Upregulation of alpha cell glucagon-like peptide 1 (GLP-1) in *Psammomys obesus*—an adaptive response to hyperglycaemia?

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Abstract

Aims/hypothesis The hormone glucagon-like peptide 1 (GLP-1) is released in response to a meal from the intestinal L-cells, where it is processed from proglucagon by the proconvertase (PC)1/3. In contrast, in the adult islets proglucagon is processed to glucagon by the PC2 enzyme. The aim of the study was to evaluate if, during the development of diabetes, alpha cells produce GLP-1 that, in turn, might trigger beta cell growth.

Methods Beta cell mass, GLP-1 and insulin levels were measured in the gerbil *Psammomys obesus* (*P. obesus*), a rodent model of nutritionally induced diabetes. Furthermore, the presence of biologically active forms of GLP-1 and PC1/3 in alpha cells was demonstrated by immunoflu-

orescence, and the release of GLP-1 from isolated *P. obesus*, mouse and human islets was investigated.

Results During the development of diabetes in *P. obesus*, a significant increase in GLP-1 was detected in the portal vein (9.8 ± 1.5 vs 4.3 ± 0.7 pmol/l, $p < 0.05$), and in pancreas extracts (11.4 ± 2.2 vs 5.1 ± 1.3 pmol/g tissue, $p < 0.05$). Freshly isolated islets from hyperglycaemic animals released more GLP-1 following 24 h culture than islets from control animals (28.2 ± 4.4 pmol/l vs 5.8 ± 2.4 , $p < 0.01$). GLP-1 release was increased from healthy *P. obesus* islets following culture in high glucose for 6 days (91 ± 9.1 pmol/l vs 28.8 ± 6.6 , $p < 0.01$). High levels of GLP-1 were also found to be released from human islets. PC1/3 colocalised weakly with alpha cells.

Conclusions/interpretation GLP-1 release from alpha cells is upregulated in *P. obesus* during the development of diabetes. A similar response is seen in islets exposed to high glucose, which supports the hypothesis that GLP-1 released from alpha cells promotes an increase in beta cell mass and function during metabolic challenge such as diabetes.

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Abbreviations

BCM	Beta cell mass
BHK	Baby hamster kidney
DPP-IV	Dipeptidyl peptidase 4
GLP-1	Glucagon-like peptide-1
GSIS	Glucose-stimulated insulin secretion
HED	High-energy diet
LE	Low-energy chow
LOCI	Luminescence oxygen channelling immunoassay

mAb	Monoclonal antibody
mBG	Morning blood glucose
PC	Prohormone convertase
P/S	Penicillin/streptomycin

Introduction

Conventional treatment of type 2 diabetes aims to control blood glucose levels through diet and exercise, drugs that target insulin secretion or resistance or by insulin injections. Despite intensive intervention therapies, the diabetic patient displays a continued decline in beta cell mass (BCM) and function [1]. The incretin hormone glucagon-like peptide (GLP)-1 is currently receiving considerable interest because of its ability not only to reduce blood glucose levels but also to potentially regenerate islet BCM [2]. Several in vitro [3, 4] and rodent in vivo studies [5–7] have demonstrated anti-apoptotic and proliferative effects of GLP-1 on pancreatic beta cells. Clearly, restoration of beta cell function will be of a great therapeutic value in the treatment of diabetes.

GLP-1 is a product of the pro-glucagon gene. The pro-glucagon precursor hormone is produced in intestinal L-cells, pancreatic alpha cells and a subset of neurons. The presence of different processing enzymes allows cell-specific products to be generated from the same precursor. In the L-cells, prohormone convertase (PC)1/3 is responsible for the generation of GLP-1 and GLP-2, whereas the alpha cells produce glucagon as a result of processing by PC 2, leaving the active form of the GLP-1 peptide trapped in a larger fragment, the major proglucagon fragment [8, 9]. After GLP-1 is released from the intestinal L-cells to the circulation, it is very rapidly degraded to an inactive metabolite (GLP-1 [9–37]) by the enzyme dipeptidyl peptidase 4 (DPP-IV) and only a small fraction reaches the pancreatic beta cells that express the GLP-1 receptor [10, 11].

