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The C-terminal extension of exendin-4 provides additional metabolic stability when added to GLP-1, while there is minimal effect of truncating exendin-4 in anaesthetized pigs

L. Simonsen [a,1], J.J. Holst [a], K. Madsen [b], C.F. Deacon [a,*]

[a] University of Copenhagen, Department of Biomedical Sciences, Panum Institute, Blegdamsvej 3, 2200 Copenhagen N, Denmark
[b] Novo Nordisk A/S, Novo Nordisk Park, 2760 Maaloev, Denmark

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The most striking sequence difference between glucagon-like peptide-1 (GLP-1)[2] and the longer-acting GLP-1 receptor agonist, exendin-4 (Ex-4),[3] is the nine-amino acid COOH-terminal extension of Ex-4. We investigated the contribution of this extension to the survival time of Ex-4.

We assessed the overall metabolism of GLP-1, Ex-4, a COOH-terminally extended GLP-1 peptide (GLP-1 + Ex(31–39); GLP-Ex),[4] and a COOH-terminally truncated exendin peptide (Ex(1–30)) in anaesthetized, catheterized pigs, with focus on the extraction across the kidneys and a peripheral tissue (a hindleg, representing muscle, adipose- and connective tissue). Peptide analysis was carried out with assays against the mid-region of the peptides, whereby the role of dipeptidyl peptidase-4 (DPP-4)[5] mediated NH_2-terminal degradation could be disregarded.

The half-life of GLP-1 was signi cantly increased when the COOH-terminal extension of Ex-4 was added (GLP-1 4.8 ± 3.3 min; GLP-Ex 19.5 ± 3.3 min). In contrast, there was no effect of truncating Ex-4 (Ex-4 32.4 ± 4.1 min; Ex(1–30) 28.4 ± 1.7 min). Ex-4 and Ex(1–30) were cleared solely by the kidneys at rates corresponding to the glomerular ltration rate (GFR),[6] while GLP-1 and GLP-Ex were cleared by both the kidneys and peripheral tissues. Both extraction rates were, however, signi cantly reduced with GLP-Ex compared to GLP-1. The renal clearance rate of GLP-1 greatly exceeded GFR, while GLP-Ex was cleared at a rate resembling GFR.

In conclusion, the COOH-terminal extension of Ex-4 contributes minimally to the increased survival time of Ex-4, while addition of this sequence to GLP-1 signi cantly reduces its clearance.

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1 Introduction

Glucagon-like peptide-1 (GLP-1) is a product of proglucagon gene expression and is released from the L-cells of the distal small intestine in response to nutrients. It is an incretin hormone, but has a number of additional anti-diabetic effects [1]. GLP-1 is very rapidly degraded in vivo, primarily by dipeptidyl peptidase-4 (DPP-4) [2]. In a search for long-acting GLP-1 analogues, Ex-4 (exendin-4), isolated from the saliva of the Gila monster, turned out to be not only a potent GLP-1 receptor agonist [3–5], but to have a signi cantly longer half-life [6], partly due to its DPP-4 resistant primary structure [7]. GLP-1 and Ex-4 share 53% sequence homology in the rst 30 amino acids, being most pronounced in the NH_2-terminal part of the peptides [8]. COOH-terminally, the peptides differ radically by the addition of a nine amino acid extension to Ex-4, which conformationally has been shown to exhibit a compact tertiary structure known as a tryptophan-cage motif [9].

In spite of the pronounced sequence homology between Ex-4 and GLP-1, their rates of metabolism differ signi cantly. Ex-4 is resistant to DPP-4 mediated degradation due to the presence of glycine as the penultimate NH_2-terminal amino acid versus alanine in GLP-1. Other enzymes, such as neutral endopeptidase 24.11 (NEP),[7] may cleave GLP-1 in the central and more COOH-terminal part of the peptide [10], whereas Ex-4 seems to have a more general resistance towards enzyme-mediated degradation, and is remarkably stable when incubated in the presence of kidney brush border membranes, which are particularly rich in a number of ectopeptidases [11].

