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Proglucagon-Derived Peptides Do Not Significantly Affect Acute Exocrine Pancreas in Rats

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Abstract

Objectives—Reports have suggested a link between treatment with glucagon-like peptide 1 (GLP-1) analogues and an increased risk of pancreatitis. Oxyntomodulin, a dual agonist of both GLP-1 and glucagon receptors, is currently being investigated as a potential anti-obesity therapy, but little is known about its pancreatic safety. The aim of this study was to investigate the acute effect of oxyntomodulin and other proglucagon-derived peptides on the rat exocrine pancreas.

Methods—GLP-1, oxyntomodulin, glucagon and exendin-4 were infused into anaesthetised rats to measure plasma amylase concentration changes. Additionally, the effect of each peptide on both amylase release and proliferation in rat pancreatic acinar (AR42J) and primary isolated ductal cells was determined.

Results—Plasma amylase did not increase post peptide infusion, compared to vehicle and cholecystokinin (CCK); however, oxyntomodulin inhibited plasma amylase when co-administered with CCK. None of the peptides caused a significant increase in proliferation rate or amylase secretion from acinar and ductal cells.

Conclusions—The investigated peptides do not have an acute effect on the exocrine pancreas with regard to proliferation and plasma amylase, when administered individually. Oxyntomodulin appears to be a potent inhibitor of amylase release, potentially making it a safer anti-obesity agent regarding pancreatitis, compared to GLP-1 agonists.

Keywords

pancreatitis; GLP-1; glucagon; adverse effects; amylase

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Introduction

Proglucagon-derived peptide GLP-1 receptor agonists, such as the Food and Drug Administration (FDA)-approved exenatide and liraglutide, are widely used in the treatment of type 2 diabetes due to their ability to induce glucose-stimulated insulin secretion, also known as the incretin effect, as well as their beneficial weight-lowering properties. Pancreatic safety became a subject of debate when a number of case reports linked the use of incretin therapies to pancreatitis. Subsequently, a number of post-marketing studies were published supporting this association (1-6), and a histological study of human pancreata from organ donors with type 2 diabetes treated with incretin therapy demonstrated an increase in pancreatic size accompanied with elevated ductal proliferation and dysplasia (7). However, a number of chronic studies in rodents and non-human primates found no association between incretin therapies and pancreatic abnormalities (8-14). The discrepancy might be explained by confounding factors such as high fat diet and type 2 diabetes contributing to the development of pancreatitis, irrespective of the use of incretin based medications (15, 16). Indeed, in July 2013, the European Medicines Agency's Committee for Medicinal Products for Human Use and the FDA concluded that the data available did not indicate an increased incidence of pancreatitis with GLP-1 receptor agonists, and the benefits of their use in patients with type 2 diabetes outweighed any possible risks (17).

However, GLP-1 analogues are currently undergoing regulatory evaluation for use as weight loss agents in obese people without diabetes, increasing the target population of patients potentially eligible for treatment (18). Oxyntomodulin is another proglucagon-derived peptide which acts at both GLP-1 and glucagon receptors. It has been demonstrated to have beneficial effects on energy homeostasis via reduction of food intake and increase in energy expenditure in rodents and in man, and its therapeutic potential in the treatment of obesity is currently being assessed (19-26). However, little is known about the pancreatic safety of this peptide.

The purpose of this work was to evaluate the acute effect of oxyntomodulin and other proglucagon-derived peptides including GLP-1 and glucagon, as well as the GLP-1 analogue exendin-4, on the exocrine pancreas, and potentially provide additional information on their safety. To that end, *in vivo* and *in vitro* peptide-induced amylase secretion, as well as peptide-induced acinar and ductal proliferation was assessed, in the rat model.

Methods

Study of plasma amylase release in vivo

Animals—All animal procedures undertaken were approved by the British Home Office under the UK Animal (Scientific Procedures) Act 1986. Adult male Wistar rats (Charles River Ltd., Margate, Kent, UK) weighing 300-500 g were maintained under controlled temperature (21-23°C) and lights (12:12 hr light-dark schedule, lights on at 0700). Animals were fasted for 18 hours before the experiment while allowed *ad libitum* access to water.

