-1r agonist exendin-4 causes paradoxical hyperglycemia in rats, possibly mediated by activation of the sympathetic nervous system [91, 93]. The immensely high plasma concentration of GLP-1(7-36)amide of about 450 pmol/l seen with the highest dose infusion into the jugular vein could be sufficient to induce symptoms of aversion, activate the sympathetic nervous system and possibly limit the insulin release from β-cells [94]. Because this unexpected drop in insulin towards the end of the clamp was seen consistently across the whole cohort a random effect or technical problems with the GLP-1 infusion seem unlikely.

We employed a complex and labor intensive study design to test our hypothesis. The hyperglycemic clamp is the most rigorous experiment to test insulin secretion. However, a more physiological setting with primary glucose infusion into the portal vein may have been better for the purpose of the study. Following the previous publications using GLP-1r antagonists and the hypothesis of a constitutive activated receptor, portal vein infusion of exendin-9 may have shown interesting results. On the other hand the incretin effect should be mediated by increases in GLP-1 and not decreased receptor agonism. Although our study was generally well powered, some results reached only borderline significance (GIR, insulin in clamps with vildagliptin, GLP-1 plasma levels) and a bigger sample size would have likely shown clearer results. Still, with greater power the general

conclusions most likely would not have differed from the reported results. Finally, measurements of GLP-1 plasma concentrations during the clamp experiments would have allowed generating individual dose-response curves and correlations for portal and jugular GLP-1 infusion. Unfortunately, it was not possible to draw the necessary amount of blood during the hyperglycemic clamp without causing severe anemia and significantly elevate stress levels in this experimental setup with conscious animals.

The importance and physiological relevance of portal vein GLP-1r signaling remains elusive and study results are conflicting. While some studies have found effects of disrupted portal vein GLP-1r signaling, others - including this one - have not shown an incretin effect specific for portal GLP-1. However, particularly the studies involving genetic disruption of neurally mediated GLP-1r signaling make it difficult to argue towards a relevant physiological action. On the other hand, this study is in line with the growing body of evidence against the endocrine mode of action of GLP-1 and alternative models like intra-islet GLP-1r signaling seem worthwhile to follow-up on. Most importantly, the effort should be directed towards transporting these studies of physiological effects from pre-clinical models into humans to further explore and optimize the treatment of diabetes and obesity.

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. However, we demonstrated that high, pharmacological doses of GLP-1 need to be infused into the portal vein to reach significant arterial plasma concentrations of the active peptide and consequently insulin release from pancreatic β-cells. We also confirmed that the hepatoportal bed is a major site of GLP-1's inactivation *in vivo* and furthermore retains significant activity towards GLP-1 degradation in the presence of a pharmacological DPP-IV inhibitor. Altogether, our findings give further support to the reservations about an exclusive endocrine mechanism of action of intestinally released GLP-1 and pharmacological DPP-IV inhibition.

5 Summary

Glucagon-like peptide-1 (GLP-1) is an enteral hormone secreted by L-cells in the small and large bowel after meal ingestion. GLP-1 stimulates insulin secretion in a glucose-dependent manner and accounts together with glucose-dependent insulinotropic peptide (GIP) for the incretin effect. GLP-1 mediates its effects through a specific receptor (GLP-1r) expressed in a variety of tissues including the β-cells of the pancreatic islets. The commonly accepted endocrine model of the insulinotropic action of GLP-1 suggests that the peptide hormone is secreted from the intestine after luminal contact of nutrients and reaches the β-cell through the systemic circulation. However, its short half-life due to rapid degradation by Dipeptidylpeptidase-IV (DPP-IV), and the barely detectable plasma concentrations even in the postprandial state, are conflicting with an endocrine mechanism of action and alternative models have been investigated. Several studies have been indicating an essential role of the GLP-1r in the portal vein and the involvement of a neuroendocrine signal to stimulate insulin secretion.

This study was the first to compare the effect of portal GLP-1 infusion with systemic infusion through the jugular vein during constant hyperglycemia in rats, allowing a direct comparison of insulin levels. We hypothesized that GLP-1 infusion into the portal vein would result in higher plasma insulin levels during a hyperglycemic clamp than a jugular infusion of the same dose. Catheters were placed into the carotid artery, jugular, and portal vein during general anesthesia. Hyperglycemic clamps were performed in conscious, freely moving rats with a graded infusion of GLP-1 (1.5 – 5 µg/kg/h) into the portal or jugular vein. To test the degree of hepatic degradation of GLP-1, the same experiments were repeated with a DPP-IV inhibitor. In addition, arterial plasma concentrations of the active peptide were measured after site-specific infusion of GLP-1 with or without a DPP-IV inhibitor.

Contrary to our hypothesis, GLP-1 infusion into the jugular vein resulted in higher insulin concentrations than an equimolar dose of GLP-1 in the portal vein, contradicting an important role of portal GLP-1r signaling for insulin secretion. This also suggests that the hepatoportal bed is a major site of GLP-1 inactivation and GLP-1 infused into the portal vein is more susceptible to degradation by

DPP-IV than an infusion into the jugular vein. After DPP-IV inhibition the insulin response to portal and jugular GLP-1 infusion did not differ significantly. However, there remained a numerically higher insulin response with jugular compared to portal GLP-1 infusion and hence a left shift of the dose-response-curve. Correspondingly, the arterial plasma concentrations of active GLP-1 were 2-3-fold higher with jugular compared to portal infusion of the peptide and this difference persisted even after administration of a potent pharmacological DPP-IV inhibitor.

