GLP-1 REGULATES PROLIFERATION OF GLP-1 – SECRETING CELLS THROUGH A FEEDBACK MECHANISM

Master of Science Degree project, 30 credits point

Accomplished at the

Department of Clinical Science and Education, Södersjukhuset

Unit for Diabetes Research, Karolinska Institutet, Stockholm, Sweden

Author: Mohamed Abdullahi
2010

Supervisor: Senior researchers, Qimin Zhang: Karolinska Institutet
Examinator: Senior Lecturer, Sven Hamp: Märladalen University

CONTENTS

Abstract

Background and aim: Diabetes mellitus (DM) is a chronic and progressive illness that affects all type of populations and ages. According to World health organization (WHO) by 2030 it will be 366 million people effected world wild. Many new drugs are Glucagon-like peptide-1 (GLP-1) based therapy for treatment of type 2diabetes. GLP-1 is released from the intestinal L-cells, and is a potent stimulator of glucose-dependent insulin secretion. The aim of this study was to investigate the effect of GLP-1 and its stable analogs on cell proliferation of GLP-1 secreting GLUTag cells.

Material and methods: GluTag cells were incubated for 48h in DMEM medium containing (0.5% fetal bovine serum and 100 IU/ml penicillin and 100 μg/ml streptomycin and 3mM glucose concentration) in the present or absence of the agents. DNA synthesis was measured using ^{3}H- thymidine incorporation and Ki67 antigen staining. Western blot were performed to investigate the present of GLP-1 receptor in GLUTag cells.

Results/conclusions: GLP-1(7-36) increased cell proliferation in GLUTag cells, an effect which was blocked by the GLP-1 receptor antagonist exendin(9-39). The GLP-1 receptor was expressed in GluTag cells.

These results suggest that GLP-1 regulates proliferation of the GLP-1-secreting cell through a feedback mechanism via its receptor. Since serum GLP-1 levels are decreased in type 2 diabetic patients, the effect of GLP-1 on the GLP-1-secreting cell proliferation suggested here provides a novel beneficial long-term effect of the incretin-based drugs in clinical practice i.e. through increase of the GLP-1-secreting cell mass, augmenting the incretin effect. In addition, the feedback mechanism action of GLP-1 reveals a new insight in regulation manner of the L-cell proliferation.

Keywords: Incretin hormone*, GLP-1, GLP-1 receptor, Exendin-4, Diabetes*

List of abbreviations

BSA	Bovine Serum Albumin
DPP-IV	dipeptidyl peptidase-4
DM	Diabetes mellitus
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
Ex4	Exendin4
FBS	Fetal bovine serum
GLP-1	Glucagon-like peptide 1
GLP-1R	Glucagon-like peptide 1 receptor
GIP	Gastric inhibitory polypeptide
TBS	Tris Buffered Saline
PBS-T	Phosphate Buffered Saline/Tween 20
PBS	Phosphate Buffered Saline
Pest	Penicillin/streptomycin

Introduction

Diabetes mellitus

Diabetes mellitus (DM) is a chronic and progressive illness that affects all type of populations and ages. According to World health organization (WHO) the prevalence of diabetes for all age-groups worldwide which was estimated to be 2.8% in 2000 will increase to 4.4% by 2030. This means that the total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030 [1].

There are different types of diabetes such as gestational diabetes, diabetes that are associated with hormonal disorders, but the two main type of diabetes are type1 and type 2. Type 1 is accounted for 5-10% of all diagnosed cases, while type 2 accounts for 85-90% of all patients with DM [2]. DM is a metabolic disorder where the cells of the body cannot utilize glucose properly. In type 1 DM, the pancreatic beta cells are gradually destroyed by the immune system resulting in reduced insulin production and an increased peripheral resistance to insulin. The blood glucose level rises (hyperglycemia) due to lack of insulin required for systemic glucose metabolism and glycogen synthesis in the liver [3]. Type 2 diabetes results from a combination of genetic and acquired factors that impair beta-cell function and tissue insulin sensitivity [4]. There are at least three factors contribute to hyperglycemia in type 2 diabetes (1) peripheral insulin resistance, (2) impaired insulin secretion, and (3) increased liver (hepatic) glucose production. One of the first sign that a metabolic abnormality is present in type 2 DM patient is the presence of insulin resistance which presents as an overproduction of glucose by the liver, impaired peripheral glucose utilization and increased breakdown of fat (5).

In the beginning as insulin resistance develops the pancreas compensates by increasing insulin secretion, as a result blood glucose levels remain normal. But over time the pancreas fails to produce enough insulin to meet the body´s demands resulting in insulin resistance and glucose intolerance progressing to type 2 DM [6, 7]. Hyperglycemia is only evident many years after insulin resistance begins, and as result tissue cells and the vasculature can be exposed to the negative impact of insulin resistance, hyperglycemia and hyperlipidemia for years before there is clinical evidence of a problem.

Incretins

Incretins are hormones that are released from the gut into the bloodstream in response to ingestion of food, thereby modulating insulin secretory response. The incretin effect is described as the ability of the gastrointestinal hormones such as GLP-1, released in response to food intake, to stimulate insulin release from the endocrine pancreas. The incretin effect accounts for 20 to 60 % of the overall postprandial insulin secretion in healthy subjects [8]. The two most important increatin hormones are glucose-dependent insulintropic peptide (GIP) and glucagon-like peptide 1 (GLP-1).

GIP

GIP is a 42 amino acid peptide produced by the K- cells and they are often found in the duodenum but also in the small intestine, the active form is GIP (3-42). The main stimulators for the secretion of GIP are fat and carbohydrate rich meals [9]. GIP secretion reaches peak