The prevailing view is that in adults, all circulating GLP-1 is produced by the intestinal L-cells, with no contribution from the alpha cells as they are thought not to produce PC1/3, thus excluding GLP-1 production. However, several reports have demonstrated the production of a small amount of fully processed GLP-1 from alpha cells [12–15]. Furthermore, during specific stages of fetal development glucagon-positive cells in the pancreas co-produce PC1/3 and hence potentially also secrete GLP-1, which may be of importance during islet cell differentiation [16, 17]. These findings raise the possibility that GLP-1, produced within the islets under certain circumstances, acts as a paracrine factor facilitating beta cell protection and function as well as regeneration when needed. The alpha cells are in close proximity to the GLP-1 receptors on the beta cells, and alpha cells may, if GLP-1 production is switched on, provide a continuous beneficial supply of GLP-1 to the

neighbouring beta cells. In support of this view it has recently been shown that rats with streptozotocin-induced beta cell loss display increased islet GLP-1 production [18, 19]. Recently it was elegantly shown by adenovirus introduction of PC1/3 in islet glucagon cells that GLP-1 can be released and act locally to improve islet function [15].

The gerbil *Psammomys obesus* is an animal model of nutritionally induced type 2 diabetes [20–22]. When these animals are transferred from their natural low-energy saltbush diet to more energy-dense rodent laboratory chow, the initial response is an increase in BCM to compensate for the increased insulin demand, followed by development of hyperglycaemia. This animal model is characterised by fast depletion of insulin stores as hyperglycaemia becomes manifest [23, 24]. Not all animals become hyperglycaemic as some are resistant to the high-energy diet and stay normoglycaemic (non-responders). In this paper, we used *P. obesus* to investigate the hypothesis that production of GLP-1 within the islets serves as a natural mechanism employed by the islets to promote beta cell function and increase BCM under situations of sustained metabolic pressure, such as hyperglycaemia. The findings are supported by in vitro studies using isolated *P. obesus*, human and mouse islets.

Methods

Animals Male and female *P. obesus*, aged 7–9 weeks (Harlan, Jerusalem, Israel) were fed low-energy chow (LE 10 kJ/g) until the age of 14–15 weeks, when all animals except for an ‘LE’ group, were transferred to an ad libitum high-energy diet (HED 13 kJ/g). Body weight (BW), morning blood glucose (mBG) and HbA_{1c} were measured once weekly. After 4–5 weeks, animals were killed and based on mBG the animals were categorised as responders (mBG ≥ 10 mmol/l) or non-responders (mBG < 10 mmol/l). This experimental protocol was repeated in order to obtain pancreases for characterisation of insulin content and BCM as described below. The study was approved by the Animal Experiments Inspectorate, Ministry of Justice, Denmark.

mBG Blood samples were collected from the tail and mBG was measured as previously described by Vedtofte et al. [25].

Measurement of insulin and GLP-1 in *P. obesus* plasma Blood was collected from the aorta and vena porta when the animals were killed and the plasma was stored at –80°C until analysis of insulin and GLP-1 was carried out.

Detection of insulin was by luminescence oxygen channeling immunoassay (LOCI) using two monoclonal antibodies (mAbs; mAb HUI-018 and mAb OXI005-03B) that recognise two different epitopes. The principle of the LOCI method is described in the electronic supplementary material (ESM).

Intact GLP-1 was detected with a method similar to that carried out for insulin determination by LOCI. However, two different mAbs were used: acceptor beads were conjugated with mAbF5 (which recognises amino acids 25–33 of GLP-1 [7–37]) and for biotinylation, mAb26.1 (which recognises amino acids 7–8 of GLP-1 [7–37]).

Measurements of GLP-1 and insulin in culture medium or KRB GLP-1 from *P. obesus* islets in culture medium and GLP-1 from mouse and human islets in KRB were measured as described above, with the difference that the calibrators and controls were prepared in the corresponding matrix. Human insulin levels in KRB were measured by LOCI as described above with the following modifications: acceptor beads were conjugated with mAb RDI-TRK2IP10-D6C4, and mAb RDI-TRK2IP10-D3E7 was used for biotinylation. Calibrators and controls were prepared in the corresponding matrix.