Procedure—GLP-1, glucagon, oxyntomodulin and exendin-4 were purchased from Bachem, Ltd. (Merseyside, UK). Gelofusine was used as the vehicle. CCK8 (Sigma-Aldrich,

Dorset, UK), the main stimulus to amylase secretion from the exocrine pancreas, was used as a positive control. Animals were anaesthetised with isoflurane at 1.5 - 2% flow rate. The technical aspects of the operation have been previously described by Christakis et al (27). A catheter was introduced into the left femoral vein in order to introduce a 30nmol bolus injection of peptide. To avoid sample contamination, the route of injection was different to the route of sampling. The dose was based on previous unpublished studies performed in the lab in order to achieve detectable levels of each peptide and is 6.25-fold higher than the average therapeutic dose of exendin-4, to maximise the potential side effect. The procedure length and sampling times were chosen in order to detect early amylase release post CCK administration, as well as cover the maximum time spectrum of potential amylase release in response to peptide injection. Blood samples were collected through another catheter placed in the right jugular vein. The catheters used were polyethylene tubing (internal diameter (ID) 0.46 mm, outside diameter (OD) 0.91 mm) (Instech Solomon, PA USA). The operating time for cannulation of the jugular and femoral vein and the unexpected events were recorded in a database. Glucose levels were measured before and during operation to monitor animal stress. At the end of the experiment, animals were killed by CO₂ inhalation and neck dislocation (Schedule 1 method under the Animals (Scientific Procedures) Act 1986).

Sampling—Two baseline samples were obtained from the jugular vein prior to peptide injection. Post injection, a 0.1 ml sample was obtained every 2 min for the first 10 min, then at 15 min, and every 10 min until 50 min. Total blood volume withdrawn was within recommended volume limits. Plasma amylase activity was determined using an Amylase assay kit (Abcam, Cambridge, UK) according to the manufacturer's instructions.

Study of amylase secretion in vitro

The rat pancreatic acinar cell line AR42J was purchased from Sigma-Aldrich (Dorset, UK). AR42J cells were routinely maintained in RPMI 1640 media, supplemented with 10% Foetal Bovine Serum (FBS), 100 IU/ ml penicillin, 100 µg/ ml streptomycin and 2 mM glutamine (Sigma-Aldrich, Dorset, UK). Cells were incubated with 20 nM dexamethasone for 48 h before secretion assay to induce CCK responsiveness, as described by Logsdon et al (28). Cells from passage 11-18 were used throughout this study and were routinely incubated in a humidified incubator at 37°C with 95% air and 5% CO₂.

To assess amylase secretion, cells were plated at approximately 10⁵ cells/ ml onto 24-well plates (500µl/ well) 24hr before the experiment. Immediately prior to treatment, cells were washed with 0.1M phosphate buffered saline. Cells were treated for 50 min at 37°C with ascending concentrations of the peptides diluted in serum-free RPMI 1640 media. Following this, cells were similarly treated with 10nM of each investigated peptide and CCK. The dose was in agreement with previously published research (29, 30). The incubation medium was collected and cells were lysed. Lysates were collected for total amylase content. Amylase secretion was then quantified as a percentage of amylase activity in incubation medium compared to total activity (incubation medium plus lysate) using an Amylase assay kit (Abcam, Cambridge, UK) according to the manufacturer's instructions.

Primary isolation of epithelial pancreatic duct cells

Rats were killed using a Schedule 1 method under the Animals (Scientific Procedures) Act 1986. An abdominal midline incision was performed in order to expose the contents of the abdominal cavity. By displacing the liver superiorly and the intestine inferiorly, the duodenum was accessible. Curved dissecting scissors were used to isolate and dissect the pancreas free from its attachments to the duodenum and the rest of the abdominal organs. The organ was then transferred to a 6 cm tissue culture dish with ice-cold Leibovitz (Gibco, Paisley, UK). Using a dissecting microscope, the surrounding pancreatic tissue was cleaned away and the duct was transferred in a tube containing 5ml of collagenase XI (Sigma-Aldrich, Dorset, UK) solution for 12 minutes. Following incubation, the remaining fibroblasts were removed and the treatment is repeated. The biliopancreatic duct in rat is a distinct structure under the dissecting scope, which ensures that it is neatly isolated with minimum contamination of surrounding tissue.