concentration within 15-30 min after intake of a meal [10]. Shortly after GIP (3-42) peptide is released into the circulation it is cleaved by the enzyme dipeptidyl-peptidase IV(DPP IV) at the NH_2 - terminal part resulting in GIP(3-42). In type 2 DM, the response of the pancreatic β-cell to GIP is reduced. The reduced β-cell response to GIP remains unclear. It has been proposed that hyperglycemia alters the physiological response as a result of down-regulation of GIP receptor [11]. Therefore GLP-1 becomes more important target for treating type 2 DM.
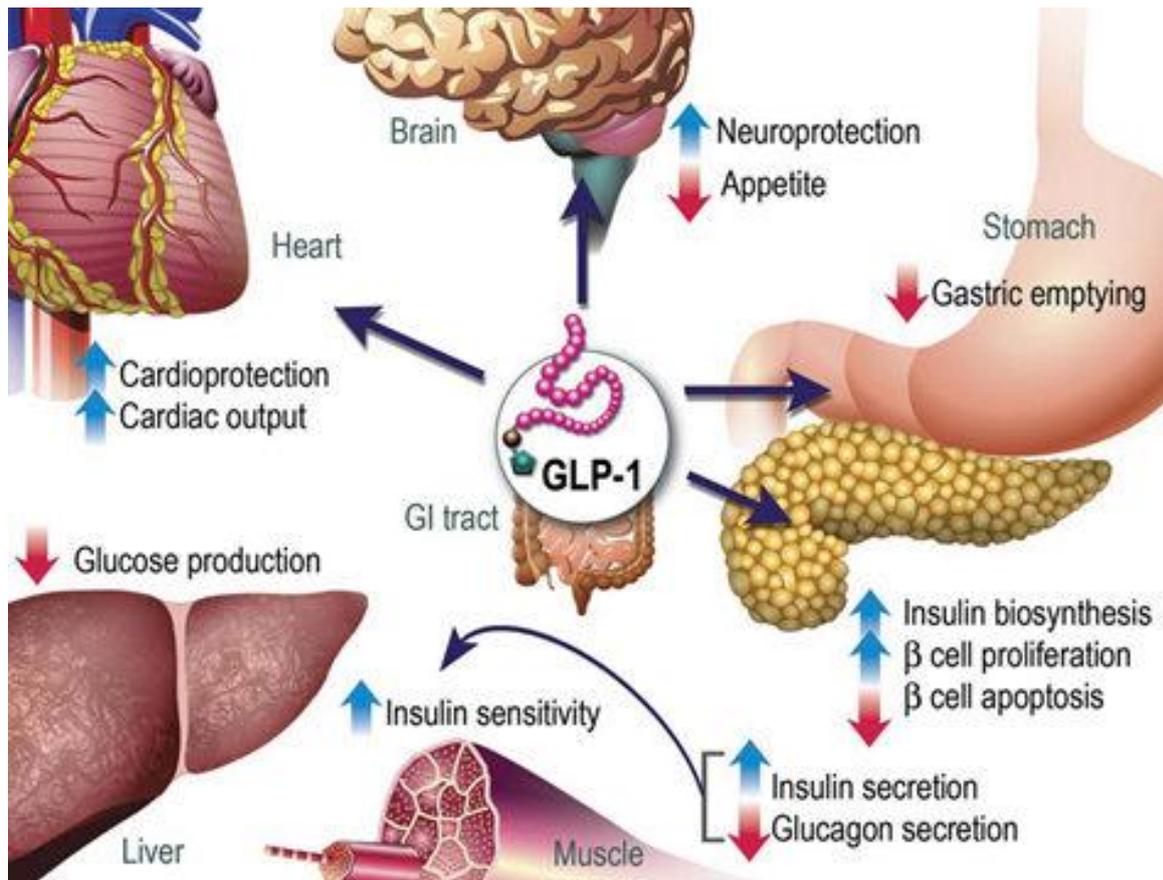
GLP-1

GLP-1 is a 30 amino acid peptide hormone product in the L-cells,which are distributed in the small and lager intestine, with the majority of L-cells are localized to the ileum and colon[12,13,14]. It is produced by posttranslational cleavage of proglucagon, which is processed by the coexpressed prohormone convertase PC1/3 in the L-cells [15, 16, 17]. The L- cells is endocrine cells. Ingestion of meal, particularly rich in carbohydrates and fat, is the primary stimulator for GLP-1 secretion, although the sizes of the meal and the gastric emptying rate have an impact on the secretion [12].

In human, GLP-1 is release rapidly into the circulation after oral nutrient ingestion and oral glucose administration but not by intravenous glucose administration, and its secretion occurs in two phases starting with an early phase within 10-15min, followed by a longer 30-60 min secondary phase[18.19].Even though little is known about the exact mechanisms where nutrients stimulate the GLP-1 secretion the early phase of GLP-1 secretion may be due to the L-cells located in more proximal regions of the small intestine, or the autonomic nervous systems neurotransmitters and GIP [14,20 ,21].

There are several forms of GLP-1 secreted in vivo such as GLP-1(1-37) and GLP-1(1-36)-NH_2 which are thought to be biologically inactive, but the essential and biologically active forms of GLP-1 are GLP-1(7-37) and GLP-1(7-36)NH_2. Both of them appear to have the similar potency in their ability to stimulate insulin secretion [17, 21, 22]. The majority of GLP-1 that is in circulation in humans are GLP-1(7-36)NH_2, the addition of the amide group to GLP-1(7-36)NH_2 is believed mediated by the enzyme peptidylglycine α-amidating monooxygenase and may enhance the survival of GLP-1 in plasma [23].

GLP-1 is extremely vulnerable to the catalytic activity of the dipeptidyl peptidase-4(DPP-IV), which cleaves off the two NH_2-terminal amino acid, resulting in the formation of the metabolites GLP-1(9-37) or GLP-1(9-36)NH_2 [17]. DPP-IV is widely expressed and can be found in multiple tissues and cell type, including the kidney, lung, liver, CNS, and pancreas. But also on the luminal surface of endothelial cells those lining blood vessels that drain the intestinal mucosa which are positioned directly next to the sites of GLP-1 secretion. This leads to more than half of the GLP-1 that enters the portal circulation already has been cleave by DPP-IV before entering into the systemic circulation [21, 24, 25,]. Numerous of studies in both animals and humans have demonstrated that inhibition of DPP-IV activity prolongs the half-life of intact biologically active GLP-1, therefore a number of inhibitors has been developed or are still in a development phase. The major route for GLP-1 eliminations is through the kidney and involves a mechanism that includes glomerular filtration, uptake and catabolism [26].

Figure 1: *GLP-1 actions in deferent (peripheral) tissues. The majority of the effects of GLP-1 are mediated by direct interaction with GLP-1Rs on specific tissues. But the actions of GLP-1 in liver, fat, and muscle possibly occur through indirect mechanisms.[1]*

GLP-1 Receptor

The GLP-1 receptor (GLP-1R) is a G-protein coupled receptor and belongs to the same family as glucagon, GLP-2 and GIP receptors [27]. The human and rat GLP-1R were cloned and sequenced in the early 1990s by Bernard Thorens from their respective pancreatic islet cDNA libraries. Both receptors are 463amino acid in length and have 90% amino acid sequence identity [28, 29]. GLP-1R has been identified to be expressed in a widely range of tissues including pancreatic islets α-, β-, and δ-cells but also in the brain, heart, kidney and the gastrointestinal tract although its function is not known for all these locations [30, 31, 32, 33, 34]. The N-terminal which is a extracellular region of the GLP-1R is necessary for the GLP-1 binding, while distinct domains within the third intracellular loop are critical for efficient coupling of the receptors to its specific G-proteins [35]. GLP-1R agonists produces several biological actions in the pancreas β-cells including stimulation of glucose-dependent insulin secretion, where the most important component is cAMP which mediates its stimulating effect on insulin secretion via two distinct mechanisms (1) Protein kinase A (PKA)-dependent phosphorylation of the downstream targets (2) PKA- independent activation of cAMP-regulated guanine nucleotide exchange factor II also known as Epac 2 [36].

Physiological action of GLP-1

The most important action of GLP-1 is its incretin function. The incretin effects imply the amplification of insulin secretion caused by the incretin hormones. GLP-1 enhances both the biosynthesis and secretion of insulin from pancreatic β-cells. The incretin effect is estimated to account for approximately 50%–70% of the total insulin secreted after oral glucose administration [37]. The circulating GLP-1 concentration is low in the fasting state, but rises within 15 min after ingestion normally peaking within 30-45 min and returning to basal levels over 2-3 hours [38]. The incretin effect is demonstrated by comparing insulin responses to oral and intravenous administration glucose, where the intravenous infusion is adjusted so it results in the same plasma glucose concentration [27, 39]. The outcome of a healthy person, oral administration causes a three to fourfold lager insulin response comparing with the intravenous method. Similar increases are revealed by measuring the insulin´s C-peptide, ruling out the possibility that the differences caused by a liver insulin uptake, since the C-peptide is not taken up by the liver. This proves that the increase in insulin secretion in oral administration is mainly due to the actions of insulinotropic gut hormones where the two most important are GLP-1 and GIP [27, 40, 41, 42]. In addition its incretin effects, GLP-1 is involved in the maintenance of pancreatic β -cell mass by stimulating β -cell proliferation and inhibiting β -cell apoptosis [43,44]. In the gastrointestinal tract the GLP-1 effects include inhibition of meal-stimulated gastric acid secretion and gastric emptying. The inhibition of gastric empting reduce the increases in meal associated blood glucose levels by slowing the transit of nutrients from the stomach to the small intestinal, and therefore contributes to the normalization of blood glucose levels. GLP-1 also has several other effects such as suppressing liver glucose production, inhibit glucagon secretion and increasing insulin sensitivity and secretion [45, 46, 47, 48, 49].

