

# A local glucagon-like peptide 1 (GLP-1) system in human pancreatic islets

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## Abstract

**Aims/hypothesis** Glucagon-like peptide 1 (GLP-1) is a major incretin, mainly produced by the intestinal L cells, with beneficial actions on pancreatic beta cells. However, while in vivo only very small amounts of GLP-1 reach the pancreas in bioactive form, some observations indicate that GLP-1 may also be produced in the islets. We performed comprehensive morphological, functional and molecular studies to evaluate the presence and various features of a local GLP-1 system in human pancreatic islet cells, including those from type 2 diabetic patients.

**Methods** The presence of insulin, glucagon, GLP-1, proconvertase (PC) 1/3 and PC2 was determined in human pancreas by immunohistochemistry with confocal microscopy. Islets were isolated from non-diabetic and type 2 diabetic donors. GLP-1 protein abundance was evaluated by immunoblotting and matrix-assisted laser desorption–ionisation-time of flight (MALDI–TOF) mass spectrometry. Single alpha and beta cell suspensions were obtained by enzymatic dissociation and FACS sorting. Glucagon and GLP-1 release were measured in response to nutrients.

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**Results** Confocal microscopy showed the presence of GLP-1-like and PC1/3 immunoreactivity in subsets of alpha cells, whereas GLP-1 was not observed in beta cells. The presence of GLP-1 in isolated islets was confirmed by immunoblotting, followed by mass spectrometry. Isolated islets and alpha (but not beta) cell fractions released GLP-1, which was regulated by glucose and arginine. *PC1/3* (also known as *PCSK1*) gene expression was shown in alpha cells. GLP-1 release was significantly higher from type 2 diabetic than from non-diabetic isolated islets.

**Conclusions/interpretation** We have shown the presence of a functionally competent GLP-1 system in human pancreatic islets, which resides in alpha cells and might be modulated by type 2 diabetes.

**Keywords** Alpha cells · GLP-1 · Glucagon · Pancreatic islets

### Abbreviations

DPP-4	Dipeptidyl peptidase IV
GLP-1	Glucagon-like peptide 1
MALDI-TOF	Matrix-assisted laser desorption/ionisation–time of flight
PC	Proconvertase

### Introduction

Glucagon-like peptide 1 (GLP-1) is encoded in the proglucagon gene in the long arm of chromosome 2, and is mainly expressed in pancreatic alpha cells, intestinal L cells and the caudal brain stem [1–4]. The primary translation product is processed differently, so that proconvertase (PC)2 leads to glucagon production in alpha cells, whereas PC1/3 cleaves proglucagon to GLP-1 in L cells and the brain, with GLP-1(7–37) and GLP-1(7–36)amide representing the bioactive peptides [1–4]. As an incretin, GLP-1 lowers glucose levels by modulating glucose-stimulated insulin secretion from pancreatic beta cells [1–3]. It also has or may have roles in the maintenance of beta cell mass, regulation of gastric motility, reduction of plasma glucagon, protection of endothelium, promotion of satiety and stimulation of glucose disposal [1–3, 5, 6]. The beneficial effects of GLP-1 and its mimetics on beta cells have made them potential therapeutic agents for type 2 diabetes [1–3, 5–7]. However, the action of GLP-1 is limited in duration due to cleavage by dipeptidyl peptidase IV (DPP-4), resulting in a GLP-1 half-life of approximately 2 min [1–3, 5, 6]. Indeed, more than 50% of GLP-1 is inactivated on its release from L cells, and a large amount of the remaining intact peptide is inactivated as it passes through the liver, with only very small amounts of bioactive GLP-1 reaching the pancreas [8].

Some experimental evidence suggests that GLP-1 may also be produced in pancreatic islets. In the mid-1980s, investigators using antibodies against GLP-1 and electron microscopic

immunocytochemistry observed molecular forms of GLP-1 in the human pancreas [9, 10]. Later, fully processed GLP-1 was visualised in rat pancreatic extracts by chromatographic analysis and radioimmunoassays [11]. In another study [12], monoclonal antibodies to GLP-1(7–37) were produced for immunocytochemistry and radioimmunoassay experiments, and immunoreactive GLP-1 was detected in rat alpha cells, with increased release upon glucose stimulation [12]. Accordingly, GLP-1(7–36)amide was found in the pancreas of fetal and neonatal rats [13], and fully processed GLP-1 was produced by insulinoma cell lines and primary rat alpha cells [14]. Furthermore, when partial beta cell loss was induced in neonatal rats by streptozotocin, islet cell regeneration was accompanied by alpha cell hyperplasia, with an altered phenotype of increased GLP-1 synthesis [15]. More recently, a study mainly focused on the alpha cell line,  $\alpha$ TC1-6, showed the processing of proglucagon to GLP-1, with the incretin also found in rat and human islets [16], while investigators working on the *Psammomys obesus* gerbil incidentally observed that human islets could release GLP-1 [17]. Finally, an extensive study in rats showed that interleukin-6 could increase GLP-1 release from L cells and alpha cells, with some of the results reproduced with human islets [18].

With all this in mind, we performed a more thorough investigation of the presence of a local GLP-1 system in human islets, using morphological, molecular and functional studies of intact islets and human alpha and beta cell-enriched fractions. Part of the studies was also done on islets from type 2 diabetic individuals.

### Methods

**Pancreas and islet samples** In total, 34 pancreases and/or isolated islet preparations from non-diabetic (18 men, 16 women; age  $57 \pm 18$  years; BMI  $24.7 \pm 2.7$  kg/m<sup>2</sup>) multiorgan donors and 16 pancreases and/or isolated islet preparations from type 2 diabetic (eight men, eight women; age  $60 \pm 8.0$  years; BMI  $26.1 \pm 2.5$  kg/m<sup>2</sup>) multiorgan donors were obtained, with approval of the local Ethics Committee.