The ductal cells were isolated as previously described by Chen et al (31, 32). In order to dissociate ductal cells from the extracellular matrix, the ducts were incubated with dispase solution (Sigma-Aldrich, Dorset, UK). To dissociate the duct into cellular aggregates, it was subjected to a series of trypsinization steps, each one stopped by adding 0.5ml of ice-cold FBS. The collected fractions were then centrifuged and the resulting pellet was re-suspended in pancreatic medium (1:1 Hams F12/DMEM, 100 ng/ml cholera toxin, 100 µg/ml soybean trypsin inhibitor, 20% FBS, penicillin 100 IU/ml and streptomycin 100 mg/ml) plated in MaxGel ECM (Sigma-Aldrich, Dorset, UK) matrix coated wells in 6 well-plates and incubated at 37°C and at 5% CO₂.

RNA extraction and quantitative PCR in AR42J and ductal cells

Cells were cultured 2 hours post isolation to ensure maximum viability and replication detection, in the presence or absence of 10nM of each peptide for 18 hours. Medium was changed every 9 hours. Peptides were diluted in RPMI 1640 (AR42J) or pancreatic (ductal) medium, supplemented with 2% FBS. Total RNA was isolated from AR42J cells using TRI Reagent (Sigma-Aldrich, Dorset, UK) by referring to the manufacturer's protocol. 1-Bromo-3-chloropropane (Sigma-Aldrich, Dorset, UK) was added to precipitate the total RNA and the extracted RNA pellet was then washed with 75% ethanol. RNA was purified using PureLink RNA kit and DNA was digested from total RNA using PureLink DNase (Thermo Fisher Scientific, Paisley, UK). The purified RNA was dissolved in RNase and DNase free distilled water (Thermo Fisher Scientific, Paisley, UK) and was immediately stored at -80°C until further analysis.

Complementary DNA was synthesized from total RNA with High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Paisley, UK) according to the protocol recommended by the manufacturer. The protocol conditions were 10 minutes at 25°C for primer annealing, 120 minutes at 37°C for reverse transcription, and 5 minutes at 85°C. Quantitative PCR analysis was used to quantify the expression level of proliferation marker Ki-67. For the detection of Ki-67, QPCR primers which crossed a splice junction were designed using Primer Express (Invitrogen, Paisley, UK) (Table 1)). The expression levels were measured using a 7500 Fast Real-Time PCR System (Applied Biosystems, Paisley,

UK). The reaction mixture contained 6 µl of Fast SYBR Green Master Mix (Invitrogen, Paisley, UK), 0.35 µl of each primer (10 µM; Thermo Fisher Scientific, Paisley, UK), 3 µl water and 2 µl of cDNA in a final volume of 12 µl. Samples were amplified in duplicates. The reaction was started with 2 min at 50°C followed by the activation and pre-denaturation step at 95°C for 10 min. The reaction run consisted of 40 cycles comprising 15 s at 95°C and 1 min at 60°C.

Data analysis

Data are presented as mean ± Standard Error Mean (S.E.M.). All reported p-values were based on two-sided tests. Student t-test with Bonferroni correction was used in all experiments and one way ANOVA with Tukey's post-hoc test was used to compare amylase secretion levels of peptide treated AR42J cells against untreated control group. Ki-67 mRNA data were normalised against β-actin levels. The analytical method used was 2^{-(ΔΔCt)}. The level of statistical significance was set at p < 0.05. Prism 5.01 (GraphPad Software Inc. San Diego, USA) statistical software was used for all statistical analysis.

Results

Study of amylase secretion in vivo

A small percentage of amylase that is released in the gut leaks into the bloodstream and its concentration can be used as indicator of pancreatitis. To assess any pathogenic effect of peptides on the exocrine pancreas, rats were infused with GLP-1, exendin-4, glucagon or oxyntomodulin and plasma amylase levels were determined at regular time intervals. CCK was used as a positive control at a 2.4nmol/ rat dose that could induce significant amylase secretion. The generated curve is used as a comparison in figures 1 and 2. Rats treated with CCK demonstrated an almost 2-fold increase in plasma amylase during the 50 minute post injection period (Figures 1a, b). CCK increased plasma amylase significantly from 30 min onwards compared to time 0 (p < 0.01), reaching approximately 18.00± 0.70 unit/ l. Plasma amylase concentration did not increase post GLP-1, glucagon, oxyntomodulin or exendin-4 intravenous injection, compared to basal amylase and vehicle controls. No significant increase in plasma amylase was observed following treatment with the rest of the peptides and the vehicle as amylase levels reached approximately 12.00± 0.21 unit/ l throughout the 50 min of sampling (Figures 1a, b). Average basal amylase was 10.50± 0.39 unit/ l for all groups (n= 4-5).