GLP-1 has neural effects since both GLP-1 and it´s receptors are present in regions of the CNS that regulate many functions including feeding behavior resulting in loss of appetite, gastric motility and cardiovascular function. It also has a neuroprotective effects and has been proposed as a new therapeutic agent for neurodegenerative diseases. The cardiac effects associated with GLP-1 are for example an increase in cardiac output and other cardio protections [17, 45].

GLP-1 in diabetes treatment

While other insulinotropic agents such as sulfonylureas have an increasing effect on pancreatic β-cell apoptosis. Studies in different animal models have provided evidence that GLP-1 can delay or even reverse the loss of β-cell mass by inhibiting apoptosis even in human β-cells [49, 50, 51]. Even do β-cell replications rarely occur in human inhibitions of apoptosis may be important for the treatment of diabetes, since the normal number of β-cells is maintained in a balance between apoptosis and proliferation [49, 52, 53]. Furthermore GLP-1 stimulates the transcription of genes coding for β-cell components involved in the process of glucose sensing and insulin synthesis and secretion [54]. GLP-1 has an effect on appetite and food intake therefore reducing the body weight and benefiting the treatment of the disease [55].

Incretin effect of GLP-1 in type 2 diabetes

The incretin effect seems to be absent or at least reduced in patient with type 2diabetes, since, unlike a healthy person, there were no significant differences in C-peptide concentration between the experiments where oral and intravenous glucose was given [56].GLP-1 has been shown to have a significantly reduction in type 2 diabetes, although the insulin response to GLP-1 is retained [57,58,59].Both defects in the secretion and action of incretin hormones seems be responsible for the reduced incretin effects on diabetes type 2 patients even though the molecular mechanisms underlying this is currently unknown.

GLP-1 and its stable analogues

There are several reasons way GLP-1 is used for treatment of diabetes, the fact that insulin secretion can be restored to a normal level by administration of GLP-1 is the most important one. The major challenge for development of GLP-1 based drugs has been that the hormone is rapidly inactivated by DPP-IV. There are now two approaches used to overcome this problem. The first consist in the development of GLP-1 analogs, also called incretin mimetics that bind to the GLP-1 receptors with the same affinity as GLP-1 but resist the degradation by DPP-IV. The second is to design drugs that inhibit the action of DPP-IV so called incretin enhancers witch prolong the effect of native GLP-1 and increase their serum levels. The two available incertin enhancers are sitagliptin(Januvia) and vildagliptin (Galvus).

Exenatide (synthetic exendin 4) was isolated from the lizard venom, *Heloderma suspectum* and shares approximately 50% of its amino acid sequence with mammalian GLP-1 [60, 61]. Exendin-4 is a GLP-1 receptor agonist and unlike GLP-1 it is not degraded by DPP-IV. It was approved for diabetes treatment in 2005 and is available under the name Byetta. Exendin 4 has several incretin effect which include lowering blood glucose without a risk for hypoglycaemia, increases insulin secretion (beta-cell function), and lowers glucagon release [58, 62, 63, 64]. Exendin (9-39) which is an N-terminally reduced peptide copied of the Lizard GLP-1R exendin(9-39) , binds to the GLP-1R and functions as a specific GLP-1 antagonist[65].

DPP-IV cleavage site
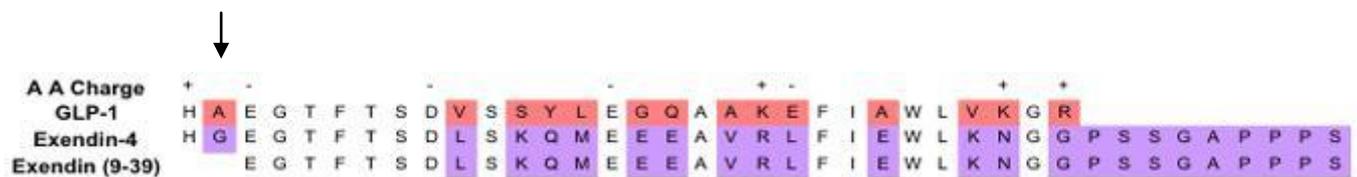
Figure 2: *Amino acid sequences for GLP-1, Ex4 and the GLP-1 receptor antagonist, Exendin (9-39). The purple shading represents the amino acid substitutions in the Ex4 sequence relative to the GLP-1 sequence (shaded red). Replacement of the alanine with glycine in position 8 renders the peptide protease resistant and improves stability.*

The limitations of older therapies for diabetes have intensified the request for new medications and better understanding of both the physiology and pathophysiology of glucose metabolism. The effect of GLP-1 and its agonists on proliferation of the GLP-1-secreting cells are unknown. In this study, we have studied the role of GLP-1 on proliferation of the GLP-1-producing cell GluTag.

Aim of the study

- To investigate the roles of anti-diabetic drugs GLP-1 (7-36), exendin-4, and its metabolite GLP-1 (9-36) on proliferation of the GLP-1 secreting GluTag -cells.

- To study the involvement of the GLP-1 receptor on GLP-1/exendin-4-induced proliferation of GluTag cells.

Materials and methods

Cells

The cells used for all experiments were GLUTag cells which are a stable immortalized murine enteroendocrine cell line that secretes GLP-1. This cell line was isolated from a glucagon producing entroendocrine cell tumour that occurs in glucagon gene SV40 T-antigen transgenic mice. The GLUTag cells were grown in DMEM medium containing 5.5mM glucose concentration, 10% (vol/vol) fetal bovine serum (FBS) and 1% penicillin/streptomycin (Pest) at 37°C in a humidified (5% CO_2, 95% air) atmosphere. The medium was changed every 2days.

Experimental methods

Anti-diabetic agents

The concentration of the drugs during the incubation was GLP1-1(7-36) 10nM (Polypeptide Group), GLP-1(9-36) 10nM (PolyPeptide Laboratories, Torrance, CA), Exendin4 1nM (Sigma Aldrich, St Louis, MO) Exendin (9-39) 100nM (PolyPeptide Laboratories, Torrance, CA). Due to the degradation of GLP-1(7-36) was added every 12 h during the 48 h incubation.

^{3}H-thymidine incorporation

400,000-450 000 Glutag cells was grown in 6-well culture plate with 2 ml medium/well comntaining DMEM, supplimentated with 10% FBS and 5,5 mM glucose, and allowed to reach 80-90% confluence. The cells was then incubated overnight with starvation medium (DMEM 0,5%FBS and 5.5 mM glucose), followed by an incubation for 48 hours with the starvation medium containing the different anti-diabetic agents. Two wells of cells were used as controls where no anti-diabetic agent was present. Six hours before the end of the incubation, all wells were added with 1µCi/ml ^{3}H-thymidine (Amersham Biosciences #TRKQ7090). At the end of the incubation, the cells were trypsinized, centrifuged and the supernatant was discarded, the cells were washed three times in1 ml PBS . ^{3}H-thymidine incorporated into DNA was measured using microplate scintillation and luminescence counter (Wallac MicroBeta® Trilux 1450-024, PerkinElmer with software: MicroBeta windows workstation version: 3.1) in the presence of 200µl scintillation fluid (PerkinElmer). The result was normalized with the protein concentration of the sample determined using BCA Protein Assay kit.

