

## Important species differences regarding lymph contribution to gut hormone responses



Marie Hansen<sup>1</sup>, Karina R. Hjøllund<sup>1</sup>, Bolette Hartmann, Astrid Plamboeck, Carolyn F. Deacon, Nicolai J. Wewer Albrechtsen, Jens J. Holst\*

NNF Center for Basic Metabolic Research and Department of Biomedical Sciences, Section for Translational Metabolism, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

### ARTICLE INFO

#### Article history:

Received 28 January 2015

Received in revised form 21 May 2015

Accepted 25 May 2015

Available online 3 June 2015

#### Keywords:

GLP-1

Lymph

DPP-4

Portal vein

Lamina propria

Neuromedin C

### ABSTRACT

**Introduction:** GLP-1 is secreted from the gut upon nutrient intake and stimulates insulin secretion. The lymph draining the intestine may transport high levels of GLP-1 to the systemic circulation before it is metabolized by DPP-4. The aims of this study were to investigate to what extent the lymphatic system might contribute to the final level(s) of systemic circulating intact GLP-1 and, in addition, whether secretory profiles in intestinal lymph might reflect lamina propria levels of GLP-1 i.e. before capillary uptake and degradation by endothelial dipeptidyl peptidase-4 (DPP-4).

**Method:** 7 pigs of the YDL-strain were catheterized in the portal vein, carotid artery and cisterna chyli (lymph). Neuromedin C (NC) was infused through an ear vein catheter, before and after injection of a selective DPP-4 inhibitor (vildagliptin). Total and intact GLP-1 levels were measured throughout the 150 min experiments using specific sandwich ELISAs. DPP-4 activity was measured spectrophotometrically.

**Results:** Concentrations of both total and intact GLP-1 were markedly lower in lymph compared to plasma samples, and did not increase significantly in response to stimulation with NC in the absence/presence of vildagliptin. In contrast, total and intact GLP-1 levels increased significantly in the portal vein and carotid artery. DPP-4 activity was lower in lymph than plasma, and was reduced further by vildagliptin.

**Conclusion:** Our observations indicate that the lymphatic system does not transport high levels of intact GLP-1 to the systemic circulation, and that GLP-1 levels in cisternal lymph do not reflect the hormone levels in the intestinal lamina propria.

© 2015 Elsevier Inc. All rights reserved.

### Introduction

Glucagon-like peptide-1 (GLP-1), secreted from intestinal L-cells, regulates glucose levels by potentiating glucose-stimulated insulin secretion (the incretin effect), suppressing glucagon secretion, and inhibiting appetite [4,14,19]. Because of these features, GLP-1 receptor agonists are currently used as treatment in patients with type 2 diabetes (T2DM). After secretion, intact (active) GLP-1 (7–36 NH<sub>2</sub>) diffuses to the lamina propria, and enters the local capillaries, where it is rapidly degraded by dipeptidyl peptidase

4 (DPP-4) located on the capillary endothelium to its metabolite (9–36 NH<sub>2</sub>). This process is highly efficient, with only 20–30% reaching the portal vein in the intact form, and less than 15% circulating as the intact peptide [5]; indeed, it has been calculated that only 8% of newly secreted GLP-1 actually reaches peripheral targets in the intact form [13]. Circulating GLP-1 levels are, therefore, low, which complicates measurement and assessment of potential GLP-1 stimulating drugs (secretagogues). However, it has also been suggested that GLP-1 might enter the lymphatic capillaries (lacteals) together with other nutrients e.g. chylomicrons [17], with the lymph system acting as a conduit for transporting high levels of GLP-1 from the intestinal mucosa to the systemic circulation [17]. Moreover, since DPP-4 activity was reported to be less in lymph compared to plasma [17], measurement of intact GLP-1 in lymph has also been suggested as a novel model for studying the effects of GLP-1 secretagogues [15].

The primary aim of this study was to investigate whether the lymphatic system in anaesthetized pigs carries high levels of intact GLP-1 to the systemic circulation. In addition, we hypothesized that

**Abbreviations:** DPP-4, dipeptidyl peptidase 4; GLP-1, glucagon-like peptide-1; NC, bombesin/neuromedin C.

\* Corresponding author. Tel.: +45 35327518.