Measurement of pancreatic GLP-1 and insulin content in *P. obesus* Pancreases were removed from a subset of animals, weighed and immediately frozen in liquid nitrogen. For peptide extraction the tissue was dissolved in 1% (vol./vol.) trifluoroacetic acid and peptide material was isolated from using Sep-Pak C18 cartridges (Waters, Milford, MA, USA). Bound material was eluted with 70% (vol./vol.) ethanol and 0.1% (vol./vol.) trifluoroacetic acid, and lyophilised. Lyophilised material was dissolved phosphate buffered saline (+ albumin 0.1% [wt/vol.]) and the content of GLP-1 and insulin was measured by radioimmunoassay. Carboxy-terminal GLP-1 immunoreactivity was determined using antiserum 89390 [26], which has an absolute requirement for the intact amidated carboxy terminus of GLP-1 7–36 amide and has cross-reactivity of less than 0.01% with fragments truncated at the carboxy terminus and 89% with GLP-1 9–36 amide, the primary metabolite of DPP4-mediated degradation. Sensitivity was below 5 pmol/l, and the intra-assay coefficient of variation was below 10% [26]. Insulin was measured as previously described by Brand et al. [27].

cAMP measurements Cells were harvested from a stable transfected baby hamster kidney (BHK) cell line producing the human GLP-1 receptor or the human glucagon receptor, and were stimulated with GLP-1, glucagon or islet culture medium. The response of GLP-1-, glucagon- or islet-medium-induced cAMP production was monitored using the FlashPlate Assay from PerkinElmer (Skovlunde, Denmark). The basic principle of the FlashPlate Assay is a competition between radioactive and non-radioactive cAMP for a fixed number of binding sites. Islet medium was incubated overnight at 4°C with or without a high-affinity glucagon mAb [27] or GLP-1 mAb (Mab26.1) prior to the cAMP assay.

Islet isolation Islets were isolated from mice and *P. obesus* as described in the [ESM](#). Human islets were obtained from Prodo Laboratories (Irvine, CA, USA). From one donor, a 43-year-old man, the islet purity was 75% and islet viability was 85%. From another donor, a 60-year-old man, the islet purity was 70% and islet viability was 95%. The study conformed to the ethical guidelines of Novo Nordisk for the use of human biological material. At arrival the islets were incubated free-floating in Petri dishes in RPMI 1640 medium (5.5 mmol/l glucose) supplemented with 5% (vol./vol.) human serum (Lonza, Basel, Switzerland) and 1% (vol./vol.) penicillin/streptomycin (P/S) and the perfusion experiment was performed the day after arrival.

In vitro islet studies Freshly isolated *P. obesus* islets were handpicked and incubated in extracellular-matrix-coated 96 well plates (Biological Industries, Kibbutz Beit Haemek, Israel) with 10–15 islets/well in 150 µl RPMI 1640 (Invitrogen, Carlsbad, California) supplemented with 1% (vol/vol) penicillin/streptomycin, 10% (vol/vol) newborn calf serum and D-glucose at varying concentrations. The islets were incubated in a 5% CO₂ humidified atmosphere at 37°C. After incubation the buffer was carefully removed from the islets and filtered through a 96 well filter plate (Multiscreen-DV from Millipore, Billerica, MA, USA) and stored at –20°C until analysis.

Mouse and human islets were placed in 96 well plates with 25 islets per well. The mouse islets were placed in the same medium as the *P. obesus* islets. Human islets were placed in RPMI 1640 (5.5 mmol/l glucose) supplemented with 5% (vol./vol.) human serum (Lonza) and 1% (vol./vol.) P/S. After 3 days, the islets were washed and incubated with 200 µl KRB (115 mmol/l NaCl, 4.7 mmol/l KCl, 2.6 mmol/l CaCl₂, 1.2 mmol/l KH₂PO₄, 1.2 mmol/l MgSO₄, 10 mmol/l HEPES, 0.2% (wt/vol.) BSA, 2 mmol/l glutamine, 5 mmol/l NaHCO₃, 1% (vol./vol.) P/S, pH 7.4) for 1 h. After incubation the buffer was carefully removed from the islets and filtered through a 96-well filter plate and stored at –20°C until analysis.