Immunocytochemistry with Ki67 staining

Approximately 100 000-150 000 cells/well was transfer to 24-well plate containing 1 ml DMEM medium with 10%FBS and 5,5mM glucose. The cells were grown to 80-90% confluence. The medium was then change to the starvation medium with or without anti-diabetic agents and the cells were incubated for 48 hours in an incubator. At the end of the incubation, the medium was removed and the cells were rinsed twice with PBS before fixed with ice cold 3.7% formaldehyde (Sigma, F8775) in PBS for 10 minutes at room temperature. The cells were then washed three times with PBS before proceeding with immunolabeling.

Immunolabeling

The fixed cells were blocked in PBS-0.1% Tween 20 (PBS-T) with 10% rabbit serum for 2 hours at 4° C. Immunolabeling was performed by incubation of the fixed cells with the primary rabbit- polyclonal anti-Ki67 antibody (Leica Biosystems #NCL-Ki67p, 1:500 in PBS-T, 200 μl/well) for 1 hour. The cells was washed four time with PBS-T before a two-hour incubation with the secondary antibody anti-rabbit FITC-conjugated (Invitrogen , #21206, 1:200 in PBS-T/10% rabbit serum) The cells were washed four times in PBS-T. Nuclear was stained with the fluorescent marker 4',6-diamidino-2-phenylindole (DAPI) (SIGMA #D8417, 1: 50000, 5 min). The cells were washed several times with PBS-T before images were captured on an Olympus microscope (CKX41 with a KX-85 Olympus camera and software: Cell^A Olympus).

Protein measurement - BCA-assay

The protein content in each sample was determined using a BCA Protein Assay (Micro BCA Protein Assay Kit Thermo Scintific #23235) following the manufacturer instructions. Briefly, a standard curve was prepared as follows, 6 ml PBS solution was diluted with 315 μl lysis buffer and bovine serum albumin (BSA) with the concentration 2 mg/ml to obtain a series of dilutions (0,0,5, 1, 2.5, 5, 10, 20, and 40 μg/ml). 20 μl from each cell samples was also diluted with 380 μl PBS solution and both standards and samples were transferred to the microplate, subsequently, 150 μl reagent mixture from the kit were added to each well. The mixture was then allowed to incubate at 37° C in a humidified (5% CO_2, 95% air) atmosphere for 2-3 hours. Absorbance was measured at 562 nm using a microplate reader (Labsystems iEMS Reader MF, Finland with software: Ascent software version 2.6)

Western blotting

Cell lysate preparation

Two culture flasks containing GLuTag and MIN6 cells with a confluence of 80-90% were harvested by trypsinization.. The cells were washed twice with PBS and lysed in lysis buffer (PBS 1%Tritorn, 2.5μl 200 μM PMSF and protease inhibitor cocktail). After 30 minute incubation on ice, the cell lysate was centrifuged at 5000 × g for 20 min, at 4° C. The supernatant was collected into fresh tubes and stored at -80° C pending analysis. Ten μl of the supernatant was taken for protein content determination. The protein content in each sample was determined using BCA protein assay.

SDS-PAGE and Western blotting

A gel for SDS-PAGE containing 7.5% polyacrylamide was prepared.Same amount of protein i.e. 20 μg from each of the two samples was loaded to the gel and run for approx 1 h at 150 V. Separated proteins in the gels were electrophoretically transferred onto nitrocellulose membranes (100 V for 1 hour). The membrane was rinced with TBS-T(20 mM Tris base,137 mM NaCL pH 7.6, with, 0.05% Tween20) and blocked overnight in 5% non-fat-milk(Bio-Rad Laboratories #1706405) in TBS-T. The membrane was washed again with TBS-T and further incubated overnight with the primary anti-GLP-1 receptor antibody (Santa

Cruz Biotechnology #Sc-66911) diluted 1:500 in TBS-T containing 1% BSA. The membrane was washed 6 times on a plate shaker. The nitrocellulose membrane was then incubated for 1 hour with the secondary antibody goat anti-rabbit IG-HRP (Santa Cruz Biotechnology #Sc-2030) diluted 1:10000 in TBS-T-1%BSA. The membrane was washed in TBS-T six times and protein bands were visualized by ECL (Amersham, GE Healthcare UK # RPN2132). In order to normalize the band densities, the membrane was stripped and re-blotted with monoclonal β-actin mouse antibody (SIGMA #A1978) diluted 1: 2000 in TBS-T and 1%BSA. The band densities were measured using densitometry (Bio-Rad Laboratories Segrate, Milano, Italy Id no: 101865-0 software : Quantiy one 4.6.3).

Statistic methods

Statistical analysis was conducted using ANOVA or Student´s t test. The result were expressed as mean ±Standard error (SEM) it was considered to be statistically significant at $P<0.05$.

Results and Discussion

1/. GLP-1 (7-36), but not its metabolite GLP-1(9-36) and exendin-4, stimulates proliferation of the GLP-1-secreting cell GluTag.

Effect on cell proliferation was measured using two different methods that are widely used for studying cell proliferation. 3H- thymidine incorporation is standard method to monitor rates of DNA synthesis and cell proliferation, were Ki67 is used as a proliferation marker since the polyclonal antibody labels Ki67 antigen in the granular components of the nucleolus during proliferation stages.
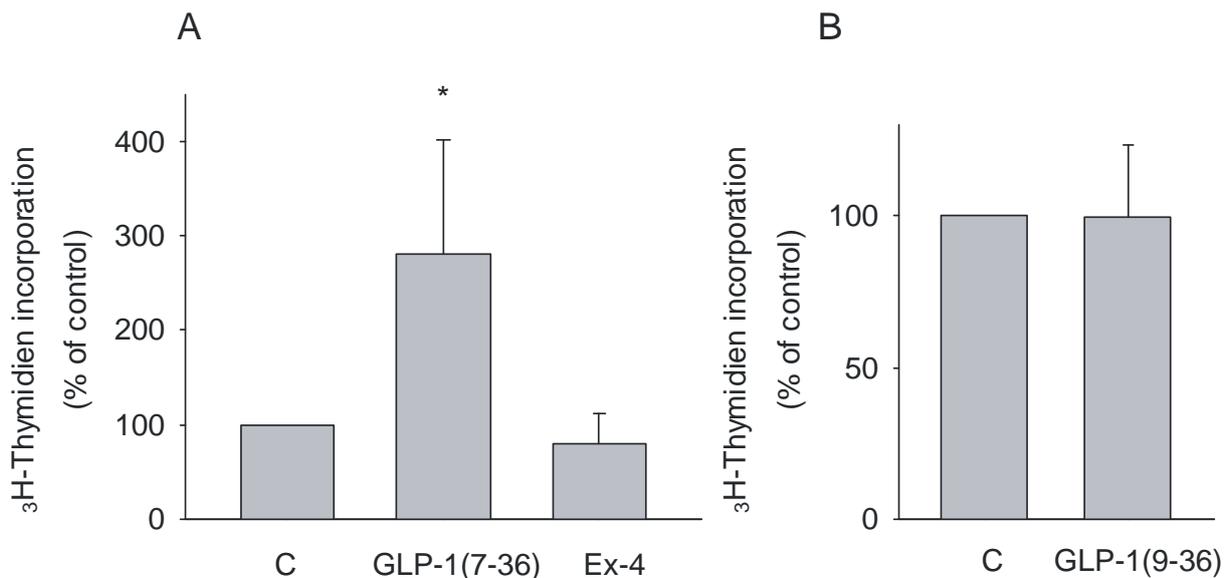
Figure1*: Cell was incubated for 48 h in DMEM medium contaning 3mM glucose, .5% FBS in the present or absent of the agents. Cells were pulsed with ^3H –thymidine 6 h prior to the end of the incubation. The result were normalized by protein content in each dish and expressed as percentage of control. They are from three independent duplicate experiments (* P<0.05).*