E-mail addresses: [mariehansendk@gmail.com](mailto:mariehansendk@gmail.com) (M. Hansen), [KRJs@novonordisk.com](mailto:KRJs@novonordisk.com) (K.R. Hjøllund), [bhartmann@sund.ku.dk](mailto:bhartmann@sund.ku.dk) (B. Hartmann), [astridp@sund.ku.dk](mailto:astridp@sund.ku.dk) (A. Plamboeck), [deacon@sund.ku.dk](mailto:deacon@sund.ku.dk) (C.F. Deacon), [hgk795@ku.dk](mailto:hgk795@ku.dk) (N.J. Wewer Albrechtsen), [jjholst@sund.ku.dk](mailto:jjholst@sund.ku.dk) (J.J. Holst).

<sup>1</sup> Equally contributed to the study.

GLP-1 levels in lymph might reflect its concentrations in the interstitial intestinal fluid of the lamina propria i.e. its local site of action, where it is been proposed to interact with sensory afferents before it reaches the capillaries and is degraded by DPP-4 [10].

### Materials and methods

The animal studies were conducted in accordance with international guidelines (National Institutes of Health publication no. 85-23, revised 1985, and Danish legislation governing animal experimentation, 1987), and were carried out after permission had been granted by the Animal Experiments Inspectorate, Ministry of Justice, Denmark.

### Animal and surgical experiments

7 young pigs of the YDL-strain weighing  $34.7 \pm 3.4$  kg (mean  $\pm$  SD) were fasted overnight, but had free access to drinking water. After premedication with ketamine (10 mg/kg, Ketalar, Parke-Davis, Morris Plains, NJ) and induction with pentobarbital (Mebumal®, Nycomed DAK, Copenhagen, Denmark), the animals were anaesthetized with iv  $\alpha$ -chloralose (50 mg/kg, Merck & Co, Darmstadt, Germany) and ventilated with intermittent positive pressure using  $N_2O/O_2$  (AGA, Copenhagen, Denmark). Catheters were placed in the left ear vein for peptide administration and the right ear vein for DPP-4 inhibitor administration (vildagliptin; kindly provided by Novartis Institutes Biomedical Research, Inc., Cambridge, USA). Catheters were placed in the right carotid artery and in the portal vein as previously described [6]. Additionally, a non-obstructing catheter was fixed to the walls of the cisterna chyli by means of a purse-string suture for lymph sampling. The lymph flow during the entire experiment was sufficient for sample collection. After surgical preparation, the animals were heparinised and left undisturbed for 30 min.

### Experimental protocol

In order to stimulate the release of endogenous GLP-1, the animals received two intravenous boluses of neuromedin C [120 nmol] [11,13] (cat#3120, Bachem, Bubendorf, Switzerland) via the left ear vein catheter, one before and one after vildagliptin (1 mg/kg) administration. Simultaneous blood (3.5 ml) and lymph (1 ml) samples were collected over 150 min from the carotid artery, portal vein and cisterna chyli, respectively, into chilled tubes containing EDTA (7.2 nmol/l, final concentration), aprotinin (500 KIE/ml blood) and the DPP-4 inhibitor, valine-pyrrolidide (0.01 mmol/l, final concentration; a gift from Novo Nordiak, Bagsvaerd, Denmark). The flow of lymph in the cisterna chyli was sufficient to allow sampling by cautious suction on the catheter during the entire sampling time. For DPP-4 activity measurements, 0.5 ml (of each sample) was dispensed into tubes containing only EDTA. The tubes were kept on ice until centrifugation at 4 °C, and the supernatant stored at –20 °C until analysis. The blood volume taken did not exceed 10% of the total blood volume, but fluid loss was replaced after each blood sample with corresponding volumes of saline [8]. Both heart rate and blood pressure were monitored continuously and remained stable throughout the experiment.

### Peptide analysis

Total GLP-1 levels were measured using Millipore Total GLP-1 ELISA kit (cat# EZGLP1T-36K, Millipore, Billerica, MA, USA) as described previously [2]. Intact GLP-1 levels were measured using an in-house 2-site sandwich ELISA for GLP-1 as described previously [18]. DPP-4 activity was assessed kinetically by standard

procedures, using H-Gly-Pro-p-nitroaniline as substrate [16], with minor modifications.