Islet perfusion Islets were washed in KRB and subsequently used in an islet perfusion experiment (Suprafusion 2500, Brandel, Lyngø, Denmark). Four columns each with 40 islets were used. The islets were placed in Bio-gel P2 (BioRad, Copenhagen, Denmark) in the columns with a nylon mesh below and above the gel. The islets were initially perfused for 40 min with KRB containing 3 mmol/l glucose to obtain a stable baseline of insulin release. After 40 min the buffer was exchanged with 15 mmol/l glucose±0.5 µmol/l exendin (9–39) for 70 min (40–110 min). Finally, the buffer was exchanged to low glucose (3 mmol/l) for 30 min (110–

140 min). The buffer was set to flow through the perfusion system at a rate of 0.3 ml/min. Samples were taken every 5 min from time 30 to 140 min. Samples were stored at -20°C and subsequently analysed for insulin.

Immunohistochemistry Human pancreatic sections from weight- and age-matched healthy controls and from patients with type 2 diabetes ($n=7$, respectively) were obtained from the National Disease Research Interchange. The method for staining the pancreas sections is described in the [ESM](#).

Pancreas morphometry The pancreases of a subset of the animals were examined histologically, as previously described by Raun et al. [28]. Briefly, pancreases were fixed in paraformaldehyde and sectioned according to a design-based stereological sampling scheme, the smooth fractionator method [29]. The sections were stained for insulin to visualise beta cells. BCM was evaluated stereologically in two to three sections with the origin of the sections blinded to the observer. The insulin staining intensity was graded semi-quantitatively on a scale of 0–5, with 0 being equal to islets characterised by absence of insulin staining and 5 equalling islets with insulin staining as seen in LE-fed *P. obesus* and in normal rat pancreas. The grading was carried out with the origin of the sections blinded to the observer.

Statistical methods All data are expressed as mean $\pm$ SEM. Student's *t* test was used for comparison of two groups. A value of $p<0.05$ was considered statistically significant.

Results

Development of hyperglycaemia in HED-fed *P. obesus* When transferred to the HED, about two-thirds of the animals ($n=26$) became hyperglycaemic (mBG 15.5 ± 0.7 mmol/l) after 4 weeks. One-third of the animals ($n=$

15) were resistant to the HED and stayed normoglycaemic (mBG 5.7 ± 0.8 mmol/l; Fig. 1a).

BCM, insulin staining intensity, and insulin and GLP-1 content of *P. obesus* pancreases at death After 4 weeks of HED, BCM was increased in responders (70 ± 9 mg/kg, $n=26$, $p<0.05$) and non-responders (87 ± 10 mg/kg, $n=19$, $p<0.01$), compared with animals on the LE diet (35 ± 7 mg/kg, $n=10$; Fig. 1b). The intensity of the insulin staining decreased in the hyperglycaemic responders (1.1 ± 0.1 , $p<0.001$) and non-responders (2.6 ± 0.3 , $p<0.001$), as compared with LE-fed animals (5.0 ± 0.0 ; Fig. 1c). Pancreatic insulin content was higher in the non-responders compared with the hyperglycaemic animals (3.4 ± 0.5 nmol/g tissue [$n=9$] vs 1.4 ± 0.3 nmol/g tissue [$n=16$], $p<0.01$) but was not significantly different from the LE group (2.1 ± 0.2 nmol/g tissue [$n=6$]; Fig. 2a).

Portal vein levels of insulin were significantly higher in both the hyperglycaemic animals (5.5 ± 0.6 nmol/l [$n=19$], $p<0.001$) and in the non-responders (5.7 ± 0.9 nmol/l [$n=10$], $p<0.001$) compared with control LE animals (0.2 ± 0.05 nmol/l [$n=9$]; Fig. 2b).