**Immunocytochemistry** Pancreatic samples were taken from the neck of the gland (the remaining tissue being processed for islet isolation) and handled as described previously [19]. Primary antibodies were: guinea pig polyclonal anti-swine insulin (A0564; DAKO, Carpinteria, CA, USA); mouse monoclonal anti-human/mouse glucagon (MAB1249, clone 181402; R&D Systems, Abingdon, UK); rat monoclonal anti-somatostatin (ab30788; Abcam, Cambridge, UK); rabbit polyclonal anti-GLP-1 (ab22625; Abcam), which reacts with the mid to C terminal region of GLP-1 [1–19], enabling immunoreactivity with N terminal truncated and C terminally extended forms of GLP-1; mouse monoclonal anti-GLP-1 (HYB 147-06; BioPorto Diagnostic, Gentofte, Denmark), which is specific for the amidated

C-terminus of the peptide [20]; and rabbit polyclonal antibody to PC1/3 and PC2 (Chemicon, Rosemont, IL, USA). Secondary antibodies were: Alexa Fluor 488: goat anti-guinea pig IgG, goat anti-rabbit IgG, and goat anti-mouse IgG; and Alexa Fluor 594: goat anti-mouse IgG and goat anti-rabbit IgG (all from Invitrogen, Molecular Probes, Leiden, the Netherlands). NCI-H716 cells, a human L cell model [21], were obtained from American Type Culture Collection (Manassas, VA, USA) and used as GLP-1-like positive controls. Samples were analysed by laser confocal microscopy (TCS SP5; Leica Microsystems, Wetzlar, Germany), as previously reported [22].

**Islet isolation and culture** Islets were prepared by enzymatic digestion and gradient separation [22, 23], and suspended in M199 culture medium (Sigma Chemicals, St Louis, MO, USA) supplemented as detailed elsewhere [22, 23]. Islets with a diameter  $\geq 100$   $\mu\text{m}$  were handpicked and studied within 3 days of isolation.

**Immunoblotting and islet hormone content** For isolated islet western blotting, 30  $\mu\text{g}$  islet protein per lane were loaded to 4 to 20% (wt/vol.) gradient SDS-PAGE gels and subjected to electrophoresis. Proteins were then transferred to nitrocellulose membranes and probed for GLP-1 using a monoclonal anti-GLP-1 antibody (ab23472; Abcam) or  $\beta$ -actin (AC-15 antibody, A5441; Sigma). The anti-GLP-1 antibody reacts with the region corresponding to amino acids 7 to 36 of human GLP-1 and shows some cross-reactivity with GLP-1 precursors. Whole-cell lysate (A-431, SC-2201; Santa Cruz Biotechnologies, Santa Cruz, CA, USA) was used as positive control. Islet glucagon and GLP-1 content was measured from four non-diabetic and three type 2 diabetic preparations after overnight acid-ethanol (1.5% HCl, 79% ethanol [95% pure], 19.5% water) extraction [22, 23].

**Islet matrix-assisted laser desorption-ionisation-time of flight mass spectrometry** Similar to procedures previously described for other cellular models [24], 15  $\mu\text{g}$  islet protein per lane was loaded to 4 to 20% gradient SDS-PAGE gel and subjected to electrophoresis. After protein visualisation by Coomassie blue, gel bands were excised, de-stained and subjected to overnight in-gel digestion by trypsin (Sigma) at 37°C, without reduction and alkylation. Of the resulting peptides, 2  $\mu\text{l}$  was mixed with 2  $\mu\text{l}$  saturated di-hydroxybenzoic acid solution (Bruker Daltonics, Bremen, Germany) prepared in 50% acetonitrile and 50% water containing 0.1% (vol./vol.) trifluoroacetic acid. Peptide solution (1  $\mu\text{l}$ ) and di-hydroxybenzoic acid-saturated matrix were taken to a sample plate of the mass spectrometry analysis apparatus and allowed to dry. The crystallised samples were analysed using a matrix-assisted laser desorption-ionisation-time of flight (MALDI-TOF)/TOF system (Ultraflex

III; Bruker Daltonics), operating in reflector mode. Typically, 100 to 200 laser shots were summed into each mass spectrum. An external calibration of a standard peptide mixture (Bruker Daltonics) was used. For identification and determination of sequence coverage, the measured mono-isotopic  $m/z$  values were searched against the SWISSPROT database using MASCOT (Matrix Science, London, UK, [www.matrixscience.com](http://www.matrixscience.com), accessed 4 July 2012) and BIOTOOLS version 3.0 (Bruker Daltonics). Parameters of missed cleavage and peptide tolerance were set to 0 or 1 and 150 ppm, respectively.

**Preparation of alpha and beta cells** Methods previously developed and validated were used [25, 26]. The dissociation of islets into single-cell suspensions was achieved by incubation with constant agitation for 3 min at 37°C in 0.05% (wt/vol.) trypsin-EDTA (Invitrogen) supplemented with 3 mg/ml DNase I (Roche Diagnostics, Mannheim, Germany), followed by pipetting vigorously to complete dissociation. Labelling and sorting of alpha and beta cell fractions were performed by Newport Green labelling [27] followed by FACS, as described [25].

**Quantitative RT-PCR studies** The expression of genes encoding insulin, glucagon, PC1/3 and PC2 was assessed by quantitative RT-PCR experiments in alpha and beta cell fractions from eight non-diabetic and three type 2 diabetic islet preparations, according to methods detailed elsewhere [22, 23, 25]. Briefly, after RNA extraction and reverse transcription, mRNA levels of the genes of interest were quantified and normalised to  $\beta$ -actin in a bioanalyser (7700; Applied Biosystems, Foster City, CA, USA). The oligonucleotides were obtained from assay-on-demand gene expression products (Applied Biosystems).

**Functional studies** Non-diabetic islets were incubated for 60 min in KRB solution with 5.5 mmol/l glucose; then, after washing, the following conditions were tested (at 60 min incubation with KRB solution): 5.5 mmol/l glucose, 16.7 mmol/l glucose, and 5.5 mmol/l glucose plus 20 mmol/l arginine. Purified cell preparations were tested with 5.5 mmol/l glucose and 5.5 mmol/l glucose plus 20 mmol/l arginine. In addition, GLP-1 and glucagon release were measured from batches of 50 islets of similar size from non-diabetic and type 2 diabetic preparations, kept in M199 culture medium for 4 h. Finally, to test the bioactivity of islet-released GLP-1, experiments were performed similarly to methods previously reported [17, 18], by assessing insulin release from islets exposed to cell culture medium from untreated non-diabetic human islets; this was done at 11 mmol/l glucose, and in the absence or presence of the GLP-1 receptor antagonist, exendin(9–39) (0.5  $\mu\text{mol/l}$ ).