The effect of each peptide on plasma amylase levels given in combination with CCK was assessed. Co-administration of GLP-1, exendin-4 and glucagon with CCK did not inhibit plasma amylase compared to CCK alone (Figure 2a), with plasma amylase levels increasing from 9.8± 0.37 unit/ l to 17.52± 0.94 unit/ l for all groups (n= 4-5). However, oxyntomodulin suppressed CCK-induced amylase release by approximately 3.4 unit/ l ±0.5 on average, when co-administered with CCK (Figure 2b, n=5, p < 0.01). Significant inhibition was achieved both in the beginning of sampling at 6 and 8 min, and the end at 30 and 50 min.

Study of amylase secretion in vitro

AR42J cells, an in vitro model of the exocrine pancreas were treated with GLP-1, glucagon, oxyntomodulin, exendin-4 and CCK. Total amylase and secreted amylase levels were measured after 50 min of incubation. Treatment of AR42J cells with CCK, a known stimulant of amylase release, resulted in a dose responsive secretion of amylase, reaching 28% of total amylase activity in 50 min (Figure 3, $n=5$, $p < 0.001$). GLP-1, exendin-4, glucagon and oxyntomodulin did not induce amylase secretion at any of the doses tested, as amylase levels remained stable at $13.8 \pm 0.45\%$ of total, the same as untreated cells. Co-administration of CCK (1nM) with each peptide (10nM) resulted in a non-significant trend of an inhibition of CCK-induced amylase secretion (Figure 4).

Cell proliferation in AR42J and biliopancreatic ductal cells

Existence of GLP-1 and glucagon receptors on AR42J and biliopancreatic ductal cells was confirmed by competitive receptor affinity studies (data not shown). To determine if the test peptides had an effect on the rate of cellular proliferation, AR42J and ductal cells were treated with 10nM of GLP-1, glucagon, oxyntomodulin and exendin-4 for 18hr. Following treatment, mRNA was extracted and the level of proliferation marker Ki-67 was analyzed using quantitative PCR (Figure 5a, b). Quantitatively, compared to the untreated control cells, the test peptide did not cause a significantly higher expression of Ki-67 ($p > 0.05$).

Discussion

Our experiments focused on four peptides which have been identified as potential therapeutic agents for the treatment of obesity and type 2 diabetes, with regard to pancreatic safety. A number of pre-clinical chronic safety studies have been completed in rodents and non-human primates to investigate findings of incretin peptides and DPP-IV long-term. However, relatively little is known about the actions of incretin peptides on the exocrine pancreas. Incretins have been hypothesized to cause pancreatic ductal and acinar cell hyperplasia and inflammation in the exocrine pancreas (7, 33, 34). On the other hand, glucagon has been previously suggested to inhibit gastric release, a fact that made it a potential peptide for the treatment of pancreatitis in the past (35).

Amylase plasma concentration increase in pancreatitis is a result of pancreatic enzyme leakage from damaged acinars. In a CCK-induced pancreatitis model in rat, hyperamylasemia was detected 30 min post treatment while acinar damage and pancreatic oedema was observed after 60 min (36). Here, we demonstrated no change in amylase release in response to administration of GLP-1, glucagon, exendin-4 and oxyntomodulin, alone, *in vivo* and *in vitro*. Similarly, GLP-1, glucagon and exendin-4 co-administration with CCK did not mitigate against the CCK- induced increase in amylase concentration. However, co-administration of oxyntomodulin and CCK caused a significant inhibition of CCK-induced plasma amylase release *in vivo*, compared to CCK alone.

A number of *in vivo* studies have been performed, evaluating the effect of constant infusion of GLP-1, glucagon and oxyntomodulin in lower doses in direct pancreatic secretion through bile duct cannulation. Investigations have shown that both glucagon and oxyntomodulin are

known to inhibit gastric release, with oxyntomodulin being 10 times more potent than glucagon in this regard, potentially because of its tissue specificity at the gastrointestinal tract, compared to glucagon which is more specific to the liver (30, 37, 38). In previous studies in the anaesthetized rat, basal pancreatic secretion could not be inhibited by oxyntomodulin infusion, yet when stimulated with a CCK agonist, pancreatic secretion levels decreased. During these studies, an oxyntomodulin effect through vagal afferents was hypothesized, as in physiologic doses of CCK8, afferent messages transmitted in the central nervous system were produced before the observation of an efferent vagal transmission to the rat pancreas (39). It has been suggested that a possible mechanism of this oxyntomodulin function is through the activity of gastropancreatic intrinsic nerves, or by preganglionic inhibition of excitatory vagal fibers via the central nervous system (CNS) (40, 41). Nonetheless, the exact mechanism through which oxyntomodulin inhibits CCK8-induced pancreatic secretion, while glucagon doesn't, remains unclear. This study confirmed the inhibitory function of oxyntomodulin, previously shown in overall gastric secretion, and cross examined if this effect extends to GLP-1 and exendin-4 using plasma amylase measurement.