Analysis of the level of amidated GLP-1 in pancreatic extracts revealed a significant increase in the hyperglycaemic animals after 4 weeks on HED compared with LE-fed control animals (11.4 ± 2.2 pmol/g tissue [$n=9$] vs 5.1 ± 1.3 pmol/g tissue [$n=9$], $p<0.05$; Fig. 2c). The pancreas content of intact GLP-1 in the non-responders was not significantly different from the LE controls (4.6 ± 1.1 pmol/g tissue [$n=7$]). The increased GLP-1 level in the pancreases of hyperglycaemic animals was reflected in the portal vein plasma concentration, for which intact GLP-1 was also significantly higher in the hyperglycaemic group (9.8 ± 1.5 pmol/l, $p<0.05$ [$n=30$]) compared with the LE control group (4.3 ± 0.3 pmol/l [$n=14$]), while GLP-1 levels in the non-responders (5.4 ± 0.8 pmol/l [$n=20$]) were not different from LE controls (Fig. 2d). In contrast, for all animal groups plasma levels of intact GLP-1 in the aorta were below or

Fig. 1 Diabetes development in *P. obesus*. **a** Blood glucose in *P. obesus* on the HED. Responders (white circles; $n=26$); non-responders (black circles; $n=15$). **b** BCM in *P. obesus* after 4 weeks on the HED: non-responder, $n=19$; hyperglycaemic, $n=26$. Values for

LE-fed controls ($n=10$) are also shown. Non-resp, non-responder; hyperglyc, hyperglycaemic. ** $p<0.01$ compared with control. **c** Insulin score for the same animals as in **b**. *** $p<0.001$ compared with control

Fig. 2 *P. obesus* plasma levels and pancreas content of insulin and active GLP-1 (**a,c**) Pancreas content of (**a**) insulin LE-fed control animals ($n=6$), non-responders ($n=9$) and hyperglycaemic ($n=16$) animals and (**c**) GLP-1 amide of LE-fed control animals ($n=9$), non-responders ($n=7$) and hyperglycaemic ($n=9$) animals. (**b, d**) Vena porta plasma levels of (**b**) insulin LE-fed control animals ($n=9$), non-responders ($n=10$) and hyperglycaemic ($n=19$) animals and (**d**) intact GLP-1 from *P. obesus* after 4 weeks on the HED (non-responder, $n=20$; hyperglycaemic, $n=30$) or LE diet (control, $n=14$). Non-resp, non-responder; hyperglyc, hyperglycaemic. $^*p<0.05$; $^{***}p<0.001$ compared with control. $^{††}p<0.01$ relative to hyperglycaemic animals

only slightly above the quantification limit of the assay (0.7 pmol/l; data not shown).

GLP-1 release from *P. obesus* islets To characterise the GLP-1 release further, accumulated GLP-1 release was analysed over 24 h in freshly isolated islets. Islets from hyperglycaemic animals released significantly more GLP-1 than islets from control animals (28.2 ± 4.4 pmol/l vs 5.8 ± 2.4 , $p<0.01$; Fig. 3a).

Glucose-induced GLP-1 release from *P. obesus* islets As diabetes is characterised by hyperglycaemia, we investigated if GLP-1 production by *P. obesus* islets could be increased by chronically elevated glucose levels in vitro. Incubation of control islets, isolated from *P. obesus* on the LE diet, for 24 h in 3, 12 or 25 mmol/l glucose did not lead to glucose-dependent changes in GLP-1 release (Fig. 3b). However, after 6 days of incubation a significant dose-dependent increase in GLP-1 release during the last 24 h of the 6 day culture period was observed (29 ± 7 ; 60 ± 6 [$p<0.05$] and 91 ± 9 pmol/l [$p<0.01$] for 3, 12 and 25 mmol/l glucose, respectively [$n=3$]; Fig. 3b).