GLP-1(7-36) significantly enhanced thymidine incorporation into cells. At the concentration of 10 nM, GLP-1(7-36) caused approximately 300% of increase in ^3H-thymidine incorporation, compared to the controls, indicating a stimulatory effect of the drugs on cell proliferation. In contrast, Exendin4 did not show any effect on cell proliferation. (Figure 1 A) In addition, incubation of the cells with GLP-1(9-36), the major GLP-1 metabolite did not result in any changes in thymidine incorporation compared to control. (Figure 1B)
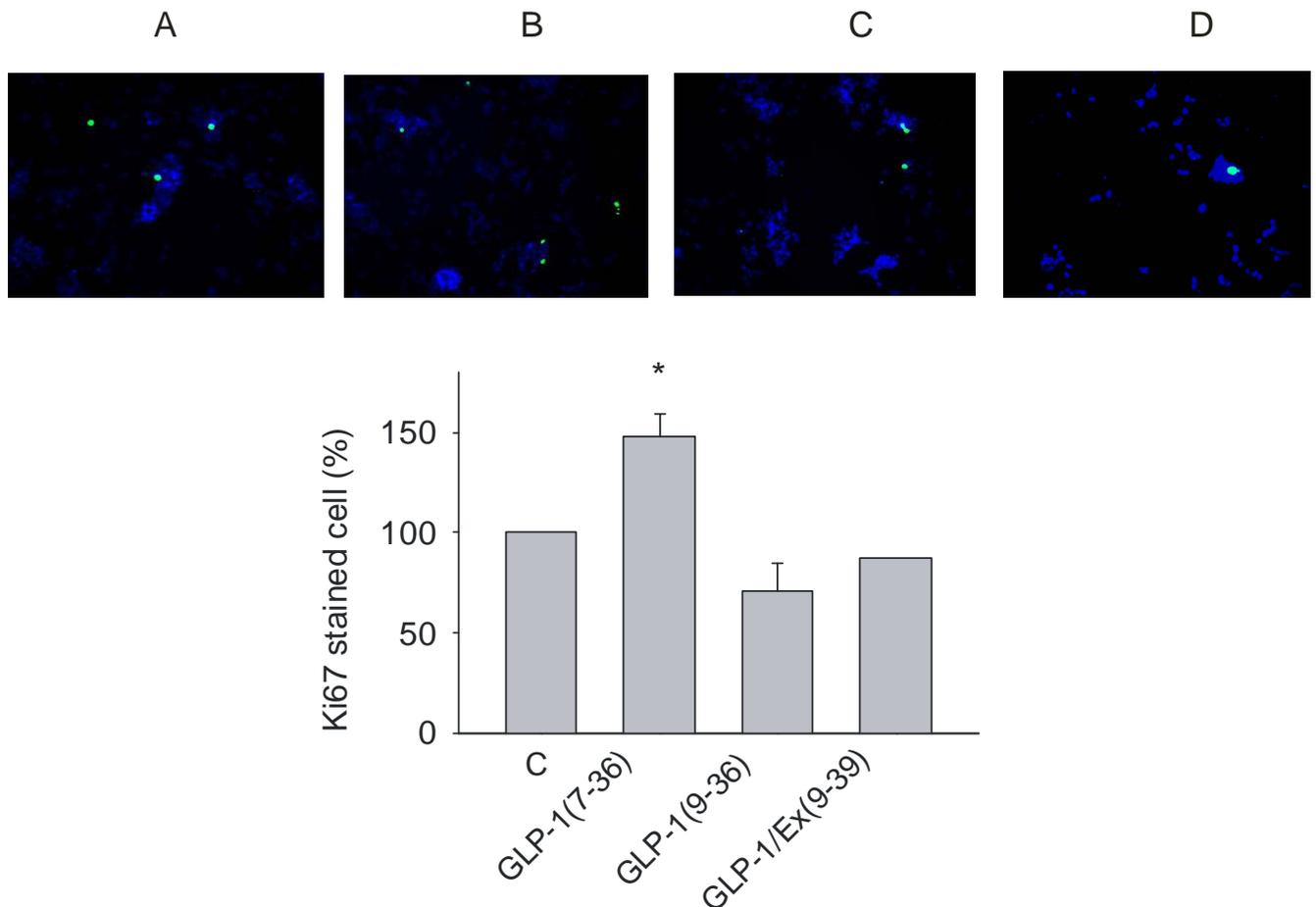
Figure 2: Cell was incubated for 48 h in DMEM medium contaning 3mM glucose, 0.5% FBS in the present or absent of the agents. Immunocytochemistry was then performed to detect cells in proliferation state. Result are from three independent duplicate experiments (P<0.05)*

To further verify the observed effect of GLP-1(7-36) on proliferation of GluTag cells, Ki67 staining was performed. The result shows that GLP-1(7-36) casued a 50% increase in proliferation compared to control. Consistent with the results from ^{3}H-thymidine incorporation, GLP-1(9-36) did not show any significant changes in the Ki67 staining (Figures 2). This result further indicates a role of GLP-1(7-36), but not its metabolite, in promoting proliferation of the cells.

The involvement of the GLP-1 receptor in the GLP-1(7-36)-induced proliferation was evaluated by co-incubation of the cells with the GLP-1 receptor antagonist Exendin (9-39). Ata concentration of 100 nM , exendin(9-39) completely blocked the GLP-1(7-36)- induced proliferation.