It has previously been shown that pancreatic ductal cell proliferation is elevated in both obese and diabetic patients, which can predispose pancreatitis, possibly due to the build-up of excessive fat in pancreatic tissue, resulting in β -cell apoptosis and inflammation (34). As β -cell numbers decrease in type 2 diabetes, it has been suggested that islet regeneration is attempted through duct-related progenitors and proliferation rate is enhanced by GLP-1, causing blockages in the pancreatic duct (42), potentially through phosphorylation of cAMP response element-binding protein (CREB) and stimulation of mitogen-activated protein kinases (MAPK) pathway (33), while Immediate Early Genes (IEGs) *egr-1* and *c-fos* were also investigated (8). Nonetheless, in this work, GLP-1 and exendin-4 did not increase pancreatic acinar or ductal cell proliferation rate when compared to untreated cells over the course of 18hr.

AR42J cells are currently the only cell line that maintains a number of normal pancreatic acinar cell characteristics, when in culture, such as the synthesis and secretion of digestive enzymes protein expression and receptor expression similar to pancreatic acinar cells. AR42J cells, used in this study, are derived from a chemically induced exocrine pancreatic tumour, therefore are not grown from a single clone and can present response differences (43). Even so, our results are in agreement with previous studies that used both AR42J and isolated rat acini treated with GLP-1, and expand the investigated effect to oxyntomodulin and glucagon (29, 30, 44).

Taken together, our results indicate that GLP-1 and exendin-4 do not have an acute effect on exocrine pancreatic release, in the *in vivo* and *in vitro* models tested. Further studies in diabetic or obese rat models should be performed to determine if failure to observe an acute effect persists in these disease states. Our finding that oxyntomodulin protects against the CCK-induced increase in the appearance of pancreatic enzymes in the blood *in vivo* supports a favourable pancreatic safety profile for this peptide, as it could be protective in cases of type 2 diabetes-induced pancreatitis. The fact that this observation was not replicated *in vitro* might suggest the peptide might not be damaging or acting directly on acinar cells, with one

possibility being this effect is mediated via vagal innervation (41). As oxyntomodulin is a dual GLP-1/ glucagon receptor agonist, the finding that neither GLP-1 nor glucagon alone produced this effect is interesting, and merit further investigation. Furthermore, longer term studies need to be performed to further understand the effect of this peptide on pancreatic exocrine tissue, and will be critical if this peptide is to be used as a weight loss agent in the future.

As the use of GLP-1 receptor agonists for the treatment of obesity has been licenced (18) it is likely there will be a rapid increase in the number of patients taking this class of medication. Whilst numerous long term safety pharmacology studies have been completed and pharmacovigilance systems are in place, questions remain regarding the pancreatic safety of this drug class. Dual GLP-1 and glucagon receptor agonists are also under development as obesity treatments. In this study, we found an inhibitory effect of oxyntomodulin on the CCK-induced increase in the appearance of pancreatic enzyme in blood, which might support that it could be useful in the treatment of diabetic obese patients, as it can have a protective effect against diabetes- induced pancreatic inflammation, in addition to weight loss induction.