GLP-1 release was measured using a GLP-1 (active) RIA kit (LINCO, Saint Charles, MO, USA) and an ELISA kit (Millipore, Billerica, MA, USA). The antibodies used in the assays are specific for the biologically active 7–36 amide and 7–37 forms of GLP-1, and do not react with GLP-1(1–36)amide, GLP-1(1–37), GLP-1(9–36)amide or GLP-1(9–37). Glucagon levels were measured using a  $^{125}\text{I}$ -labelled glucagon radioimmunoassay kit (Millipore).

**Statistical analysis** Results are expressed as mean  $\pm$  SD. Data were compared by the two-tailed Student's *t* test for unpaired data. The ANOVA test followed by Bonferroni's correction was used when more than two groups were considered.

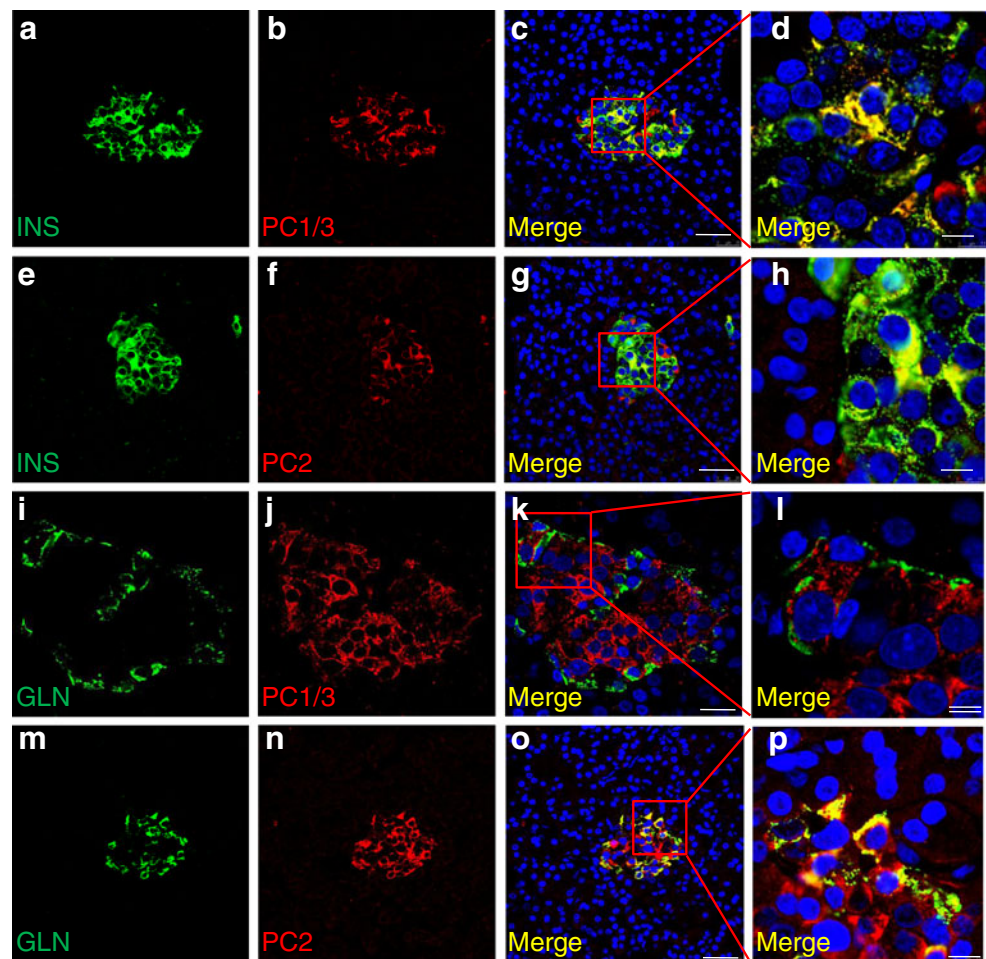
## Results

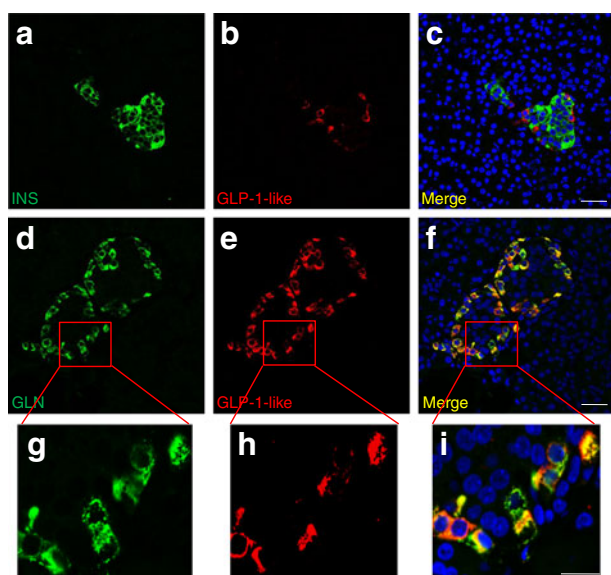
**Alpha cells have GLP-1-like and PC1/3 immunoreactivity** Unsurprisingly, insulin co-localised with PC1/3 (Fig. 1c, d) and PC2 (Fig. 1g, h). PC1/3 and PC2 cleave proinsulin at the B-chain/C-peptide and C-peptide/A-chain junctions, respectively [28]. Glucagon co-localised with PC2 (Fig. 1o, p) (PC2 cleaves proglucagon to produce glucagon [29]). However, a

few glucagon-positive cells also displayed PC1/3 immunoreactivity (Fig. 1k, l) (PC1/3 leads to the formation of GLP-1 from proglucagon [29]). Intriguingly, PC1/3 and glucagon were present in different intracellular alpha cell compartments (Fig. 1k, l). This was confirmed by superimposition analysis of glucagon and PC1/3 immunoreactivity (electronic supplementary material [ESM] Fig. 1a, b).