The GLP-1(7-36)-stimulated cell proliferation was also evaluated by cell counting. The result shows that GLP-1(7-36), but not GLP-1(9-36), showed a clear tendency in increasing the cell number, although a significant change was not reached due to a limited experiment number. The effect of GLP-1(7-36) was prevented in the presence of exendin(9-39) (Figure 3).
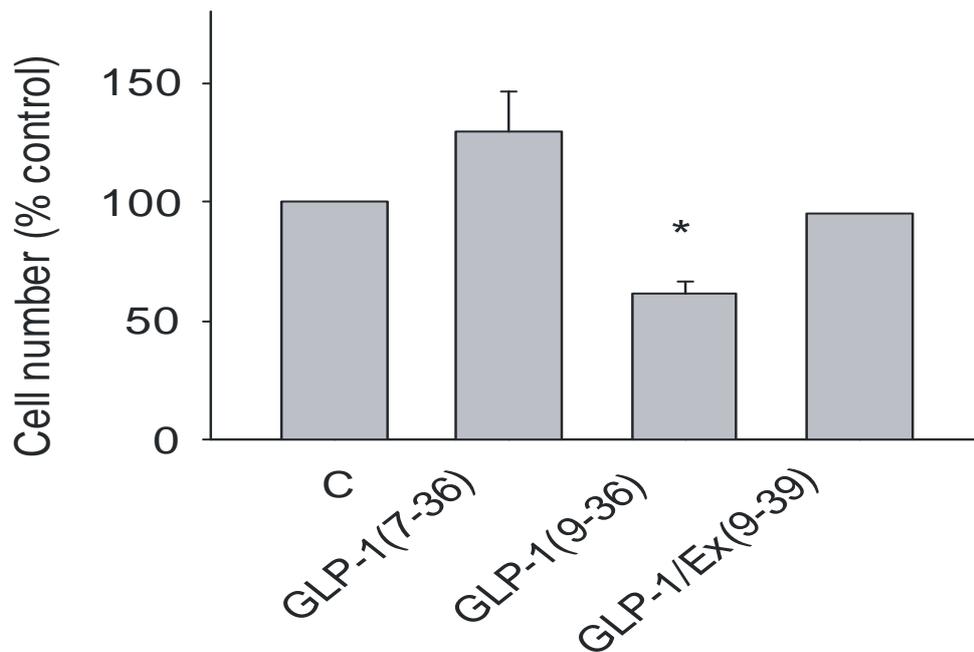
Figure 3: The cell number from Ki67 stained cells presented as percentage of control, the result are from three experiments (* P<0.05).

The above results indicate that GLP-1(7-36), secreted from the GLP-1-secreting cells is able to stimulate the cell proliferation by a feedback mechanism. Such feedback action manner has been seen in other cells, such as the pancreatic β-cell, where insulin stimulates insulin gene expression and secretion. The present results suggest that such an autocrine/paracrine action may also be functioning in the GLP-1 secreting cells in regulation of cell proliferation. Considering that the GLP-1 metabolite GLP-1(9-36), which has poor affinity to the GLP-1 receptor, was without effect on the cell proliferation; and that the effect of GLP-1(7-36) could be blocked by the GLP-1 receptor antagonist, the effect of GLP-1(7-36) on the cell proliferation is likely to be mediated through the GLP-1 receptor.

The lack of effect by exendin-4 may suggest different action manner of GLP-1 and exendin-4 [22, 23, 29]. In addition, the presence of distinct exendin receptor has been proposed [28]. This finding raised a question: whether the GLP-1 receptor is expressed in the GLP-1-secreting cells?

2/. The GLP-1 receptor is expressed in the GLP-1-secreting cell GluTag

   To verify the present of GLP-1 receptors in GLUTag cells Western blot was carry out using insulin-secreting MIN6 cells as a positive control, which are known to express the GLP-1 receptor.

   As shown in Figure 4, the GLP-1 receptor is present in the GluTag cells, with approximately 2.5 times less in abundance, compared to those in MIN6 cells, after normalized band density with β-actin. The same amount of protein (20 ug) prepared from MIN6 or GluTag cells were loaded. However, the band densities of β-actin revealed different, indicating different amount of protein loaded. The losses of MIN6 protein probably happened during sample loading process.
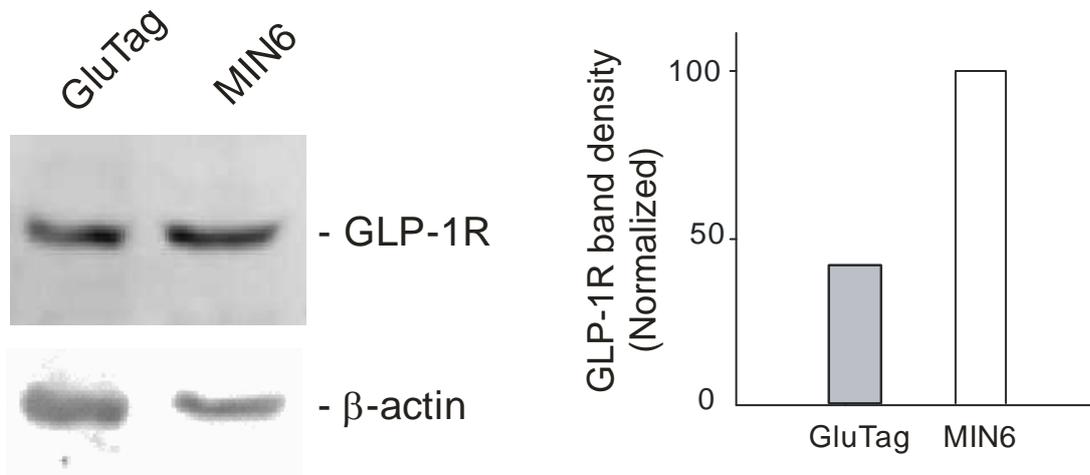
Figure 4*: **Left***) Western blot of GLP-1R express in GLUTag cells and Min6 cells (The same amount of protein was loaded to each well 20 μg per lane). **Left:** Antibody directed against GLP-1R was performed and a band with strong intensity was obtained corresponding to the GLP-1 receptor molecular mass 53kDa. **Right:** The membranes were also incubated with β-actin to establish band density.*

   Taken together, the above results suggest that GLP-1 secreted from the GLP-1-secreting cells promotes proliferation of the cells through an autocrine/paracrine mechanism mediated by the GLP-1 receptor expressed in the cells. The absence of the proliferative effect by exendin-4 may suggest different signaling and function manners of GLP-1(7-36) and exendin-4 in the cells.

Conclusion and future work

The result obtained suggests:

1. GLUTag cells express GLP-1 recepors
2. GLP-1(7-36) enhances cell proliferation in GLUtag cells
3. Exendin-4 and GLP-1(9-36) do not have effect on cell proliferation
4. The GLP-1(7-36)-induced proliferation of GluTag cells is blocked by Ex4(9-36) in GLUTag cells.

Future work:

In order to further validate these finding, further studies are required. After having investigated the agent's effect on cell proliferation, the mechanism underlying this effect needs to be studied. This includes studying whether GLP-1 receptor **actually** is activated in GLUTag cells, by investigate the signal transduction pathways activated by GLP-1(7-36) and the post- receptor activations in this event. In addition the requirement of GLP-1 receptor in the GLP-1(7-36)-induced proliferation of GluTag cells should also be studied, in cells with GLP-1 receptor knockdown using siRNA.

Acknowledgement

First and foremost I owe a great debt and gratitude to my supervisor Md. Qimin Zhang for giving me the opportunity of doing my MSc thesis here at Forskningcentrum Karolinska Institutet Södersjukhuset and more importantly, for all support, knowledge and guidance during my project.

I would also like to extend my gratitude to PhD-student Camilla Kappe for supporting my project with cells and demonstrating all the techniques. This work should not have been possible without all your help.

I also want to thanks the Head of the Unit for Diabetes Research, Åke Sjöholm for given me the opportunity to do my thesis in his group. I also want to acknowledge all the other PhD-students in Åke Sjöholm group and rest of the staff at Forskningcentrum.

Finally I also want to thank my examiner Sven Hamp from Märladalen University.