GLP-1 release from human and mouse islets To establish if GLP-1 release could also be induced in human islets, isolated islets (pre-cultured in medium containing 5.5 mmol/l glucose for 3 days) were incubated for 1 h in KRB. A considerable amount of GLP-1 (72 ± 3 fmol islet $^{-1}$ /h $^{-1}\times100$ [$n=4$]) was measured, whereas the release from mouse islets was very low

(3.0 ± 2.5 fmol islet $^{-1}$ /h $^{-1}\times100$ [$n=3$]; Fig. 4a). The functional significance of the GLP-1 released from human islets was assessed in an islet perfusion experiment. Addition of the GLP-1 receptor antagonist exendin (9–39) markedly (54%) reduced the glucose-stimulated insulin secretion (GSIS) in these islets (insulin AUC 30 ± 9 vs 65 ± 4 ng/100 islets, $p<0.05$; Fig. 4b, c). Data are shown from one experiment out of two experiments from two donors yielding similar results (52% reduction for donor two). The results indicate that the GLP-1 receptor is active in these islets.

Bioactivity of GLP-1 released from *P. obesus* islets Next we evaluated the bioactivity of the GLP-1 released from the *P. obesus* islets. Incubation of GLP-1 receptor-producing BHK cells with supernatant fraction from *P. obesus* islets cultured for 4 days in 25 mmol/l glucose-activated cAMP production to the same level as treatment of the cells with 100 pmol/l synthetic GLP-1 peptide (Fig. 5a). This receptor activation was, to a large extent, blocked by prior incubation of the islet medium with an antibody directed to the NH $_2$ -terminus of GLP-1 (mAb26.1; Fig. 5a). It is known that glucagon can activate the GLP-1 receptor with low potency ($EC_{50}\approx1$ nmol/l) [30]. Islet culture supernatant fractions contain glucagon released from alpha cells that could be responsible for activation of the GLP-1 receptor in this assay. In order to demonstrate that active GLP-1 and not glucagon was responsible for the production of cAMP, the GLP-1 receptor activation experiment was also performed in the presence of a high-affinity glucagon mAb [27]. The high-affinity glucagon mAb had no effect on the GLP-1 receptor activation by the islet medium (Fig. 5a). It did, however, completely inhibit the glucagon receptor activation (Fig. 5b). Addition of islet supernatant fraction to

Fig. 3 Release of intact GLP-1 from *P. obesus* islets in vitro. **a** Release of intact GLP-1 from freshly isolated *P. obesus* islets from control animals on the LE diet and from hyperglycaemic (hyperglyc) animals on HED over a 24 h incubation. Data represent islets isolated from four control animals and from six hyperglycaemic animals. **b** Glucose-induced release of GLP-1 from islets from *P. obesus* on the LE diet cultured for 24 h (black bars) or 6 days (white bars) at 3, 12 or 25 mmol/l glucose. Medium accumulated over 24 h; for the 6 day incubation the medium was changed between days 5 and 6. Data are mean $\pm$ SEM of three independent experiments. $^*p<0.05$; $^{**}p<0.01$ compared with control

Fig. 4 GLP-1 release from mouse and human islets. **a** Intact GLP-1 released into KRB in a 1 h incubation (human data are mean±SEM of four independent experiments representing four donors). Mouse islets, $n=3$. **b** Total insulin secreted (insulin AUC) in perfusion experiment shown in **c**. **c** Islet perfusion experiment on human islets. Effect of GLP-1 receptor antagonist exendin (9–39) on GSIS. Black circles, glucose at: (1) 3 mmol/l; (2) 15 mmol/l; and (3) 3 mmol/l. White circles, glucose at: (1) 3 mmol/l; (2) 15 mmol/l+exendin (9–39); and (3) 3 mmol/l. Data in **b** and **c** are from an experiment using islets from one donor with each condition run in quadruplicate (represented by error bars). The experiment was repeated on islets from a second donor, yielding similar results

non-transfected BHK cells resulted in no induction of cAMP production (Fig. 5c). This confirmed that GLP-1 released from the islets is an active form of the peptide.

Expression of GLP-1 in the pancreas Using antisera specific to the N-terminus of the active form of GLP-1 as well as antisera specific for the 7–36 amide form of the GLP-1 peptide, we performed immunocytochemistry to examine the localisation of GLP-1 in the islets. In most glucagon immunoreactive cells, both the C-terminal (amidated) and the N-terminal immunoreactivity of GLP-1 were detectable in control and hyperglycaemic *P. obesus* (Fig. 6a, b) and the merge is shown by a pinkish colour. There was no colocalisation of GLP-1 amide and insulin. Also in human pancreas, there was a strong colocalisation of GLP-1 amide, N-terminally derived biologically active GLP-1 and glucagon in sections from both controls and patients with type 2 diabetes and no colocalisation with insulin (Fig. 6c, d).