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References

1. Noel RA, Braun DK, Patterson RE, et al. Increased risk of acute pancreatitis and biliary disease observed in patients with type 2 diabetes: a retrospective cohort study. *Diabetes Care*. 2009; 32:834–838. [PubMed: 19208917]
2. Knezevich E, Crnic T, Kershaw S, et al. Liraglutide-associated acute pancreatitis. *Am J Health Syst Pharm*. 2012; 69:386–389. [PubMed: 22345417]
3. Alves C, Batel-Marques F, Macedo AF. A meta-analysis of serious adverse events reported with exenatide and liraglutide: acute pancreatitis and cancer. *Diabetes Res Clin Pract*. 2012; 98:271–284. [PubMed: 23010561]
4. Singh S, Chang HY, Richards TM, et al. Glucagon like peptide 1-based therapies and risk of hospitalization for acute pancreatitis in type 2 diabetes mellitus: a population-based matched case-control study. *JAMA Intern Med*. 2013; 173:534–539. [PubMed: 23440284]
5. Rouse R, Xu L, Stewart S, et al. High fat diet and GLP-1 drugs induce pancreatic injury in mice. *Toxicol Appl Pharmacol*. 2014; 276:104–114. [PubMed: 24534256]
6. Ahmad SR, Swann J. Exenatide and rare adverse events. *N Engl J Med*. 2008; 358:1970–1971. discussion 1-2. [PubMed: 18456920]
7. Butler AE, Campbell-Thompson M, Gurlo T, et al. Marked expansion of exocrine and endocrine pancreas with incretin therapy in humans with increased exocrine pancreas dysplasia and the potential for glucagon-producing neuroendocrine tumors. *Diabetes*. 2013; 62:2595–2604. [PubMed: 23524641]

8. Koehler JA, Baggio LL, Lamont BJ, et al. Glucagon-like peptide-1 receptor activation modulates pancreatitis-associated gene expression but does not modify the susceptibility to experimental pancreatitis in mice. *Diabetes*. 2009; 58:2148–2161. [PubMed: 19509017]
9. Nachnani JS, Bulchandani DG, Nookala A, et al. Biochemical and histological effects of exendin-4 (exenatide) on the rat pancreas. *Diabetologia*. 2010; 53:153–159. [PubMed: 19756486]
10. Vrang N, Jelsing J, Simonsen L, et al. The effects of 13 wk of liraglutide treatment on endocrine and exocrine pancreas in male and female ZDF rats: a quantitative and qualitative analysis revealing no evidence of drug-induced pancreatitis. *Am J Physiol Endocrinol Metab*. 2012; 303:E253–264. [PubMed: 22589391]
11. Nyborg NC, Molck AM, Madsen LW, et al. The human GLP-1 analog liraglutide and the pancreas: evidence for the absence of structural pancreatic changes in three species. *Diabetes*. 2012; 61:1243–1249. [PubMed: 22338093]
12. Tatarkiewicz K, Belanger P, Gu G, et al. No evidence of drug-induced pancreatitis in rats treated with exenatide for 13 weeks. *Diabetes Obes Metab*. 2013; 15:417–426. [PubMed: 23163898]
13. Gotfredsen CF, Molck AM, Thorup I, et al. The Human GLP-1 Analogs Liraglutide and Semaglutide: Absence of Histopathological Effects on the Pancreas in Nonhuman Primates. *Diabetes*. 2014; 63:2486–2497. [PubMed: 24608440]
14. Mondragon A, Davidsson D, Kyriakoudi S, et al. Divergent effects of liraglutide, exendin-4, and sitagliptin on beta-cell mass and indicators of pancreatitis in a mouse model of hyperglycaemia. *PloS one*. 2014; 9:e104873. [PubMed: 25119717]
15. Gonzalez-Perez A, Schlienger RG, Rodriguez LA. Acute pancreatitis in association with type 2 diabetes and antidiabetic drugs: a population-based cohort study. *Diabetes Care*. 2010; 33:2580–2585. [PubMed: 20833867]
16. Dawson DW, Hertzner K, Moro A, et al. High-fat, high-calorie diet promotes early pancreatic neoplasia in the conditional KrasG12D mouse model. *Cancer Prev Res (Phila)*. 2013; 6:1064–1073. [PubMed: 23943783]
17. Benstetter, M. Investigation into GLP-1-based diabetes therapies concluded. European Medicines Agency Committee for Medicinal Products for Human Use. Jul 26. 2013 Report No.
18. Novo-Nordisk. Phase 3a liraglutide 3 mg trial demonstrated significant weight loss and improved cardiovascular risk factors in adults with obesity and type 2 diabetes compared with placebo. International Congress of Endocrinology (ICE); 21 June 2014; Chicago.
19. Field BC, Wren AM, Peters V, et al. PYY3-36 and oxyntomodulin can be additive in their effect on food intake in overweight and obese humans. *Diabetes*. 2010; 59:1635–1639. [PubMed: 20357366]
20. Liu YL, Ford HE, Druce MR, et al. Subcutaneous oxyntomodulin analogue administration reduces body weight in lean and obese rodents. *Int J Obes (Lond)*. 2010; 34:1715–1725. [PubMed: 20531351]
21. Parker JA, McCullough KA, Field BC, et al. Glucagon and GLP-1 inhibit food intake and increase cfos expression in similar appetite regulating centres in the brainstem and amygdala. *Int J Obes (Lond)*. 2013; 37:1391–1398. [PubMed: 23337772]
22. Tan TM, Field BC, McCullough KA, et al. Coadministration of glucagon-like peptide-1 during glucagon infusion in humans results in increased energy expenditure and amelioration of hyperglycemia. *Diabetes*. 2013; 62:1131–1138. [PubMed: 23248172]
23. Day JW, Ottaway N, Patterson JT, et al. A new glucagon and GLP-1 co-agonist eliminates obesity in rodents. *Nat Chem Biol*. 2009; 5:749–757. [PubMed: 19597507]
24. Wynne K, Field BC, Bloom SR. The mechanism of action for oxyntomodulin in the regulation of obesity. *Curr Opin Investig Drugs*. 2010; 11:1151–1157.
25. Wynne K, Park AJ, Small CJ, et al. Oxyntomodulin increases energy expenditure in addition to decreasing energy intake in overweight and obese humans: a randomised controlled trial. *Int J Obes (Lond)*. 2006; 30:1729–1736. [PubMed: 16619056]
26. Wynne K, Park AJ, Small CJ, et al. Subcutaneous oxyntomodulin reduces body weight in overweight and obese subjects: a double-blind, randomized, controlled trial. *Diabetes*. 2005; 54:2390–2395. [PubMed: 16046306]