References

1. Wild S, Roglic G, Green A, Sicree R, King H. *Global prevalence of diabetes: estimates for the year 2000 and projections for 2030* Diabetes Care. 2004 May;27(5):1047-53.
2. Kumar P, Clark M *Clinical Medicine.* 6th edn. WB Saunders, London (2005)
3. Cohn RM, Roth KS *Biochemistry and Disease.* Williams and Wilkins, London (1996)
4. Stumvoll M, Goldstein BJ, Von Haeften TW. *Type 2 diabetes: principles of pathogenesis and therapy. Lencet, 365:1333-1346,*
5. John C. Pickup, Williams Gareth , 1997 *Textbook of Diabetes Volume* 1 Blackwell Science Ltd, Oxford
6. American Diabetes Association. *Standards of medical care for patients with diabetes mellitus*. Diabetes Care. 2002;25(1):213Y229
7. The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. *Follow-up report on the diagnosis of diabetes mellitus.* Diabetes Care. 2003;26(11):3160Y3167
8. Meier JJ, Nauck Ma, *Glucagon-like peptide-1 (GLP-1) in biology and pathology.* Diabetes Metab Res Rev 21:91-117,2005
9. Dupre J, Ross SA, Watson D et al. *Stimulation of insulin secretion by gastric inhibitory polypeptide in man*. The Journal of Clinical Endocrinology and Metabolism 1973; 37: 826–828
10. Cataland S, Crockett SE, Brown JC, Mazzaferri EL. *Gastric inhibitory polypeptide (GIP) stimulation by oral glucose in man.* J Clin Endocrinol Metab. 1974 Aug;39(2):223-8.
11. Lynn FC et al., Diabetes 50:1004–1011, 2001; Zhou J et al., Am J Physiol Endocrinol Metab 293:E538–E547, 2007
12. Vilsboll T, Krarup T, Sonne J, Madsbad S, Volund A, Juul AG, Holst JJ. *Incretin secretion in relation to meal size and body weight in healthy subjects and people with type 1 and type 2 diabetes mellitus.* J Clin Endocrinol Metab 88: 2706–2713, 2003
13. Miholic J, Orskov C, Holst JJ, Kotzerke J, Meyer HJ. *Emptying of the gastric substitute, glucagon-like peptide-1 (GLP-1), reactive hypoglycemia after total gastrectomy*. Dig Dis Sci 36: 1361–1370, 1991.
14. Mortensen K, Christensen LL, Holst JJ, Orskov C. *GLP-1 and GIP re colocalized in a subset of endocrine cells in the small intestine*. Regul Pept 2003;114:189–196
15. Ugleholdt R, Zhu X, Deacon CF, Orskov C, Steiner DF, HolstJJ. *Impaired intestinal proglucagon processing in mice lacking prohormone convertase 1.* Endocrinology 145: 1349–1355, 2004
16. Zhu X, Zhou A, Dey A, Norrbom C, Carroll R, Zhang C, LaurentV, Lindberg I, Ugleholdt R, Holst JJ, Steiner DF. *Disruption of PC1/3 expression in mice causes dwarfism and multiple neuroendocrine peptide processing defects*. Proc Natl Acad Sci USA 99: 10293–10298, 2002.
17. JENS JUUL HOLST.*The Physiology of Glucagon-like Peptide 1* Physiol Rev. 2007 Oct;87(4):1409-39

18. Unger RH, Ohneda A, Valverde I, Eisentraut AM, Exton J. *Characterization of the responses of circulating glucagon-like immunoreactivity to intraduodenal and intravenous administration of glucose*. J Clin Invest 1968;47:48–65.

19. Hermann C, Goke R, Richter G, Fehmann HC, Arnold R, Goke B. *Glucagon-like peptide-1 and glucose-dependent insulin-releasing polypeptide plasma levels in response to nutrients*. Digestion 1995;56:117–126.

20. Theodorakis MJ, Carlson O, Michopoulos S, Doyle ME, Juhaszova M, Petraki K, Egan JM. *Human duodenal enteroendocrine cells: source of both incretin peptides, GLP-1 and GIP*. Am J Physiol 2006;290:E550–E559.

21. Baggio LL, Drucker DJ. *GLP-1 and GIP Gastroenterology.*Biology of incretins: 2007 May;132(6):2131-57.

22. Orskov C, Wettergren A, Holst JJ. *Biological effects and metabolic rates of glucagonlike peptide-1 7-36 amide and glucagon like peptide-1 7-37 in healthy subjects are indistinguishable*.Diabetes 1993;42:658–661

23. Orskov C, Rabenhoj L, Wettergren A, Kofod H, Holst, JJ. *Tissue and plasma concentrations of amidated and glycine-extended glucagon-like peptide I in humans.* Diabetes 1994;43:535– 539.

24. Deacon CF, Johnsen AH, Holst JJ. *Degradation of glucagon-like peptide-1 by human plasma in vitro yields an N-terminally truncated peptide that is a major endogenous metabolite in vivo.* J Clin Endocrinol Metab 1995;80:952–957

25. Hansen L, Deacon CF, Orskov C, Holst JJ. *Glucagon-like peptide-1-(7-36)amide is transformed to glucagon-like peptide-1-(9-36)amide by dipeptidyl peptidase IV in the capillaries supplying the L cells of the porcine intestine.* Endocrinology 1999, 140:5356-5363.

26. Ruiz-Grande C, Alarcon C, Alcantara A, Castilla C, Lopez Novoa JM, Villanueva-Penacarrillo ML, Valverde I. *Renal catabolism of truncated glucagon-like peptide 1.* Horm Metab Res 1993;25: 612–616

27. Mayo KE, Miller LJ, Bataille D, Dalle S, Goke B, Thorens B,Drucker DJ. *The Glucagon Receptor Family.* Pharmacol Rev 55: 167–194, 2003 International Union of Pharmacology. XXXV

28. Thorens B. *Expression cloning of the pancreatic beta cell receptor for the gluco-incretin hormone glucagon-like peptide 1.* Proc Natl Acad Sci USA 89: 8641–8645, 1992.

29. Thorens B, Porret A, Buhler L, Deng SP, Morel P, Widmann C. *Cloning and functional expression of the human islet GLP-1 receptor. Demonstration that exendin-4 is an agonist and exendin- (9–39) an antagonist of the receptor.* Diabetes 42: 1678–1682, 1993

30. Alvarez E, Martinez MD, Roncero I, Chowen JA, Garcia-Cuartero B, Gispert JD, Sanz C, Vazquez P, Maldonado A, de CJ, Desco M, Pozo MA, Blazquez E. *The expression of GLP-1receptor mRNA and protein allows the effect of GLP-1 on glucose metabolism in the human hypothalamus and brainstem*. J Neurochem 92: 798–806, 2005

31. Bullock BP, Heller RS, Habener JF. *Tissue distribution of messenger Ribonucleic acid encoding the rat glucagon-like peptide-1 receptor.* Endocrinology 137: 2968–2978, 1996.

32. Campos RV, Lee YC, Drucker DJ. *Divergent tissue-specific and developmental expression of receptors for glucagon and glucagonlike peptide-1 in the mous*e. Endocrinology 134: 2156–2164, 1994.

33. Wei Y, Mojsov S. *Tissue-specific expression of the human receptor for glucagon-like peptide-I: brain, heart and pancreatic forms have the same deduced amino acid sequences*. FEBS Lett 358: 219–224, 1995.

34. Wei Y, Mojsov S. *Distribution of GLP-1 and PACAP receptors in human tissues.* Acta Physiol Scand 157: 355–357, 1996.