Ex-4 and GLP-1 also differ with respect to organ extraction. Whereas GLP-1 is cleared by the liver, the kidneys, and by the extremities (as studied across a hindleg, representing clearance by muscular, connective

* Corresponding author at: Department of Biomedical Sciences, Panum Institute 12.2.23, Blegdamsvej 3, DK-2200 Copenhagen N, Denmark. Tel.: +45 3532 7523; fax: +45 3532 7537.
E-mail address: Deacon@sund.ku.dk (C.F. Deacon).

[1] Current address: Novo Nordisk A/S, Novo Nordisk Park, 2760 Maaloev, Denmark.
[2] GLP-1: glucagon-like peptide-1.
[3] Ex-4: exendin-4.
[4] GLP-Ex: GLP-1 + Ex(31–39).
[5] DPP-4: dipeptidyl peptidase-4.
[6] GFR: glomerular ltration rate.
[7] NEP: neutral endopeptidase 24.11.

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Table 1
Peptide sequences and abbreviations.

Peptide	Abbreviation	Sequence
GLP-1(7–36)NH_2	GLP-1	HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR
Exendin-4	Ex-4	HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPS
Exendin(1–30)	Ex(1–30)	HGEGTFTSDLSKQMEEEAVRLFIEWLKNGG
GLP-1Arg^{34}(7–36), Ex(31–39)	GLP-Ex	HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRPSSGAPPPS

and adipose tissue) [12], Ex-4 is cleared exclusively by the kidneys [6]. Hepatic metabolism of GLP-1 consists of NH_2-terminal cleavage mediated by DPP-4, whereas in the peripheral tissues both DPP-4 and other enzymes are involved, as demonstrated using NH_2- and COOH-terminally directed GLP-1 assays [12]. In addition, however, the renal metabolism also differs in that the renal extraction of GLP-1 greatly exceeds what can be explained by glomerular ltration, whereas the renal extraction of Ex-4 occurs at a rate resembling glomerular ltration [6]. Thus, whereas glomerular ltration seems to be responsible for the entire renal extraction of Ex-4, additional mechanisms such as a peritubular uptake must be involved in the renal extraction of GLP-1. These differences in both the overall metabolism and the renal handling of the peptides are probably partly due to differences in the primary structure. Since the most conspicuous difference between the peptides is the nine-amino acid extension of Ex-4, it may be speculated that these additional amino acids are of major importance in adding metabolic stability to Ex-4 compared to GLP-1.

In the current study, we investigated the importance of the COOH-terminal extension of Ex-4 in contributing to the increased survival time. The study compared the elimination rates of Ex-4 and GLP-1 with the elimination of COOH-terminally truncated Ex-4 and COOH-terminally extended GLP-1. The elimination rates were investigated in anaesthetized pigs with antibodies directed towards the central part of the sequence, therefore disregarding NH_2-terminal metabolism. This study focused on the extraction across a hindleg and the kidneys, which previously have been shown to be the sites of interest.

2 Materials and methods

2.1. Animals and surgical procedure

The animal studies were conducted in accordance with international guidelines (National Institute 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 National Superintendence for Experimental Animals. Overnight fasted, female pigs (n = 18) of the strain LYD (Lars Jonssen, Lynge, Denmark), weighing approximately 30 kg were used in this study. After premedication with Ketalar (10 mg/kg ketamine hydrochloride, P zer, Ballerup, Denmark), the animals were anaesthetized with intravenous -chloralose (66 mg/kg; Sigma, Broendby, Denmark), and ventilated with intermittent positive pressure with N_2O/O_2. After midline laparotomy, catheters were placed in both ureters, with the opening of the catheters being located at the renal pelvis. Subsequently, vascular catheters were placed in the right carotid artery, the left renal vein, and the left femoral vein as previously described [12]. Both ear veins were cannulated; one was used for infusion of saline at a rate of 5 ml/min, ^{14}C-inulin, and additional -chloralose as necessary, and the other was used for peptide infusions. Following the surgical procedure, the animals were heparinized and left undisturbed for 30 min.