GLP-1-like immunoreactivity was detected by a polyclonal and a monoclonal anti-GLP-1 antibody reacting, respectively, with the mid to C terminal region of GLP-1 [1–19] and the amidated C-terminus of the peptide (see the Methods). These antibodies generated similar staining patterns with NCI-H716 cells and human islets (ESM Figs 2 and 3). We then examined cells that were double-positive for GLP-1-like and other hormone or PC immunoreactivity, observing superimposable results with both anti-GLP-1 antibodies. As a result, we decided to report only the data acquired with the monoclonal antibody. Figure 2a–c shows that no cells were double-positive for insulin and GLP-1-like immunoreactivity. Co-localisation of GLP-1-like and glucagon immunoreactivity was found in some cells (Fig. 2f, i); however, some glucagon-containing cells did not show GLP-1-like immunoreactivity (Fig. 2f, i).

**Fig. 1** Insulin, glucagon, PC1/3 and PC2 immunoreactivity in human pancreatic islet cells. Confocal microscopy analysis showed the presence of (b) PC1/3 and (f) PC2 in (a, e) insulin-containing (INS) cells, as indicated by merged images (c, g) and enlarged details (d, h). (n) PC2 positivity in glucagon-containing (GLN) cells (m), with merged images (o) and enlarged details (p). (i) A subset of glucagon-positive cells also produced PC1/3 (j), with merged images (k) and enlarged details (l). Squares (c, g, k, o) indicate the areas (d, h, l, p) shown at higher magnification. Scale bars (a–c, e–g, m–o) 30  $\mu\text{m}$ ; (i–k) 40  $\mu\text{m}$ ; (d, h, l, p) 8  $\mu\text{m}$





**Fig. 2** Insulin, glucagon and GLP-1-like immunoreactivity in human pancreatic islet cells. **(a)** Confocal microscopy analysis of insulin (INS), **(d, g)** glucagon (GLN) and **(b, e, h)** GLP-1-like immunoreactivity showed GLP-1-like positivity in the merged image of alpha cells **(f)** with enlarged detail **(i)**, but not in the merged image **(c)** of beta cells. Blue **(c, f, i)**, nuclear staining with Hoechst. Squares **(d, e, f)** indicate the areas **(g, h, i)** shown at higher magnification. Scale bars **(a–f)** 30  $\mu\text{m}$ ; **(g–i)** 15  $\mu\text{m}$

Accordingly, there were cells that were double-positive for GLP-1-like and PC1/3 or PC2 immunoreactivity (Fig. 3). GLP-1-like and PC1/3 positivity were localised in different sub-cellular compartments (Fig. 3c, d). Somatostatin-positive cells did not stain for GLP-1 or for PC1/3 (ESM Fig. 4). The main results of the confocal microscopy studies are summarised in Table 1.

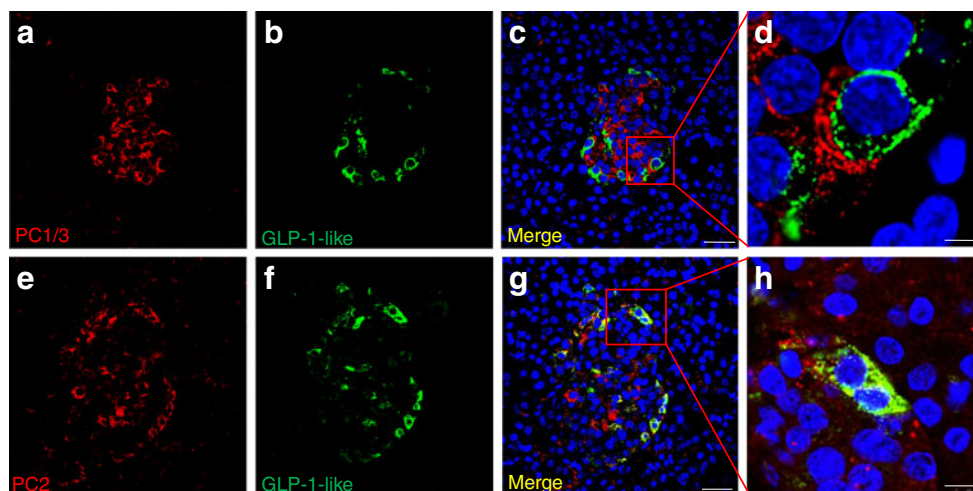
When alpha cells that were double-positive for glucagon and PC1/3 were counted, co-localisation was found in cells from 20 of 26 (76.9%) non-diabetic islets and from 21 of 26 (80.7%) type 2 diabetic islets; conversely, around 20 to 25% of the examined islets did not show glucagon and PC1/3 co-

localisation. Glucagon and PC1/3 double positivity was found in 139 of 573 alpha cells (24%) from non-diabetic islets and in 152 of 665 alpha cells (23%) from diabetic islets.

**Pancreatic islets contain GLP-1** Non-diabetic islets were used for GLP-1 immunoblotting experiments and showed a band corresponding to GLP-1 (molecular mass approximately 4 kDa), with the intensity varying among preparations (Fig. 4). A band corresponding to some GLP-1 precursors was also observed in some cases, due to partial cross-reactivity of the antibody used (see the Methods). The molecular mass of this band (10–12 kDa) is compatible with that of proglucagon-derived major proglucagon fragment. The bands of approximately 40 kDa, assigned as  $\beta$ -actin, and those of about 4 kDa, attributed to GLP-1 peptides, were submitted for identification. In both spectra, keratin and trypsin peak autolysis was subtracted. The experimental peak lists were compared with the SWISSPROT database using MASCOT. The 40 kDa band was identified as ACTB\_HUMAN (P60709) with a MASCOT score of 143, in agreement with the western blot data. Figure 5 shows the results for the 4 kDa band. In this case, the database search showed, among other species, the presence of glucagon (GLUC\_HUMAN, P01275) with a MASCOT score of 68; the sequence was covered by five peptides, starting from the amino acids in position 98 of pre-proglucagon. This corresponds to position 78 of proglucagon, known to be the position of the first amino acid of GLP-1(7–37) and GLP-1(7–36)amide. The corresponding spectra of the GLP-1(7–37) and GLP-1(7–36) tryptic sequences were matched with the putative digested sequences of the peptides using the sequence editor implemented on BIOTOOLS. GLP-1(7–37) and GLP-1(7–36) peptides were identified by the species at  $m/z$  1105 (aa 21–28) and at  $m/z$  2098 (aa 1–20).