27. Christakis I, Georgiou P, Minnion J, et al. Learning curve of vessel cannulation in rats using cumulative sum analysis. *J Surg Res.* 2015; 193:69–76. [PubMed: 25082745]
28. Logsdon CD, Perot KJ, McDonald AR. Mechanism of glucocorticoid-induced increase in pancreatic amylase gene transcription. *J Biol Chem.* 1987; 262:15765–1569. [PubMed: 2445739]
29. Zhou J, Montrose-Rafizadeh C, Janczewski AM, et al. Glucagon-like peptide-1 does not mediate amylase release from AR42J cells. *J Cell Physiol.* 1999; 181:470–478. [PubMed: 10528233]
30. Anini Y, Jarrousse C, Chariot J, et al. Oxyntomodulin inhibits pancreatic secretion through the nervous system in rats. *Pancreas.* 2000; 20:348–360. [PubMed: 10824688]
31. Pylayeva-Gupta Y, Lee KE, Bar-Sagi D. Microdissection and culture of murine pancreatic ductal epithelial cells. *Methods Mol Biol.* 2013; 980:267–279. [PubMed: 23359159]
32. Chen KL, Zheng XL, Li Y, et al. An improved primary culture system of pancreatic duct epithelial cells from Wistar rats. *Cytotechnology.* 2009
33. Gier B, Matveyenko AV, Kirakossian D, et al. Chronic GLP-1 receptor activation by exendin-4 induces expansion of pancreatic duct glands in rats and accelerates formation of dysplastic lesions and chronic pancreatitis in the Kras(G12D) mouse model. *Diabetes.* 2012; 61:1250–1262. [PubMed: 22266668]
34. Butler AE, Galasso R, Matveyenko A, et al. Pancreatic duct replication is increased with obesity and type 2 diabetes in humans. *Diabetologia.* 2010; 53:21–26. [PubMed: 19844672]
35. Hirano T, Hirano K. Glucagon ameliorates pancreatic subcellular redistribution of lysosomal enzyme in rats with acute pancreatitis of closed duodenal loop. *Dig Surg.* 1999; 16:16–21. [PubMed: 9949262]
36. Grady T, Mah'Moud M, Otani T, et al. Zymogen proteolysis within the pancreatic acinar cell is associated with cellular injury. *Am J Physiol.* 1998; 275:G1010–1017. [PubMed: 9815031]
37. Dubrasquet M, Bataille D, Gespach C. Oxyntomodulin (glucagon-37 or bioactive enteroglucagon): a potent inhibitor of pentagastrin-stimulated acid secretion in rats. *Biosci Rep.* 1982; 2:391–395. [PubMed: 6125221]
38. Biedzinski TM, Bataille D, Devaux MA, et al. The effect of oxyntomodulin (glucagon-37) and glucagon on exocrine pancreatic secretion in the conscious rat. *Peptides.* 1987; 8:967–972. [PubMed: 3441447]
39. Li Y, Owyang C. Vagal afferent pathway mediates physiological action of cholecystokinin on pancreatic enzyme secretion. *J Clin Invest.* 1993; 92:418–424. [PubMed: 8100836]
40. Ghatei MA, Uttenthal LO, Christofides ND, et al. Molecular forms of human enteroglucagon in tissue and plasma: plasma responses to nutrient stimuli in health and in disorders of the upper gastrointestinal tract. *J Clin Endocrinol Metab.* 1983; 57:488–95. [PubMed: 6874888]
41. Younes Anini CJ, Chariot Jacques, Nagain Claire, et al. Oxyntomodulin Inhibits Pancreatic Secretion Through the Nervous System in Rats. *Pancreas.* 2000; 20:348–360. [PubMed: 10824688]
42. Stoffers DA, Kieffer TJ, Hussain MA, et al. Insulinotropic glucagon-like peptide 1 agonists stimulate expression of homeodomain protein IDX-1 and increase islet size in mouse pancreas. *Diabetes.* 2000; 49:741–748. [PubMed: 10905482]
43. Christophe J. Pancreatic tumoral cell line AR42J: an amphicrine model. *Am J Physiol.* 1994; 266:G963–971. [PubMed: 7517639]
44. Zhou J, Wang X, Pineyro MA, et al. Glucagon-like peptide 1 and exendin-4 convert pancreatic AR42J cells into glucagon- and insulin-producing cells. *Diabetes.* 1999; 48:2358–2366. [PubMed: 10580424]