2.2. Compounds

The compounds used are shown in Table 1. GLP-1 and Ex-4 were obtained from Bachem (Weil am Rhein, Germany) while GLP-Ex and Ex(1–30) were synthesized at Novo Nordisk A/S according to the Fmoc strategy on an Applied Biosystems 431A peptide synthesizer (Life Technologies, Carlsbad, CA, USA). The nal puri ed products were characterized by RP-HPLC/ion spray mass spectrometry (LC–MS) (retention time and molecular mass) and analytical RP-HPLC (retention time and peptide amount). The RP-HPLC analysis was performed using UV detection at 214 nm and a Vydac 218TP54 4.66250 mm 5 mm C-18 silica column (The Separations Group, Hesperia, CA, USA). The LC–MS analysis was performed on a PE-Sciex API 100 mass spectrometer (AB Sciex, Framingham, MA, USA). The molecular masses were in agreement with the expected structures within the experimental error of the method (+1 amu) and peptide purities were above 95%.

All peptides were dissolved in 0.04 M phosphate buffer pH 7.5 containing 1% human serum albumin (Merck, Darmstadt, Germany) and administered using an automatic syringe pump. GLP-1 was administered at a rate of 2 pmol/kg/min; GLP-Ex and Ex(1–30) were infused at a rate of 1 pmol/kg/min, preceded by a bolus injection of 20 pmol/kg; and Ex-4 was given at a rate of 0.4 pmol/kg/min preceded by a bolus injection of 10 pmol/kg. ^{14}C-inulin (Perkin Elmer, Wellesley, USA) was dissolved in 0.04 M phosphate buffer pH 7.5 and was infused at a rate of 2.3 μCi/h preceeded by a bolus injection of 3 μCi.

2.3. Experimental procedure

In a cross-over design, six animals received infusions of GLP-1 and GLP-Ex, and another six animals received Ex-4 and Ex(1–30) infusions. Prior to each peptide infusion, basal blood samples were taken from the carotid artery (3 ml). GLP-1 was administered for 30 min during which time arterial blood samples (3 ml) were taken every 5 min, GLP-Ex, Ex-4 and Ex(1–30) were all administered for 40 min with blood sampling (3 ml) every 10 min. During the last 10 min of each infusion period, when steady state arterial peptide concentrations had been attained, simultaneous blood samples (3 ml) were taken from the carotid artery, the femoral vein and the renal vein, followed by a duplicate set of samples 5 min later. Following cessation of the infusions, further arterial blood samples were taken at time 32, 35, 40, 45, 50, and 60 min (GLP-1); 42, 45, 50, 55, 60, 70, 85, 100, and 130 min (Ex(1–30) and GLP-Ex); or 50, 60, 70, 85, 100, and 130 min (Ex-4). Blood samples were collected into chilled tubes containing EDTA (Merck) (7.4 mmol/l nal concentration). The tubes were stored on ice until centrifugation at 4 °C, after which, plasma was separated and kept at 20 °C until analysis.

2.4. Renal hemodynamics

All animals received continuous ^{14}C-inulin (Perkin Elmer) infusion, which was started immediately after insertion of the ureter catheters, allowing steady state levels to be reached while the rest of the catheters were being placed. Urine samples from one kidney were collected for 20-minute periods during and between the peptide infusions. At the end of each urine collection period, an extra 0.5 ml of blood was taken from the renal vein and the carotid artery. Urine volumes were recorded, and both urine and plasma samples were measured for β-radioactivity (4–156 keV), with a 2000CA Tri Carb liquid scintillation analyzer (Perkin Elmer, Hvidovre, Denmark), to allow calculation of the renal plasma flow (RPF)[8] and the glomerular ltration rate (GFR) (see below).

[8] RPF: renal plasma flow.

The total volume of blood taken (150 ml) did not exceed 5% of the total blood volume for a 30 kg animal, and this fluid loss was replaced with saline, following each blood sample. Blood pressure and heart rate were monitored throughout the experiment, and remained stable, as seen in previous experiments [12].