The presence of GLP-1 in isolated islets was also confirmed by islet extraction measurements. Non-diabetic islets contained  $57.7 \pm 25.9$  pg/islet GLP-1 ( $n=4$ ), with a trend ( $p=0.5$ ) towards higher levels in the type 2 diabetic samples ( $72.3 \pm 19.0$  pg/

**Fig. 3** PC1/3, PC2 and GLP-1-like immunoreactivity in human pancreatic islet cells. Confocal microscopy analysis of PC1/3 **(a)**, PC2 **(e)** and GLP-1-like **(b, f)** immunoreactivity, showing cells double-positive for GLP-1-like and PC1/3 immunoreactivity **(c)**, with enlarged detail **(d)**, and **(g)** cells double-positive for GLP-1-like and PC2 immunoreactivity, also with enlarged detail **(h)**. Squares **(c, g)** indicate the areas shown at higher magnification **(d, h)**. Scale bars **(a–c, e–g)** 30  $\mu\text{m}$ ; **(d)** 5  $\mu\text{m}$  and **(h)** 8  $\mu\text{m}$



**Table 1** Summary of the results of the immunohistochemistry studies performed with human pancreatic islets

Variable	INS <sup>+</sup>	GLN <sup>+</sup>	GLP-1 <sup>+</sup>	PC1/3 <sup>+</sup>	PC2 <sup>+</sup>
INS <sup>+</sup>		No double positivity	No double positivity	Double positivity	Double positivity
GLN <sup>+</sup>	No double positivity		Double positivity	Subset with double positivity	Double positivity
GLP-1 <sup>+</sup>	No double positivity	Double positivity		Subset with double positivity	Double positivity

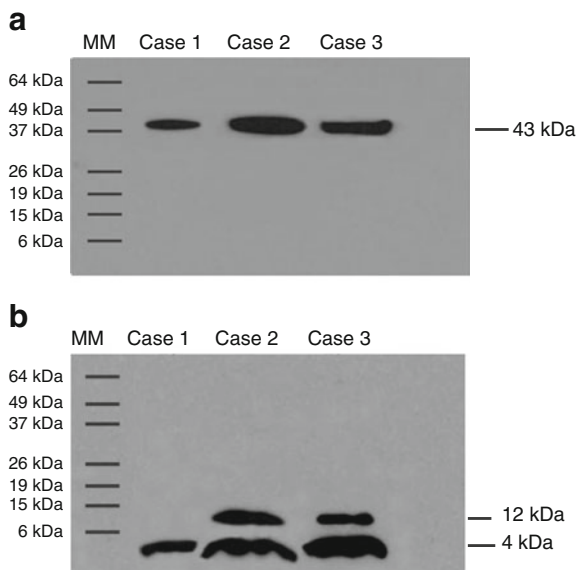
INS, insulin; GLN, glucagon; GLP-1, GLP-1-like

islet;  $n=3$ ). In the same preparations, the glucagon content was  $255\pm 119$  and  $273\pm 53$  pg/islet ( $p=0.8$ ), respectively.

**Alpha cells express PC1/3** Quantitative RT-PCR experiments showed that insulin gene expression in alpha cell preparations was less than 12% of glucagon gene expression, and that the expression of glucagon in beta cells was less than 9% of that of insulin ( $n=3$  preparations each). This is in line with protein abundance data previously reported by our group [26]. Beta cells expressed PC1/3 (also known as PCSK1) and PC2 (also known as PCSK2) (Fig. 6a), with PC2 expression tending to be lower (not statistically significant). In type 2 diabetic beta cells, PC1/3 and PC2 appeared to be upregulated, compared with non-diabetic samples (Fig. 6a). Alpha cells also expressed both PC genes (Fig. 6b); however, in non-diabetic alpha cells PC2 was upregulated compared with PC1/3, whereas in type 2 diabetic alpha cells PC1/3 expression was greater (Fig. 6b).

**Pancreatic islets release GLP-1** Glucagon release from non-diabetic islets decreased significantly at glucose concentrations that stimulate insulin release and increased by approximately twofold in response to arginine (Fig. 7a). In turn, GLP-1 secretion increased significantly in response to a high glucose and arginine challenge (Fig. 7a). In these three different conditions, the glucagon:GLP-1 molar ratio was approximately 4.7, 0.8 and 2.6, showing that the two hormones were released with some degree of independence of each other. Islets from type 2 diabetic donors tended to release more glucagon ( $0.72\pm 0.16$  vs  $0.55\pm 0.11$  pmol islet<sup>-1</sup>h<sup>-1</sup>) and secreted a significantly higher amount of GLP-1 ( $0.44\pm 0.11$  vs  $0.17\pm 0.10$  pmol islet<sup>-1</sup>h<sup>-1</sup>,  $p<0.01$ ) than non-diabetic islets (Fig. 7b). The respective glucagon:GLP-1 ratios were 1.6 and 3.2, suggesting that, at least in basal conditions, the diabetic islet phenotype is more oriented to the release of GLP-1. We also evaluated the bioactivity of islet cell-released GLP-1 by measuring insulin secretion from islets exposed to cell culture medium from non-diabetic human islets; this was done at 11 mmol/l glucose, and in the absence or presence of the GLP-1 receptor antagonist, exendin(9–39) ( $n=3$  experiments each). Figure 8 shows that the conditioned medium elicited a stronger insulin release in response to glucose, and that this was prevented by blocking GLP-1 receptors with exendin(9–39).