### Statistical methods

Total GLP-1 and intact GLP-1 responses were evaluated by AUC (total AUC: whole experiment, incremental AUC: stimulation period with NC) calculated using the trapezoidal rule, tested by paired *t*-test, and two-way ANOVA for repeated measurement followed by Bonferroni post hoc analysis as appropriate. DPP-4 activity was evaluated using a paired *t*-test. All data are presented as mean  $\pm$  standard deviation (SD), *P*-values of <0.05 were considered significant. Statistical analysis was carried out using Graphpad Prism version 6.00 for Windows, GraphPad Software, La Jolla, California, USA.

### Results

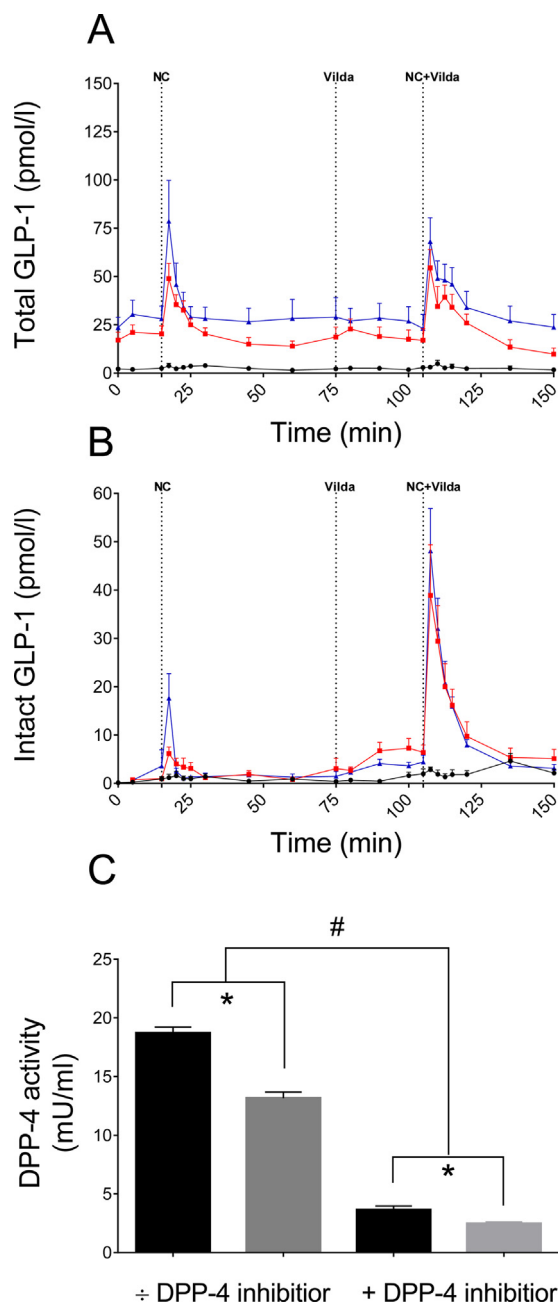
Total GLP-1 levels (see Fig. 1A) were higher ( $P < 0.05$ ) in the portal vein compared to the carotid artery, and each was markedly ( $P < 0.05$ ) higher compared to lymph ( $4680 \pm 2381$  pmol/l min,  $3067 \pm 1251$  pmol/l min and  $373 \pm 226$  pmol/l min AUC<sub>total</sub> respectively). Intact GLP-1 levels at all three sites were lower than total GLP-1 levels (see Fig. 1B), but the pattern was similar, with highest concentrations in the portal vein ( $P < 0.05$ ), and levels in both vascular vessels being greater ( $P < 0.05$ ) compared to lymph ( $853 \pm 277$  pmol/l min,  $689 \pm 185$  pmol/l min and  $134 \pm 99$  pmol/l min AUC<sub>total</sub> respectively).

Total GLP-1 levels measured during NC stimulation alone (AUC<sub>incremental</sub>) increased significantly ( $P < 0.05$ ) in plasma but not in lymph ( $P = 0.33$ ). The inclusion of the DPP-4 inhibitor, vildagliptin had no effect on total GLP-1 concentrations during NC stimulation (being similar without and with vildagliptin, portal vein:  $216/236$  pmol/l min; carotid artery:  $162/178$  pmol/l min; lymph:  $16/17$  pmol/l min). In contrast, intact GLP-1 (see Fig. 1B) levels in both portal vein and carotid artery ( $P < 0.05$ ), but not in lymph ( $P = 0.17$ ), increased significantly in response to NC when DPP-4 was inhibited.