2.5. Hormone analysis

Analyses were performed in order to directly compare GLP-1 with COOH-terminally extended GLP-1 and Ex-4 with COOH-terminally truncated Ex-4. Immunoreactivity of both GLP-1 and GLP-Ex were detectable with antiserum 2135 [13,14], which measures all molecules containing the central sequence of GLP-1. It recognizes both NH_2- and COOH-terminally truncated and extended analogues and cross-reacts 50% with GLP-Ex and 79% with GLP-1(9–36)amide. Since the antiserum detects all peptides containing the central sequence of GLP-1, it also cross-reacts with pancreatic major proglucagon fragment, but this contribution was expected to be low and constant throughout the experimental protocol for both peptides. Plasma samples were extracted with 70% ethanol before assay, giving recoveries of 75% [14]. The standards used were GLP-1 and GLP-Ex, respectively. The intra-assay coef cient of variation for the analysis of GLP-Ex was 10% and the detection-limit was 2.5 pmol/l. Ex-4 and Ex(1–30) were measured with an in-house antiserum (code-number 3145) raised in rabbits, which is directed against the central region of Ex-4 and cross-reacts ~50% with Ex(1–30) and 100% with Ex(9–39) [15]. The tracer used was ^{125}I-exendin(9–39) (Perkin Elmer). The intra-assay coef cients of variation were 6% and the detection limits were 0.5 pmol/l. Plasma samples were analyzed directly in unextracted plasma diluted 10-fold with assay buffer. The standards used were Ex-4 and Ex(1–30), respectively.

To ascertain whether the endogenous level of GLP-1 was affected by the experimental protocol, we measured the level of endogenous GLP-1 during the infusion period of GLP-Ex, using antiserum 89390 [16,17], which does not cross-react with COOH-terminally extended peptides, but is highly speci c for the intact amidated COOH-terminal of GLP-1(7–36)amide. This antiserum cross-reacts 0.01% with COOH-terminally truncated GLP-1 fragments, GLP-Ex, and Ex-4. The detection limit is 1 pmol/l [16,17].

2.6. Calculations and statistical analysis

The renal clearance of ^{14}C-inulin was used to estimate the GFR for each kidney using the formula $C_{in} = GFR = (U_{in}\ D)/A_{in}$, where U_{in} is ^{14}C-inulin activity in the urine, A_{in} is ^{14}C-inulin activity in the arterial plasma and D is the diuresis. Likewise ^{14}C-inulin was used to estimate the effective RPF, as the extraction fraction, $E_{in} = (A_{in}\ V_{in})/A_{in}$, where V_{in} is ^{14}C activity in the venous plasma. The effective RPF was used to calculate the true" $RPF = C_{in}/E_{in}$. Previous studies from this laboratory have shown that RPF determined in this way is similar to that obtained using ^{3}H-PAH (unpublished data).

For each animal, the extraction fractions across a hindleg and the kidney were calculated from arterio-venous concentration differences, as previously described [12]. The plasma half-lives were determined from the elimination phase of the carotid artery plasma pro les (after subtraction of basal concentrations in the arterial sample prior to the start of the peptide infusion). The metabolic clearance rate was determined from the actual infusion rate using the formula:

$$MCR = \text{infusion rate}/A_{carotid}$$

The renal extraction of each peptide was calculated as:

$$\text{Renal extraction} = RPF^{*}(A_{carotid}\ V_{renal}$$

and the maximal amount ltered:

$$\text{Amount filtered} = GFR^{*}A_{carotid}$$

The renal clearance rate was calculated as:

$$\text{Renal clearance rate} = ((A_{carotid}\ V_{renal}\ /A_{carotid}\ ^{*}RPF$$

Data are expressed as mean ± SEM and analyzed by analysis of variance (ANOVA) and *t*-test for paired and unpaired data as appropriate. Differences resulting in p 0.05 were considered signi cant. Statistics were performed using Stastistica 8.0 for Windows (Statsoft, Tulsa, USA).

3 Results

3.1. In vivo half-life and metabolic clearance rate

Endogenous levels of GLP-1 were unaffected by the peptide infusions and remained constant throughout the experimental protocol (data not shown). As seen from Table 2, GLP-1 was cleared more quickly than Ex-4. Truncation of Ex-4 did not signi cantly affect the survival time of Ex-4, while the COOH-terminal extension signi cantly increased the survival time of GLP-1. Hence, the survival time of GLP-1 was increased by the COOH-terminal addition to a duration similar to that of COOH-terminally truncated Ex-4, but not quite as long as that of native Ex-4.