**Alpha cells release GLP-1** When GLP-1 release from non-diabetic alpha and beta cell preparations was studied, we found that glucagon and GLP-1 secretion from alpha cells increased significantly upon arginine stimulation (Fig. 9). The respective glucagon:GLP-1 secretion ratios were 3.1 (basal) and 6.8 (stimulated), again suggesting that the release of the two hormones was modulated differently under the tested conditions. Conversely, glucagon and GLP-1 secretion from purified beta cells was undetectable, with beta cell glucagon and GLP-1 content below the limits of detection of the assays used (not shown).

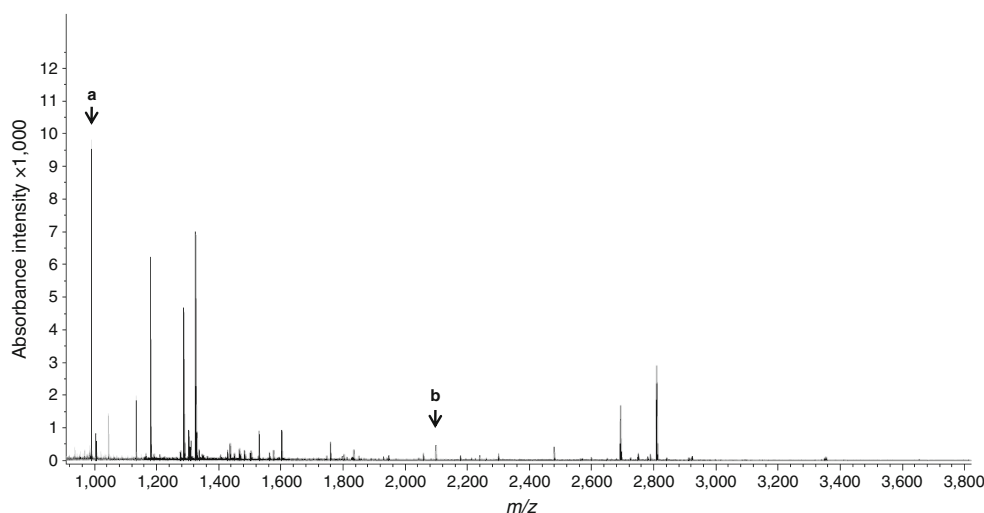


**Fig. 4** Western blot analysis of GLP-1 abundance in isolated human pancreatic islets. **(a)** β-Actin (approximately 43 kDa) was used as control. **(b)** Image showing the abundance of GLP-1 (approximately 4 kDa) and in two cases (case 2 and case 3) also the presence of a GLP-1 precursor (approximately 12 kDa). Findings for three different isolated human islet samples (case 1, case 2 and case 3) are shown. Molecular mass (MM) markers are indicated by black bars representing the different molecular mass values reported from nitrocellulose membrane loaded with Benchmark Pre-stained protein ladder (Invitrogen)

## Discussion

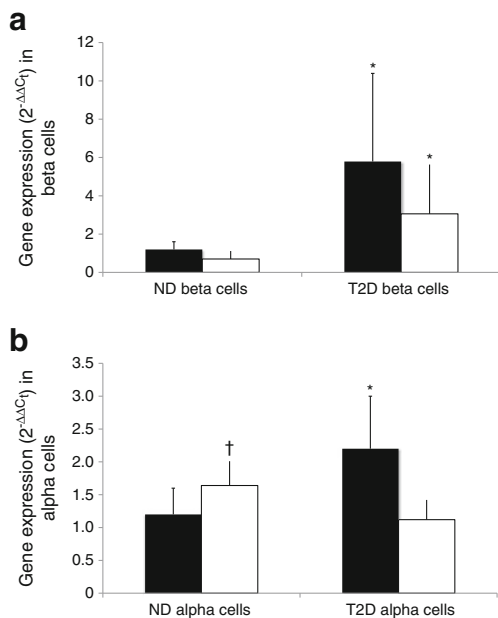
The present study shows that a local GLP-1 system is present in human pancreatic islets, residing in alpha cells, modulated by nutrients and affected by type 2 diabetes. GLP-1-like

**Fig. 5** MALDI mass spectrum of tryptic digestion of the 4 kDa band above (Fig. 4). The corresponding peaks matched to GLP-1 (7–36) and GLP-1 (7–37) partial sequences are marked by the downward arrows indicating peak value and length of amino acid sequences, i.e.: <sup>a</sup>1005.58 *m/z* (21–EFLAWLVK–28) and <sup>b</sup>2098.01 *m/z* (1–HAEGTFTSDVSSYLEGQAAK–20)

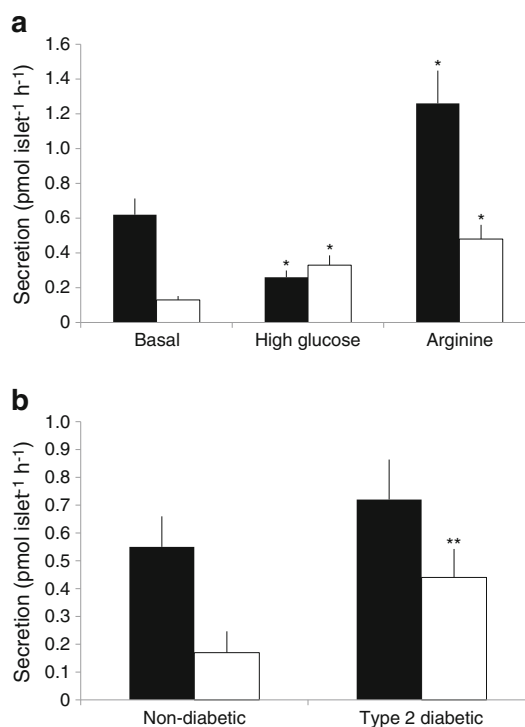


immunoreactivity was detected by immunohistochemistry, similarly to findings previously observed by others [10, 12, 17]. However, the complex processing of proglucagon by PC1/3 leads to several peptides, of which GLP-1(7–37) and GLP-1(7–36)amide are the bioactive GLP-1 forms [1–4]. Moreover, the different commercially or not commercially