Fig. 1a, b.

Amylase secretion (unit/l) post GLP-1, glucagon (GCG), oxyntomodulin (OXM) and exendin-4 (Ex-4) bolus 30nmol intravenous injection. A dose of 2.4nmol/ rat cholecystinin (CCK) was infused intravenously. Student t-test with Bonferroni correction was used to evaluate differences across time points vs. time 0. Values are shown as mean $\pm$ S.E.M. ** $p < 0.01$, *** $p < 0.001$ vs. time 0 basal sample ($n = 4-5$).

Fig. 2a, b.

Amylase secretion (unit/l) post peptide and CCK intravenous co-administration as a single bolus injection. GLP-1, glucagon (GCG), oxyntomodulin (OXM) and exendin-4 (EX-4) were co-administered at 30nmol with 2.4nmol CCK. Student t-test with Bonferroni correction was used between groups at individual time points vs. CCK. Values are shown as mean $\pm$ S.E.M. # $p < 0.05$, ## $p < 0.01$ vs. CCK (n= 4-5).

Fig. 3.

Amylase release from AR42J cells post GLP-1, glucagon, oxyntomodulin, exendin-4 and CCK treatment at ascending concentrations for 50 min (n=5). a. GLP-1 b. Glucagon c. Oxyntomodulin d. Exendin-4 e. CCK. Amylase is expressed as a percentage of the secreted amylase into the medium over the total amylase content of the cells (100%). One way ANOVA with Tukey's post-hoc test was used. Values are shown as mean $\pm$ S.E.M. *** $p < 0.001$ vs. untreated cells (0).

Fig. 4.

Amylase release from AR42J cells post peptide (10nM)/ CCK (1nM) treatment for 50 min (n=5). Amylase is expressed as a percentage of the secreted amylase into the medium over the total activity of the cells (100%). Student t-test with Bonferroni correction was used between CCK and peptides. Values are shown as mean $\pm$ S.E.M. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. CCK

A.

Fig. 5.

Relative Ki67 mRNA expression in a. AR42J cells and b. Billiopancreatic ductal cells treated with GLP-1, glucagon (GCG), oxyntomodulin (OXM) and exendin-4 (Ex-4) for 18hr (n=3-4). Student t-test with Bonferroni correction was used between untreated control group and peptides. Values are shown as mean $\pm$ S.E.M.

Table 1Primers used for the quantitative detection of Ki-67, β -actin

Analysed transcript	Sequence of forward primer	Sequence of reverse primer
Ki-67	TTGACCGCTCCTTTAGGTATGAA	GGTATCTTGACCTTCCCATCA
β -actin	CGAGTCGCGTCCACCC	CATCCATGGCGAACTGGTG