3.2. In vivo organ clearance

A signi cant extraction across the kidneys and a hindleg was observed for GLP-1, whereas Ex-4 was cleared exclusively by the kidneys. Addition of the COOH-terminal extension to GLP-1 did not affect the pattern of organ extraction, with GLP-Ex also being cleared by a hindleg (borderline signi cance) and the kidneys, but to an extent which was signi cantly less than the extraction observed for GLP-1 (Fig. 1A). COOH-terminal truncation of Ex-4 did not affect the extraction pattern, thus Ex-4 and Ex(1–30) were cleared to comparable extents exclusively by the kidneys (Fig. 1B).

3.3. Renal clearance

GFR and RPF were stable throughout the experimental period and were at rates comparable to earlier observed values in the anaesthetized pig [6]. The renal clearance rate of GLP-1 accounted for only 38.9 ± 9.5% of the overall metabolic clearance rate, whereas the renal clearance rate of Ex-4 resembled the overall metabolic clearance rate (Table 2). This clearance pattern was not affected by the COOH-terminal part of Ex-4, as GLP-Ex resembled GLP-1 (the renal clearance rate accounted for 45.8 ± 4.9% of the overall metabolic clearance rate), while Ex(1–30) resembled Ex-4 (the renal clearance rate

Table 2
Metabolic parameters.

	Ex-4	Ex(1–30)	GLP-Ex	GLP-1
T1/2 (min)	32.8 ± 4.1[c,d]	28.4 ± 1.7[d]	19.5 ± 3.3[a,d]	4.8 ± 0.8[a,b,c]
Metabolic clearance rate (ml/kg/min)	2.7 ± 0.3[c,d]	2.5 ± 0.4[c,d]	7.0 ± 0.4[a,b,d]	16.5 ± 2.1[a,b,c]
Renal clearance rate (ml/kg/min)	2.0 ± 0.4	2.1 ± 0.4	3.3 ± 0.3[e]	5.7 ± 0.7[e]

Mean ± SEM (n = 6).
[a] p 0.05 vs. Ex-4.
[b] p 0.05 vs. Ex(1–30).
[c] p 0.05 vs. GLP-Ex.
[d] p 0.05 vs. GLP-1.
[e] p 0.05 vs. metabolic clearance rate.

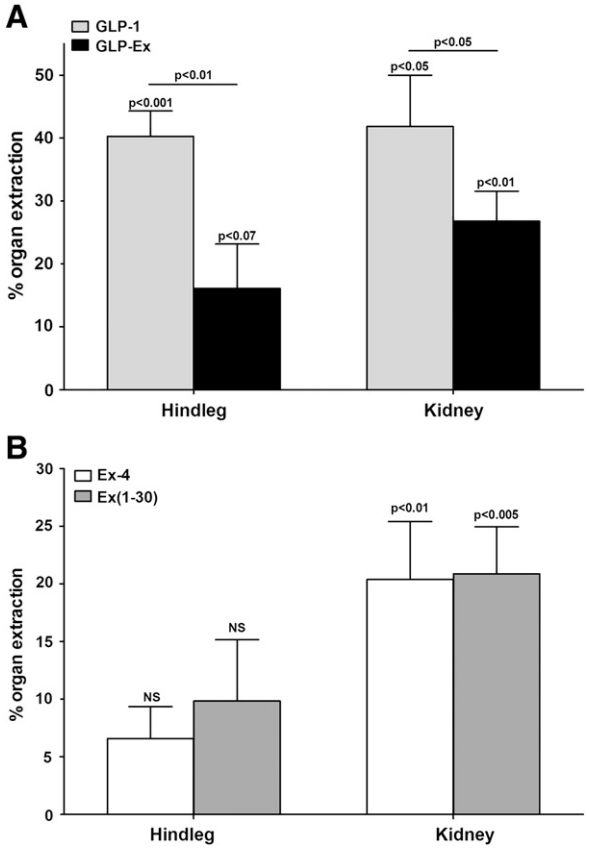

Fig 1 Extraction across a hindleg (representing muscle, adipose-, and connective tissue) and a kidney of GLP-1, GLP-Ex, Ex-4, and Ex(1–30). The p-values stated above the bars represent whether the extraction is signi cantly different from 0%, and in addition, the signi cant difference between GLP-1 and GLP-Ex is stated. No difference in organ extraction was observed between Ex-4 and Ex(1–30). Mean ± SEM (n = 6).