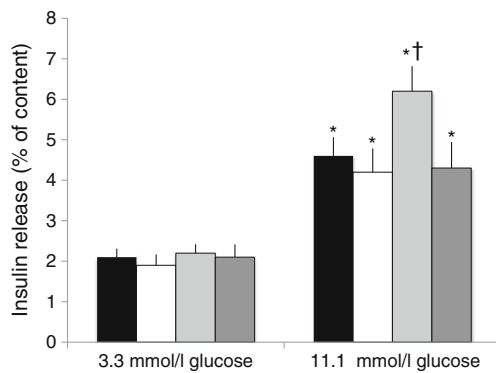
available [17] antibodies used by us and in previous studies have variable specificities. In addition, anti-glucagon antibodies may not specifically identify peptides derived from



**Fig. 6** (a) *PC1/3* and *PC2* expression in beta cell fractions from non-diabetic (ND) and type 2 diabetic (T2D) islets. Quantitative RT-PCR experiments revealed the expression of *PC1/3* (black bars) and *PC2* (white bars) in non-diabetic ( $n=8$ ) and type 2 diabetic ( $n=3$ ) beta cells, with higher expression in the latter.  $*p<0.05$  vs corresponding gene in non-diabetic. (b) *PC1/3* and *PC2* expression in alpha cell fractions from non-diabetic and type 2 diabetic islets. *PC2* (white bars) was expressed at higher levels than *PC1/3* in non-diabetic alpha cells, whereas *PC1/3* was upregulated in type 2 diabetic alpha cells, compared with non-diabetic samples.  $*p<0.05$  vs non-diabetic;  $†p<0.05$  vs non-diabetic *PC1/3*



**Fig. 7** (a) Glucagon and GLP-1 secretion from non-diabetic isolated human islets. Glucagon (black bars) and GLP-1 (white bars) secretion was measured in isolated islet incubation media as described. Conditions were: 5.5 mmol/l glucose (basal), 16.7 mmol/l glucose (high glucose) and 5.5 mmol/l glucose plus 20 mmol/l arginine (arginine). Glucagon release decreased in response to high glucose and increased in response to arginine; GLP-1 increased with high glucose and with arginine.  $*p<0.05$  vs the respective basal values (Bonferroni's correction). (b) Glucagon and GLP-1 secretion from non-diabetic and type 2 diabetic islets. Glucagon and GLP-1 secretion was measured in isolated islet incubation media as described, following incubation at 5.5 mmol/l glucose.  $**p<0.01$  vs non-diabetic (Student's *t* test)



**Fig. 8** Insulin release from non-diabetic islets exposed to unconditioned (black bars) or conditioned (light grey bars) (from islets previously incubated with 11.1 mmol/l glucose) medium without exendin (9–39) (an antagonist of GLP-1 receptors), or from islets exposed to unconditioned medium+exendin(9–39) (white bars) or to conditioned medium + exendin(9–39) (dark grey bars). Glucose-stimulated insulin release was higher than that at the basal (3.3 mmol/l glucose) condition, with a further significant potentiation by the conditioned medium, which was prevented by exendin(9–39). \* $p < 0.05$  vs the respective values at 3.3 mmol/l glucose (Student's  $t$  test); † $p < 0.05$  vs the other values at 11.1 mmol/l glucose (Bonferroni's correction)

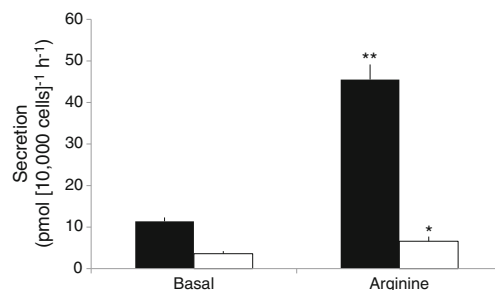
PC2 processing. Nevertheless, we did consistently find that the distribution of GLP-1 immunoreactivity follows that of glucagon in subsets of glucagon-containing cells, as observed by others [10, 12, 17]. In agreement with previous studies [30, 31], we also observed that a subset of alpha cells contained PC1/3, which cleaves proglucagon to bioactive GLP-1 [29]. Finally, no co-localisation of GLP-1-like and PC1/3 immunoreactivity with somatostatin was observed. Therefore, despite the specific arrangement of endocrine human islet cells, which may also surround each another [32], the current evidence shows that the immunoreactivity to different substances described above occurs in separate cells. In our experiments, GLP-1 and PC1/3 immunoreactivity appeared in different sub-cellular compartments. Whereas our data were unable to fully clarify this point, it cannot be ruled out that proglucagon might reach distinct compartments in the Golgi apparatus containing either PC2 (yielding glucagon) or PC1/3 (generating GLP-1).

Islet GLP-1 peptides were detected in the present study by immunoblotting, MALDI-TOF mass spectrometry and measurements in cell extracts. A band corresponding to 10 to 12 kDa molecular mass was observed by western blotting in some, but not all, islet preparations, possibly due to partial cross-reactivity of the antibody used. This suggests that islets may be heterogeneous in the way they process proglucagon to GLP-1, which is further confirmed by the observation that approximately 20 to 25% of islets did not show cells that were double-positive for glucagon and PC1/3. As for the precursor, it could be the major proglucagon fragment, which has a molecular mass compatible

with that of the observed band [1, 8, 28]. The presence of GLP-1 and an intermediate form was also found in rat pancreas [13].

In a seminal article [4], Holst et al reported that GLP-1 (1–37) or GLP-1(1–36)amide is present in pancreatic extracts from human and porcine pancreases. In our study, mass spectra generated by tryptic sequences of GLP-1 peptides contained partial segments ascribable to GLP-1(7–37) and GLP-1(7–36)amide. Although the amide form of GLP-1 (7–36) could not be identified (probably because the peptide containing arginine amidation that is generated by trypsin digestion has a very low mass [ $m/z$ ]), islet production of bioactive GLP-1 was confirmed by the GLP-1 receptor-dependent potentiation of glucose-stimulated insulin release, which was observed with medium deriving from previous islet cultures. This is in agreement with the observation that GLP-1 secreted from isolated human islets could indeed potentiate insulin release in vitro [18].