In conclusion, these findings do not support an important role of hepatoportal GLP-1r signaling in mediating the incretin effect of GLP-1. However, the clearly lower potency of portal compared to jugular GLP-1-infusion to elicit an insulinotropic response together with the persistently lower arterial plasma concentrations after portal infusion even in the presence of a DPP-IV inhibitor, argue against an endocrine mechanism of action of endogenously released GLP-1. Furthermore, the failure of the DPP-IV inhibitor vildagliptin to protect the majority of GLP-1 infused into the portal vein from degradation does not support the concept that this drug class acts by augmenting GLP-1 levels in the circulation. Other alternatives to an endocrine model of GLP-1 action, like paracrine GLP-1 signaling within the islets, seem promising and should be further investigated.

6 Zusammenfassung

Glucagon-like peptide-1 (GLP-1) ist ein enterales Hormon, das von den L-Zellen des Dünn- und Dickdarms nach oraler Nahrungsaufnahme sezerniert wird. GLP-1 stimuliert glukoseabhängig die Insulinsekretion und vermittelt zusammen mit dem Glucose-dependent insulinotropic polypeptide (GIP) den Inkretineffekt. GLP-1 wirkt über einen spezifischen GLP-1 Rezeptor (GLP-1r), der in einer Vielzahl unterschiedlicher Gewebe exprimiert wird, z.B. in den β-Zellen der Langerhans-Inseln des Pankreas. Der bisher angenommene endokrine Wirkmechanismus von GLP-1 sieht vor, dass Nahrungsbestandteile im Lumen des Darms zur Sekretion des Peptidhormons in die Zirkulation führen und GLP-1 hierüber an seine Rezeptoren auf der β-Zelle gelangt. Allerdings geben die extrem kurze Halbwertszeit von GLP-1, aufgrund der raschen Inaktivierung durch Dipeptidylpeptidase-IV (DPP-IV), sowie die kaum messbaren postprandialen Plasmaspiegel von GLP-1, Anlass an einer endokrinen Wirkweise zu zweifeln und alternative Wirkmechanismen wurden erforscht. Mehrere Studien haben einem neuroendokrinen Mechanismus, ausgehend von GLP-1r in der Portalvene, eine wesentliche Rolle in der Vermittlung des insulinotropen Effekts von GLP-1 zugeschrieben.

Die hier beschriebene Studie ist die erste, die die Insulinantwort einer portalen GLP-1 Infusion mit der einer systemischen (jugulären) Infusion unter stabilen hyperglykämischen Bedingungen vergleicht und damit eine Beurteilung des insulinotropen Effekts unabhängig von Glukosespiegeln erlaubt. Unsere Hypothese war, dass eine portale Infusion von GLP-1 während eines hyperglykämischen Clamps, einen stärkeren insulinotropen Effekt haben würde, als die gleiche Dosis in der Jugularvene. Hierfür wurden Long-Evans Ratten operativ Katheter in die Arteria carotis, die Jugularvene und die Pfortader implantiert. Hyperglykämische Clamps wurden an wachen und sich frei bewegenden Tieren durchgeführt und mit einer GLP-1 Infusion mit steigender Dosierung (1.5-5 µg/kg/h), wahlweise in die Portal- oder Jugularvene, kombiniert. Um das Ausmaß der hepatischen Inaktivierung von GLP-1 zu bestimmen, wurden dieselben Clamps nach Gabe eines pharmakologischen DPP-IV-Hemmers wiederholt. Zudem wurden die arteriellen Plasmaspiegel des aktiven

GLP-1 nach Infusion des Peptids in die Portal- oder Jugularvene mit und ohne DPP-IV-Hemmer gemessen.

Im Gegensatz zu unserer Hypothese führte die juguläre GLP-1 Infusion zu höheren Insulinspiegeln, als die gleiche Dosis in der Portalvene. Dies spricht gegen eine wichtige Rolle des portalen GLP-1r in der Vermittlung des insulinotropen Effekts von GLP-1. Darüber hinaus bestätigen die Ergebnisse, dass die hepatoportale Zirkulation einen wesentlichen Anteil zum Abbau von GLP-1 beiträgt. Unter DPP-IV-Hemmung unterschieden sich die Insulinspiegel zwischen portaler und jugulärer Infusion nicht mehr signifikant. Dennoch zeigten sich numerisch höhere Insulinkonzentrationen nach jugulärer GLP-1-Infusion verglichen mit portaler Infusion und eine entsprechende Linksverschiebung der Dosis-Wirkungskurve. Korrelierend dazu waren die arteriellen Plasma-konzentrationen von aktivem GLP-1 2-3-mal höher wenn es in die Jugularvene infundiert wurde. Interessanterweise blieb dieser Unterschied auch nach der Gabe eines potenten DPP-IV-Hemmers bestehen.

Zusammenfassend sprechen die Daten dieser Studie nicht für einen relevanten Effekt von GLP-1r in der Portalvene bei der Vermittlung des Inkretineffekts. Ein endokriner Mechanismus von endogenem GLP-1 scheint aufgrund des ausgeprägten intrahepatischen Abbaus und den entsprechend niedrigeren arteriellen Plasmaspiegeln des aktiven Peptids nach portaler Infusion dennoch unwahrscheinlich. Die ausgeprägte Inaktivierung von portalem GLP-1 trotz der Gabe von Vildagliptin spricht darüber hinaus dagegen, dass DPP-IV-Hemmer über zirkulierendes GLP-1 ihre insulinotrope Wirkung vermitteln. Daher scheinen Alternativen zu einem endokrinen Wirkmechanismus, wie z.B. eine parakrine Wirkung von GLP-1 innerhalb der Langerhans-Inseln, vielversprechend und sollten weiter verfolgt werden.

7 Literature

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