PC2 and PC1/3 are produced in alpha cells of hyperglycaemic *P. obesus* and of patients with type 2 diabetes To further explore the production of GLP-1 in the islets and to establish whether changes in the processing enzymes responsible for processing proglucagon to glucagon (PC2) or GLP-1 (PC1/3) were changed in hyperglycaemic vs control

islets from *P. obesus* as well as in islets from humans, histochemical analyses were performed. Pancreas sections from *P. obesus* and humans were stained for insulin, glucagon and PC1/3. The beta cells stained strongly for PC1/3 while the majority of alpha cells were weakly positive for PC1/3, and the surrounding exocrine pancreas showed no immunoreactivity (Fig. 6e–h). As seen in Fig. 6f, h the diabetic state results in a very different staining pattern. In hyperglycaemic *P. obesus*, nearly all the alpha cells now stained more intensely for PC1/3 (Fig. 6f) and some beta cells lost immunoreactivity. In sections from humans with type 2 diabetes, either a low level was observed, but not in every glucagon immunoreactive cell, or the staining was not much different from the non-diabetic state (Fig. 6g, h).

PC2 staining (co-staining with glucagon and insulin) of human and *P. obesus* normal and diabetic pancreas tissue is shown in ESM Fig. 1. In LE-fed *P. obesus* pancreas, PC2 was colocalised with both insulin and glucagon, while in the hyperglycaemic state, PC2 was only found in the alpha cells. This contrasts with the human pancreas, where PC2 was found in both insulin and glucagon cells in pancreases from control and type 2 diabetes patients.

Fig. 5 Bioactivity of GLP-1 released by islets into medium. **a** cAMP production by activation of the GLP-1 receptor expressed in BHK cells by islet medium with and without pre-incubation with glucagon antibody or GLP-1 antibody; 100 pmol/l GLP-1 serves as positive control **b** cAMP production by activation of the glucagon receptor produced in BHK cells by islet medium with and without pre-incubation with glucagon antibody; 100 pmol/l glucagon serves as positive control **c** cAMP production by non-transfected BHK cells stimulated with islet medium. The data shown are mean±SEM from a single representative experiment performed in duplicate. Glu Ab, high-affinity glucagon antibody

Fig. 6 Pattern of glucagon, insulin and PC1/3- and biologically active forms of GLP-1 in pancreas. **a–d** Colocalisation of glucagon with GLP-1 amide (**a**, **c**) and N-terminal GLP-1 (**b**, **d**) in normal and hyperglycaemic *P. obesus* and human pancreas. Nearly all glucagon cells are co-immunoreactive as denoted by a pink-purple colour. **e–h** Colocalisation of PC1/3 in insulin and some glucagon immunoreactive cells in normal and diabetic *P. obesus* and human pancreas. The white arrow marks glucagon-positive cells producing PC1/3. Scale bars 20 μ m. N-Term, N-terminal

Discussion

We have provided evidence that pancreatic islets from *P. obesus* produce fully processed GLP-1 during the development of hyperglycaemia and that this production can also be induced in vitro following culture of *P. obesus* islets in high glucose. Pancreatic alpha cells from patients with type 2 diabetes and *P. obesus* stained positive for GLP-1, and

human islets released GLP-1 in vitro. Based on our observations we hypothesise that this paracrine GLP-1 system may play a role in adult islets in regulating BCM and function when metabolic stress is imposed on the beta cells e.g. during the development of diabetes.