DPP-4 activity (see Fig. 1C) was significantly higher (1.5 fold,  $P < 0.05$ ) in plasma (carotid artery) compared to lymph. Vildagliptin significantly ( $P < 0.05$ ) reduced DPP-4 activity in both plasma and lymph (by  $80 \pm 6\%$  and  $81 \pm 4\%$ , respectively; values not corrected for dilution in the assay), but absolute activity still remained higher in plasma.

### Discussion

In the current study, we investigated whether the lymphatic system transports high levels of intact GLP-1 from the intestine (lamina propria) to the systemic circulation, using anaesthetized pigs. We used the pig as a model because of its physiological similarities to human, in terms of digestive and associated metabolic processes [12], and since it allowed us to collect multiple larger (volume) samples compared to rodents. By estimating both intact and total GLP-1 in lymph and in plasma from portal vein and carotid artery simultaneously, we were able to identify the systemic, mesenteric and lymph GLP-1 ratios under basal conditions and during stimulation with NC (a powerful GLP-1 secretagogue), with or without simultaneous inhibition of the DPP-4 enzyme. This allowed us to estimate the sites of important DPP-4-mediated inactivation of the hormone. In particular we hypothesized that DPP-4-mediated degradation would be small in intestinal lymph, because GLP-1 is not expected to come into contact with DPP-4 before it enters the capillaries of the gut (and again in the liver).



**Fig. 1.** Total GLP-1 (A) and intact GLP-1 (B) levels (pmol/l) in the portal vein (blue), carotid artery (red) and the cisterna chyli (black) during NC (first vertical stippled line), vildagliptin (second vertical stippled line) and vildagliptin+NC (third vertical stippled line). (C) illustrates the DPP-4 activity (mU/ml) in plasma (black) and in lymph (gray) before (left bars) and after (right bars) infusion with vildagliptin. Data are means  $\pm$  SD,  $n = 7$ . \* indicates a significant difference ( $P < 0.05$ ) between plasma and lymph; # indicates a significant difference ( $P < 0.05$ ) in DPP-4 activity after vildagliptin for plasma and lymph using a paired  $t$ -test (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

Measurement of GLP-1 in lymph fluid has been proposed to provide a reliable estimate of GLP-1 levels in the lamina propria of the gut. This is important to know, because GLP-1 has been proposed to interact with local sensory nerves in addition to acting via the classical endocrine route [10,14,19]. Our lymph concentrations were considerably lower than the portal vein plasma levels. Given that GLP-1 must diffuse across the lamina propria from the endocrine epithelial cells to the capillaries, it follows that the