resembled its overall metabolic clearance rate). A closer look at the renal metabolism revealed that the renal extraction rate of GLP-1 exceeded the theoretical maximal amount of peptide being ltered, since only 49.9 ± 4.0% of the extraction could be accounted for by glomerular ltration, whereas Ex-4 was characterized by a total renal extraction, which equalled the maximal amount ltered (Fig. 2). Addition of the COOH-terminal tail of Ex-4 to GLP-1 reduced the renal extraction fraction to an extent resembling the amount being ltered. In contrast, truncation of the COOH-terminal part of Ex-4 did not affect the pattern of renal extraction, since Ex(1–30), like Ex-4, was characterized by a total renal extraction which could be fully explained by glomerular ltration (Fig. 2).

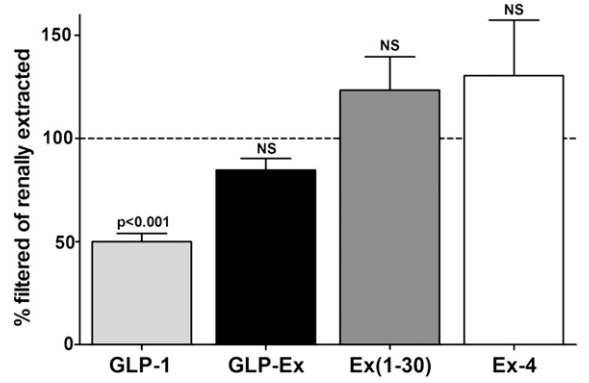

Fig 2 The bars show to which extent the overall renal extraction can be explained by glomerular ltration. The p-values indicate whether the value is signi cantly different from 100%. Values are given as mean ± SEM (n = 6).

4 Discussion

The present study investigated the importance of the nine-amino acid extension of Ex-4 in contributing to the increased survival time of Ex-4 relative to GLP-1. Previous studies demonstrated that the COOH-terminal extension of Ex-4 contributes signi cantly to the binding af nity of Ex-4 for the GLP-1 receptor, although adding this extension to the GLP-1 molecule does not markedly affect its binding af nity. Likewise, cAMP production in Chinese hamster ovary cells stably transfected with the GLP-1 receptor was signi cantly reduced when the COOH-terminal region of Ex-4 was deleted, while there was only a marginal effect of adding the extension to GLP-1. Thus, the results of Doyle and co-workers indicated that both Ex(1–30) and GLP-Ex resemble native GLP-1 with regard to binding and activation of the GLP-1 receptor, whereas native Ex-4 is superior [18]. With regard to the importance of the nine amino acid extension in the metabolism of the peptides, we now demonstrate that this extension does influence the survival time of the peptide. Thus, although there is a minimal effect of truncating Ex-4, adding the COOH-terminal extension to GLP-1 does increase its survival time, although not to the same extent as that of native Ex-4. Nonetheless, even though the nine amino acid extension of Ex-4 in itself is able to reduce clearance, there must be additional features in the core-molecule of Ex-4 which also contribute, since the clearance of Ex(1–30) does not seem to differ from Ex-4. The differences observed in the overall metabolic stability were reflected in the organ extraction pattern. Ex(1–30), like Ex-4, was cleared solely by the kidneys, while GLP-1 and GLP-Ex were cleared by the kidneys as well as a hindleg, albeit that GLP-Ex was cleared to a lesser degree than GLP-1.