The pancreatic beta cells are located quite distantly from the GLP-1 producing L-cells in the intestine. It is well established that only a fraction of the intestinal GLP-1

reaches the beta cell via the circulation because GLP-1 is exposed to inactivation by DPP-IV immediately after its release [10, 11]. Bearing in mind this rapid decline in the peripheral GLP-1 signal arising from the L-cells, it is tempting to hypothesise the existence of a local GLP-1 signalling system in the islets close to the beta cell GLP-1 receptors. Indeed, proglucagon and PC1/3 colocalise in the pancreas at certain fetal stages, suggesting an important and transient role of GLP-1 during pancreas development [16, 18]. In the present study, we have shown also that the adult islet harbours a local GLP-1 signalling system, as has also been suggested by other studies [13]. Interestingly, a paracrine system involving GLP-1 was recently reported where GLP-1 produced locally by taste cells and acting via GLP-1 receptors produced on nearby afferent nerve fibres, serves to modulate taste sensitivity [31]. To what extent GLP-1 produced by the alpha cells will reach the beta cells is controversial, based on the proposed circulation from beta to alpha cells [32]. Nevertheless, it has recently been shown that islets in which PC1/3 production is enhanced by gene transfer display improved GSIS and are less sensitive to cytokine treatment. When transplanted to a mouse model of type 1 diabetes the GLP-1-producing islets perform better than normal islets [15]. Also, cell therapy with PC1/3-producing alpha cells improves glucose handling in *db/db* mice [33].

The mechanism leading to upregulation of GLP-1 from alpha cells needs to be determined. One possibility is increased production or activity of PC1/3. Indeed it has been shown that PC1/3 is upregulated by high glucose in alpha cells [34] and in hyperglycaemic *db/db* mice [17]. Therefore, it is possible that this is also the mechanism in our studies. Our data did show heterogeneity of PC1/3 production with mainly low but detectable levels in glucagon immunoreactive cells. Using two different antisera, we were able to demonstrate N-terminally biologically active GLP-1 as well as amidated GLP-1, both of which result from PC1/3 cleavage of proglucagon, providing further evidence of PC1/3 activity in alpha cells. Interestingly, fully processed GLP-1 can be detected in the plasma of humans with PC1/3 deficiency [35] and this finding emphasises that there are some aspects of proglucagon processing that are yet to be understood. As an alternative possibility there could be an inherent presence of PC1/3 in alpha cells which, during upregulation of transcription and translation of proglucagon, would lead to increased levels of GLP-1 accompanied by increased levels of circulating glucagon. In the present study, however, we did not see an increase in circulating glucagon (data not shown) accompanying the increase in GLP-1 in *P. obesus*.

When *P. obesus* were fed a HED the BCM increased but the insulin content of the pancreases of non-responders was significantly higher than in hyperglycaemic *P. obesus*, and

so was the intensity of insulin staining, although there was no difference in BCM. The reason for this could be that BCM is a measure of the volume of the area of the endocrine pancreas that has any positive staining for insulin, and is not related to the quantity of insulin in the cells. If there is an expansion of the islet area with peripheral weak insulin staining, this will be reflected in an increased BCM but not indicative of the level of functionality. Insulin content of the pancreas and intensity of insulin immunoreactivity could be a better estimate for a functional BCM. We also saw the same level of increase of insulin in vena porta vessels from non-responders and hyperglycaemic animals compared with the LE controls, despite profound differences in mBG levels. This suggests that the responders are not able to compensate for elevated mBG by increasing insulin secretion, probably because of an inadequate functional BCM [25].

The marked inhibition by exendin (9–39) on GSIS in the isolated human islets demonstrates that the GLP-1 receptors in these islets are already active. This could be explained by constitutively active receptors [36] or by an adequate amount of GLP-1 present locally to activate the receptor. The level of active GLP-1 measured in the islet medium of 1 h incubations as well as the blunting of the GSIS by the GLP-1R antagonist exendin (9–39) favours the latter possibility. In addition, in similar experimental setups we failed to see the expected increment in insulin release by addition of exogenous GLP-1 (data not shown). So why is alpha cell GLP-1 production switched on in healthy human islets? There could be several reasons for this. For example, they may have experienced metabolic stress, such as hypoxia before or during the isolation procedure, shipment or subsequent culture before the experiments were undertaken. Further studies are needed to explore whether GLP-1 secretion from alpha cells in humans is a real phenomenon under normal or diabetic conditions and to what extent failure in this process could be involved in the pathogenesis of diabetes.

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