concentration here, in the interstitial fluid, must be higher than in the plasma draining the gut, leading us to conclude that GLP-1 concentrations in lymph in this study do not necessarily reflect the lamina propria concentrations. This raises the question of the relationship between the lymph and blood vessels in the villus. It is generally thought that the lymph vessels of the villi (the lacteals) are located in the core of the villus, whereas the blood capillaries form a dense network immediately below the basal membrane of the epithelium. The intestinal blood flow is high (in the pig, receiving about 1/3 of the cardiac output) and it is, therefore, conceivable that a large fraction of newly released hormone from the epithelium will be removed by solvent drag in the capillary layer. This could explain that much lower concentrations are found in the central core [7]. Tso et al. previously found food stimulated GLP-1 levels in lymph from rats to be 10-fold higher than plasma (v.portae) levels increasing from about 15 (basal levels) to 300 pmol/l (total GLP-1), and Ohlsson et al. recently reported increases in intact GLP-1 during glucose or fat stimulation from 0.5 to around 40 pmol/l in lymph from anaesthetized mice [1,3,17]. In the present study, NC resulted in robust increases in arterial GLP-1 concentrations (reaching 50 pmol/l), indicating that we achieved a similar degree of stimulation of secretion as in the rodent studies. This raises the question of whether the lymph drainage in pigs differs from that of rodents [7]. The low concentrations were not due to excessive DPP-4 activity – on the contrary, the activity measured in lymph was lower than that found in plasma. In addition, although DPP-4 inhibition, as expected, increased portal as well as arterial plasma levels of intact GLP-1, there was little influence on lymph concentrations. For the present study, we used assays for intact and total GLP-1 that have been thoroughly validated [2,18], also regarding their reliability for use in intestinal lymph. Thus, we obtained linear and complete recoveries of low picomolar amounts of added GLP-1 with these assays in pig lymph. The lack of a response, therefore, is unlikely to represent erroneous analytical results. Whether similar methodological scrutiny was exercised in the previous studies is unclear. In the present study, we used non-obstructing catheters draining the cisterna chyli, whereas the rodent studies involved indwelling obstructing catheters in a major mesenteric lymph duct. It is difficult to see that this should provide a significant difference (although the use of obstructing catheters may present a problem), since the intestinal lymph flows through the cisterna chyli before emptying into the thoracic duct (at least in pigs and humans). It has been reported that intestinal lymph flow may be reduced by anesthesia; but to mitigate this we used an anesthetic ( $\alpha$ -chloralose), which is mainly a hypnotic with minimal effect on gastrointestinal motility and secretion and enteric reflexes in pigs [9]. While our findings with respect to GLP-1 levels in lymph clearly contrast to the cited studies [1,3,17], D'Alessio et al. reported, similar to our findings, lower DPP-4 activity in lymph when compared activity levels in plasma [3]. Thus, despite stimulating the secretion of GLP-1 to a similar extent as in the rodent studies (as evidenced from the prompt and robust increases in plasma concentrations), we were unable to demonstrate a corresponding increase in lymph levels, which remained low and relatively unchanged throughout the study. However, based on the available data we are hesitant to conclude whether this is due to species variation or perhaps technical issues.

It cannot be excluded that a very small fraction of measured circulating total GLP-1 may be derived from the pancreatic alpha cells; however, there is little doubt that the majority is derived from the intestinal L-cells. Anesthesia is another limitation, although the secretory response obtained by us was clearly adequate. Finally, we cannot exclude that GLP-1 stimulation by orally administered nutrient may result in higher concentrations of GLP-1 in the lymphatic fluid draining the gut compared to i.v. administration of neuromedin C.

## Conclusion

In conclusion, this study indicates that the lymphatic system in pigs does not per se seem to function as a significant alternative route for GLP-1 delivery to the systemic circulation. In addition, the lymph concentrations of GLP-1 are unlikely to reflect those of the interstitial fluid of the lamina propria in the small intestine given that these must be higher than those observed in plasma from the portal vein.

## Funding

The study was supported by a grant to J.J. Holst from the NNF Center for Basic Metabolic Research (Novo Nordisk Foundation, Denmark), Augustinus foundation 14-0962. The planning and conduct of the study, writing of the manuscript or other matters related to this study were completely independent of the funding sources.

## Duality of interest

The planning and conduct of the study, writing of the manuscript or other matters related to this study were completely independent of the funding sources. The authors declare no financial or otherwise conflicts of interest.

## Contribution statement

JJH, MH, KRH and AP provided substantial contribution to the concept and design. JJH, MH, KRH, BH, AP, CFD, NWA substantially contributed to data analysis and interpretation. NWA, MH and KRH drafted the manuscript. JJH, AP, BH and CFD critically revised the manuscript for intellectual content. All authors gave final approval of the version to be published.

JJH is responsible for the integrity of the work as a whole.

## Acknowledgements

The authors thank Lene Albaek and Sofie Pilgaard for outstanding technical assistance. Affiliation: NNF Center for Basic Metabolic Research and Department of Biomedical Sciences, Faculty of Health Science, University of Copenhagen, DK-2200 Copenhagen, Denmark.

## References

- [1] Arnold M, Dai Y, Tso P, Langhans W. Meal-contingent intestinal lymph sampling from awake, unrestrained rats. *Am J Physiol Regul Integr Comp Physiol* 2012;302:R1365–71.