Extraction across the hindleg represents extraction by muscle, connective- and adipose-tissue, and was only observed for GLP-1 and GLP-Ex. This extraction is probably largely due to enzymatic degradation. One of the major differences between GLP-1 and Ex-4 is the susceptibility of GLP-1 to DPP-4 mediated degradation, which is explained by the simple substitution of the penultimate NH_2-terminal amino acid in Ex-4 providing resistance to DPP-4. The present study, where assays against the mid-region of the peptides were used, disregarded the NH_2-terminal metabolism, which explains the relatively long half-life and is in agreement with previous ndings in the anaesthetized, catheterized pig [12]. Other enzymes, such as NEP which may play a role in the in vivo metabolism of GLP-1 [19], may be involved in the degradation at other sites of the molecule. Since previous studies have shown Ex-4 to be resistant to a number of enzymes to which GLP-1 is susceptible [11], it may be speculated that the central sequence of Ex-4 in itself is enough to provide this enzymatic resistance. However, the COOH-terminal extension may also be involved, since adding this to GLP-1 does provide some additional protection, albeit incomplete.

It is clear from this study and previous ndings [6,20–22], that the renal metabolism is the point of major difference between the metabolism of GLP-1 and Ex-4, since GLP-1 is cleared at a rate exceeding the glomerular ltration rate, while the clearance of Ex-4 can be fully accounted for by glomerular ltration [6]. It is, therefore, very interesting to investigate what mechanisms might be involved in this additional uptake of GLP-1. The present study clearly indicates that the COOH-terminal extension of Ex-4 must provide some protection, since the renal clearance of GLP-1 was reduced to an amount closer to and not signi cantly different from the glomerular ltration rate when this sequence was added to GLP-1. The precise mechanisms explaining the additional renal extraction of GLP-1 have not been elucidated, but it has been speculated that a peritubular uptake could take place [20]. It is, however, intriguing which pathways mediate this extraction. Some degree of enzymatic degradation has been suggested to take place in the kidneys independent of the enzymatic activity related to the brush border membrane, as has been described for the related hormone, glucagon [23]. On the other hand, a receptor mediated uptake could

be speculated to take place. The GLP-1 receptor has been reported to be located to the proximal tubule cells, but has been suggested to be located towards the tubulus lumen [24]. This observation is still controversial, however, since it is hard to envisage the relevance of a GLP-1 receptor located luminally, where the ltered peptide will be immediately degraded by brush border enzymes [11]. A pharmacodynamic study with subchronic administration of Ex-4 to GLP-1 receptor knock-out mice reported an eight-fold increase in Ex-4 plasma concentration in GLP-1 receptor knock-out mice as compared to their wild-type counterparts [25]. These ndings suggest that the GLP-1 receptor might be involved in the clearance of Ex-4. It could be speculated that a receptor located basolaterally could be involved in the additional renal extraction of GLP-1. The difference in the renal handling of GLP-1 and Ex-4 is more dif cult to explain, since Ex-4 has similar af nity for the GLP-1 receptor as the native peptide itself [5]. However, this could be explained if the receptor–ligand complex is internalized after binding, since it has been argued that receptor activation and internalization involve interaction with different domains in the receptor [26]. Thus, Widman and co-workers showed that whereas GLP-1 is internalized following binding, Ex(9–39) is not internalized even though it binds to the receptor [26]. It could, therefore, be speculated that the additional renal extraction of GLP-1 is due to a receptor mediated internalization of the peptide which does not take place for Ex-4 and is prevented if the COOH-terminal tail from Ex-4 is added to GLP-1. However, it cannot be excluded that a GLP-1 receptor subtype could exist; thus, cardiovascular effects of GLP-1 are still apparent in GLP-1 receptor knock-out mice [27], and Ex(9–39) is able to block all the cardioprotective effects of Ex-4, but not GLP-1, in the rat heart [28].

In summary, we found that the nine amino acid extension of Ex-4 does not contribute to the increased survival time of Ex-4, whereas adding this sequence to GLP-1 signi cantly increases the survival time by decreasing the renal extraction fraction to a level equivalent to the glomerular ltration rate.

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Author contributions

KM synthesized and delivered the non-commercially available peptides. LS, CD and JJH designed the study. LS performed the experimental work and analyzed data. LS, CD and JJH interpreted data. LS drafted the manuscript. CD and JJH revised the manuscript. All authors have seen and approved the nal manuscript.

Disclosure statement

The authors have no conflicts of interest in relation to the present study.

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