35. Hallbrink M, Holmqvist T, Olsson M, Ostenson CG, Efendic S, Langel U. *Different domains in the third intracellular loop of the GLP-1 receptor are responsible for Galpha(s) and Galpha(i)/ Galpha(o) activation.* Biochim Biophys Acta 2001;1546:79–86.

36. Holz GG. *Epac: a new cAMP-binding protein in support of glucagon-like peptide-1 receptor-mediated signal transduction in the pancreatic beta-cell.* Diabetes 53: 5–13, 2004.

37. Baggio LL *Gastroenterology* 2007;132:2131–2157).

38. Deacon CF. *What do we know about the secretion and degradation of incretin hormones?* Regulatory Peptides 2005; 128:117–124.

39. Perley M, Kipnis DM. *Plasma insulin responses to oral and intravenous glucose: studies in normal and diabetic subjects*. J Clin Invest 46: 1954–1962, 1967.

40. Nauck MA, Homberger E, Siegel EG, Allen RC, Eaton RP, Ebert R, Creutzfeldt W. *Incretin effects of increasing glucose loads in man calculated from venous insulin and C-peptide responses*. J Clin Endocrinol Metab. 1986 Aug;63(2):492-8

41. McIntyre N, Holdsworth CD, Turner DS. *Intestinal factors in the control of insulin secretion.* Secretion.*J Clin Endocrinol Metab* 25: 1317–1324, 1965.

42. Vilsboll T, Holst JJ. *Incretins, insulin secretion and Type 2 diabetes mellitus.* Diabetologia 47: 357–366, 2004.

43. Buteau J, Foisy S, Joly E, Prentki M, *Glucagon-like Peptide-1 Induces Pancreatic β-cell proliferation via transactivation of the epidermal growth factor receptor.* Diabetes 2003, 52:124-132

44. Buteau J El-Assaad W, Rhodes CJ, Rosenberg L, Joly E, *Glucagon-like peptide-1 prevents beta cell glucolipotoxicity.* Diabetologia 2004, 47:806-815

45. During MJ, Cao L, Zuzga DS, Francis JS, Fitzsimons HL, Jiao X, Bland RJ, Klugmann M, Banks WA, Drucker DJ, Haile CN. *Glucagon-like peptide-1 receptor is involved in learning and neuroprotection.* Nat Med 9: 1173–1179, 2003.

46. Willms B, Werner J, Holst JJ, Orskov C, Creutzfeldt W, Nauck MA. *Gastric emptying, glucose responses, and insulin secretion after a liquid test meal: effects of exogenous glucagon-like peptide-1 (GLP-1)-(7-36) amide in type 2 (noninsulin-dependent) diabetic patients.* J Clin Endocrinol Metab 1996;81:327–332.

47. Meier JJ, Gallwitz B, Salmen S, Goetze O, Holst JJ, Schmidt WE, Nauck MA. *Normalization of glucose concentrations and deceleration of gastric emptying after solid meals during intravenous glucagon-like peptide 1 in patients with type 2 diabetes*. J Clin Endocrinol Metab 2003;88:2719–2725.

48. Fehmann HC, Goke R, Goke B. *Cell and molecular biology of the incretin hormones glucagon-like peptide-I and glucose-dependent insulin releasing polypeptide*. Endocr Rev 16: 390–410, 1995.

49. Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC. *Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes*. Diabetes 2003; 52: 102–110.

50. Buteau J, El-Assaad W, Rhodes CJ, Rosenberg L, Joly E, Prentki M. *Glucagon-like peptide-1 prevents beta cell glucolipotoxicity*. Diabetologia 47: 806–815, 2004.

51. Farilla L, Bulotta A, Hirshberg B, Li CS, Khoury N, Noushmehr H, Bertolotto C, Di MU, Harlan DM, Perfetti R. *Glucagon-like peptide 1 inhibits cell apoptosis and improves glucose responsiveness of freshly isolated human islets*. Endocrinology 144: 5149–5158, 2003.

52. Bonner-Weir S. *Beta-cell turnover: its assessment and implications*. Diabetes 50 Suppl 1: S20–S24, 2001.

53. Tyrberg B, Ustinov J, Otonkoski T, Andersson A. *Stimulated endocrine cell proliferation and differentiation in transplanted human pancreatic islets: effects of the ob gene and compensatory growth of the implantation organ*. Diabetes 2001; 50: 301–307

54. Wang Y, Egan JM, Raygada M, Nadiv O, Roth J, Montrose Rafizadeh C. *Glucagon-like peptide-1 affects gene transcription and messenger ribonucleic acid stability of components of the insulin secretory system in RIN 1046-38 cells*. Endocrinology 1995; 136: 4910–4917.

55. Hu FB, Manson JE, Stampfer MJ, *et al*. *Diet, lifestyle, and the risk of type 2 diabetes mellitus in women*. New Engl J Med 2001; 345: 790–797

56. Nauck M, Sto¨ckmann F, Ebert R et al. *Reduced incretin effect in type 2 (non-insulin-dependent) diabetes*. Diabetologia 1986; 29: 46–54.

57. Nauck MA, Heimesaat MM, Orskov C, Holst JJ, Ebert R, Creutzfeldt W. *Preserved incretin activity of glucagon-like peptide 1 [7–36 amide] but not of synthetic human gastric inhibitory polypeptide in patients with type-2 diabetes mellitus*. J Clin Invest 91: 301–307, 1993.

58. Toft-Nielsen MB, Damholt MB, Madsbad S, Hilsted LM, Hughes TE, Michelsen BK, Holst JJ. *Determinants of the impaired secretion of glucagon-like peptide-1 in type 2 diabetic patients*. J Clin Endocrinol Metab 86: 3717–3723, 2001

59. Vilsboll T, Krarup T, Deacon CF, Madsbad S, Holst JJ. *Reduced postprandial concentrations of intact biologically active glucagon-like peptide 1 in type 2 diabetic patients*. Diabetes 50: 609–613, 2001.

60. ChenYE, Drucker DJ. *Tissue-specific expression of unique mRNAs that encod proglucagon-derived peptides or exendin 4 in the lizard*. J Biol Chem 1997; 272: 4108–15.

61. Thorens B, Porret A, Buhler L, Deng SP, Morel P, Widmann C. *Cloning and functional expression of the human islet GLP-1 receptor. Demonstration that exendin-4 is an agonist and exendin- (9–39) an antagonist of the receptor*. Diabetes 42: 1678–1682, 1993

62. Vilsboll T, Krarup T, Madsbad S, Holst JJ. *Defective amplification of the late phase insulin response to glucose by GIP in obese Type II diabetic patients.* Diabetologia 45: 1111–1119, 2002

63. Kendall, D.M., et al., *Effects of exenatide (exendin-4) on glycemic control over 30 weeks in patients with type 2 diabetes treated with metformin and a sulfonylurea.* Diabetes Care, 2005. 28(5): p. 1083-91.

64. Egan, J.M., A.R. Clocquet, and D. Elahi, *The insulinotropic effect of acute exendin-4 administered to humans: comparison of nondiabetic state to type 2 diabetes*. J Clin Endocrinol Metab, 2002. 87(3): p. 1282-90.

65. Thorens B, Porret A, Buhler L, Deng SP, Morel P, Widmann C.*Cloning and functional expression of the human islet GLP-1 receptor. Demonstration that exendin-4 is an agonist and exendin- (9–39) an antagonist of the receptor*. Diabetes 42: 1678–1682, 1993