Our results show that glucose and arginine increased GLP-1 release. This supports the concept that GLP-1-secreting cells possess the machinery needed to recognise substrates and release the hormone [1–4, 8]. Accordingly, Whalley et al found that high glucose (25 mmol/l) could increase GLP-1 production in  $\alpha$ TC1-6 cells and rat islets exposed to streptozotocin [16]. Furthermore, Ellingsgaard et al reported that arginine increased GLP-1 secretion from human islets and that this was potentiated by interleukin-6 [18]. We, moreover, observed here that purified alpha cells express *PC1/3* and secrete GLP-1, and that this secretion is regulated by arginine. All this is in agreement with experimental evidence that alpha cells have variable functionality and can, indeed, produce GLP-1, as observed with the alpha cell line,  $\alpha$ TC-1-6, the glucagonoma MSL-G-AN cell line, primary rat alpha cells and primary human alpha cells [16, 18, 33, 34]. Human alpha cells can also produce glucose-dependent insulinotropic peptide, the



**Fig. 9** Glucagon and GLP-1 secretion from purified human alpha cells. Glucagon (black bars) and GLP-1 (open bars) secretion was measured in alpha cell incubation medium as described. Conditions were: 5.5 mmol/l glucose (basal) and 5.5 mmol/l glucose plus 20 mmol/l arginine (arginine). Both hormones increased significantly in response to arginine. \* $p < 0.05$  and \*\* $p < 0.01$  vs the respective basal values (Student's  $t$  test)



other major incretin [35]. Therefore, although cell fractions from isolated islets usually show some degree of contamination, as shown here and by others [25, 26], the available evidence suggests that alpha cells are the source of islet GLP-1.

However, the quantification of GLP-1 and glucagon release from human islet cells has yielded conflicting results. Some authors found that islet GLP-1 abundance was in the femtomolar range and that the hormone was not measurable in islet incubation medium [16]. However, Hansen et al were able to measure GLP-1 in islet culture medium (at 5.5 mmol/l glucose), with the amount again in the femtomolar range [17]. Another study [18] and we, here, have reported GLP-1 values in the picomolar range, suggesting intrinsic islet biological variability and/or differences in experimental conditions. In addition, in our study, glucagon release from isolated islets was similar to that reported by some other authors [36, 37], but several fold lower than that observed during perfusion experiments [38]. All this led, in our hands, to a glucagon/GLP-1 molar secretion ratio (basal condition) of approximately 5.7, similar to findings previously reported for  $\alpha$ TC1-6 cells [16]. In addition, when we measured glucagon and active GLP-1 in islet extracts, the values and proportions were close to those found by others [18, 37]. Interestingly, however, the bi-hormone secretion ratio with alpha cell fractions tended to decrease (approximate value of 3), suggesting that primary human alpha cells might rapidly change their phenotype. Accordingly, human alpha cells in culture released 50% more GLP-1 than glucagon, basally and upon stimulation [18]. We did not investigate the molecular mechanisms that cause alpha cells to modulate, to different degrees, their sensitivity to various stimuli, such that glucose, for example, causes reduced glucagon release and increased GLP-1 secretion. However, this seems to be a more general phenomenon, as previous authors have also observed that, with primary human alpha cells, incubation with interleukin 6 caused a twofold increase of GLP-1 secretion, while not affecting that of glucagon [18].

Somewhat surprisingly, we observed that GLP-1 release was higher from type 2 diabetic than from non-diabetic islets. We did not study the GLP-1 secretion of purified alpha cells from type 2 diabetic donors, due to the limited tissue availability. However, when we counted islet cells that were double-positive for glucagon and PC1/3, no major difference was observed between non-diabetic and type 2 diabetic samples. Instead, *PC1/3* gene expression was found to be higher in alpha cells from type 2 diabetic donors. This suggests functional variations in the two conditions, resembling what happens with glucagon, the release of which is altered in type 2 diabetic islets, despite similar amounts of glucagon-containing cells [38–40]. Although type 2 diabetic patients may release less GLP-1, which could contribute to

decreased incretin effects in this patient group [1, 41–43], it cannot be ruled out that the reduced concentrations of active GLP-1 in such patients might be partly due to increased DPP-4 activity. Increased plasma DPP-4 activity in type 2 diabetic patients has been reported [44], and chronic hyperglycaemia induced higher DPP-4 activity, with reductions of circulating active GLP-1 [45].

The increased GLP-1 production from type 2 diabetes islets could represent an attempt to protect beta cells in a condition tending to lead to beta cell dysfunction [46, 47]. In line with this concept, the activation of PC1/3 in alpha cells has been observed in mouse models of insulin resistance [34]. It has also been reported that, following beta cell injury, the biological function of alpha cells switches from glucagon to GLP-1 production, promoting beta cell function and survival [48]. In addition, adenovirus-mediated production of PC1/3 in mouse alpha cells increased islet GLP-1 secretion, with improved glucose-stimulated insulin secretion and enhanced survival after cytokine treatment [49], whereas the production of PC1/3, rather than PC2, in  $\alpha$ TC1-6 cells induced GLP-1 production and converted alpha cell function from hyperglycaemia-promoting to blood glucose-lowering after transplantation [50]. Finally, GLP-1 release from alpha cells is upregulated in the *Psammomys obesus* gerbil during the development of diabetes, which has been interpreted as an adaptive response to increased glucose levels [17]. This is in agreement with the findings of the present study, which show enhanced release of GLP-1 and a lower glucagon:GLP-1 secretion ratio in islets from type 2 diabetic donors.

Overall, these results support the view that a local and functionally competent GLP-1 system is present in human islets, residing in alpha cells and possibly modulated by type 2 diabetes. This suggests that alpha cells may release more than one single hormone, depending on the needs of the islet microenvironment at a given moment. These findings supplement current knowledge on the pathophysiology of incretins and may open new paths for their use as therapeutic tools.

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