- [2] Bak MJ, Wewer Albrechtsen NJ, Pedersen J, Knop FK, Vilsbøll T, Jørgensen NB, et al. Specificity and sensitivity of commercially available assays for glucagon-like peptide-1 (GLP-1): Implications for GLP-1 measurements in clinical studies. *Diabetes Obes Metab* 2014;16:1155–64.
- [3] D'Alessio D, Lu W, Sun W, Zheng S, Yang Q, Seeley R, et al. Fasting and postprandial concentrations of GLP-1 in intestinal lymph and portal plasma: evidence for selective release of GLP-1 in the lymph system. *Am J Physiol Regul Integr Comp Physiol* 2007;293:R2163–9.
- [4] Deacon CF, Ahren B. Physiology of incretins in health and disease. *Rev Diabet Stud* 2011;8:293–306.
- [5] Deacon CF, Nauck MA, Toft-Nielsen M, Pridal L, Willms B, Holst JJ. Both subcutaneously and intravenously administered glucagon-like peptide I are rapidly degraded from the NH<sub>2</sub>-terminus in Type II diabetic patients and in healthy subjects. *Diabetes* 1995;44:1126–31.
- [6] Deacon CF, Pridal L, Klarskov L, Olesen M, Holst JJ. Glucagon-like peptide 1 undergoes differential tissue-specific metabolism in the anesthetized pig. *Am J Physiol* 1996;271:E458–64.
- [7] DeSesso JM, Jacobson CF. Anatomical and physiological parameters affecting gastrointestinal absorption in humans and rats. *Food Chem Toxicol* 2001;39:209–28.
- [8] Diehl K-H, Hull R, Morton D, Pfister R, Rabemampianina Y, Smith D, et al. A good practice guide to the administration of substances and removal of blood, including routes and volumes. *J Appl Toxicol* 2001;21:15–23.
- [9] Fahrenkrug J, Galbo H, Holst JJ, Schaffalitzky de Muckadell OB. Influence of the autonomic nervous system on the release of vasoactive intestinal polypeptide from the porcine gastrointestinal tract. *J Physiol* 1978;280:405–22.
- [10] Hansen L, Deacon CF, Orskov C, Holst JJ. Glucagon-like peptide-1-(7–36)amide is transformed to glucagon-like peptide-1-(9–36)amide by dipeptidyl peptidase IV in the capillaries supplying the L cells of the porcine intestine. *Endocrinology* 1999;140:5356–63.
- [11] Hansen L, Hartmann B, Bisgaard T, Mineo H, Jørgensen PN, Holst JJ. Somatostatin restrains the secretion of glucagon-like peptide-1 and -2 from isolated perfused porcine ileum; 2000.
- [12] Heinritz SN, Mosenthin R, Weiss E. Use of pigs as a potential model for research into dietary modulation of the human gut microbiota. *Nutr Res Rev* 2013;26:191–209.
- [13] Højlund KR, Deacon CF, Holst JJ. Dipeptidyl peptidase-4 inhibition increases portal concentrations of intact glucagon-like peptide-1 (GLP-1) to a greater extent than peripheral concentrations in anaesthetised pigs. *Diabetologia* 2011;54:2206–8.
- [14] Holst JJ. Incretin hormones and the satiation signal. *Int J Obes* (2005) 2013;37:1161–8.
- [15] Kohan AB, Yoder SM, Tso P. Using the lymphatics to study nutrient absorption and the secretion of gastrointestinal hormones. *Physiol Behav* 2011;105:82–8.
- [16] Nagakura T, Yasuda N, Yamazaki K, Ikuta H, Tanaka I. Enteroinular axis of db/db mice and efficacy of dipeptidyl peptidase IV inhibition. *Metabolism* 2003;52:81–6.
- [17] Ohlsson L, Kohan AB, Tso P, Ahrén B. GLP-1 released to the mesenteric lymph duct in mice: effects of glucose and fat. *Regul Pept* 2014;189:40–5.
- [18] Wewer Albrechtsen NJ, Bak MJ, Hartmann B, Christensen LW, Kuhre RE, Deacon C, et al. Stability of glucagon-like peptide-1 and glucagon in human plasma. *Endocr Connect* 2015;4:50–7 <http://www.endocrineconnections.com/content/4/1/50.long>
- [19] Wewer Albrechtsen NJ, Kuhre RE, Deacon CF, Holst JJ. Targeting the intestinal L-cell for obesity and type 2 diabetes treatment. *Expert Rev Endocrinol Metab* 2014;9:61–72.