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**MOLECULAR REGULATION  
OF HORMONE SECRETION,  
GROWTH AND APOPTOSIS  
OF GLP-1-PRODUCING  
CELLS**

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## ABSTRACT

Type 2 diabetes (T2D) spreads like an epidemic in today's society, and there is a great need for new and improved treatments. T2D is characterized by hyperglycemia, resulting from impaired insulin production and insulin resistance in peripheral tissues. Incretin hormones, such as glucagon-like peptide-1 (GLP-1), secreted from L-cells dispersed along the intestinal tract, potentiate meal-stimulated insulin secretion in a glucose-dependent manner. Defective GLP-1 secretion has been indicated in T2D and administration of GLP-1 to T2D patients restores glucose-induced insulin secretion and normalizes glycemia, making stable analogs of GLP-1 among the best available treatments for T2D today. However, enhancing endogenous GLP-1 production/secretion by direct stimulation of GLP-1 secretion/promotion of growth and viability of L-cells may be a novel and more physiological option in incretin-based diabetes therapy. The aim of this work was to determine the effect of diabetic conditions and anti-diabetic agents on GLP-1-producing cells, in order to unravel some of the mechanisms regulating growth, survival and function of this cell type.

Studies I-III were performed *in vitro* using the murine GLUTag cell line as a model. In study I, direct effects of metformin on apoptosis, and function of GLP-1-secreting cells were determined. Simulated diabetic hyperlipidemia resulted in increased caspase-3 activity and DNA fragmentation, indicating lipoapoptosis. Metformin treatment significantly decreased this lipoapoptosis in conjunction with increased phosphorylation of AMPK. In addition, metformin treatment stimulated GLP-1 secretion.

In study II, we determined molecular mechanisms mediating lipotoxicity and metformin-induced lipoprotection in GLP-1-secreting cells. Diabetic hyperlipidemia was simulated in this cell system by addition of the fatty acid palmitate. Palmitate increased ROS production in GLP-1-secreting cells, and the lipotoxic effects of palmitate were abolished in the presence of the antioxidant Trolox. Further, palmitate phosphorylated p38 MAPK and inhibition of this enzyme significantly reduced lipoapoptosis. Pre-incubation with metformin further increased palmitate-induced ROS production, while significantly reducing the expression of p38 MAPK.

Study III focused on direct effects of insulin and exendin-4/GLP-1 on lipoapoptosis and function of GLP-1-secreting cells. The GLP-1R was found to be expressed in the GLUTag cells, and diabetic lipotoxicity was partially inhibited by pre-incubation with insulin or the stable GLP-1 analog exendin-4. The lipoprotective effect of exendin-4 was GLP-1R-dependent, while independent of PKA activity. In addition, both insulin and exendin-4 significantly stimulated acute and long term GLP-1 secretion in the presence of glucose. In study IV, we investigated if a high fat diet (HFD) reduces the number of enteroendocrine GLP-1-secreting L-cells in C57/Bl6 mice. We also determined the effects of a HFD on GLP-1 plasma levels and possible effects on these parameters by metformin treatment. A HFD rapidly induced a diabetic phenotype with increased HbA<sub>1c</sub> levels, as well as fasting plasma insulin levels in conjunction with reduced oral glucose tolerance – indicating

the manifestation of insulin resistance. A 14 day oral administration of metformin reduced HbA<sub>1c</sub>, fasting insulin and prandial FFA levels. The number of L-cells was significantly reduced after 12 weeks on a HFD, while - - in contrast -- there was a clear trend toward increased prandial plasma GLP-1 levels despite reduced food intake in HFD-fed mice.

These findings may be of pathogenic significance not only in understanding mechanisms of the impaired incretin response characterizing T2D patients, but may also be harnessed to therapeutic advantage in efforts to enhance endogenous GLP-1 production. Such an approach has hitherto received little attention but may be superior to contemporary incretin-based antidiabetic therapy, which does not faithfully mimic physiologic GLP-1 release in for instance terms of secretory pattern (*e.g.* pulsatility) and actions on topographically adjacent hormone receptors (*e.g.* in the portal vein).

## LIST OF PUBLICATIONS

*This thesis is based on the following original papers, which will be referred to in the text by their Roman numerals.*

- I. Kappe, C., Patrone C., Holst, J.J., Zhang, Q., Sjöholm, Å. Metformin protects against lipoapoptosis and enhances GLP-1 secretion from GLP-1-producing cells. *J Gastroenterol* 2012
- II. Kappe, C., Holst, J.J., Zhang, Q., Sjöholm, Å. Molecular mechanisms of lipoapoptosis and metformin protection in GLP-1-secreting cells. *Biochem Biophys Res Commun* 2012; 427:91-95.
- III. Kappe, C., Holst, J.J., Zhang, Q., Sjöholm, Å. Evidence for paracrine/autocrine regulation of GLP-1-producing cells (Submitted).
- IV. Kappe, C., Holst, J.J., Zhang, Q., Sjöholm, Å. Regulation of GLP-1-producing cells *in vivo*: Effects of high-fat diet and the anti-diabetic drug metformin (Manuscript).

*Other publications, not included in the thesis:*

- I. Darsalia, V., Mansouri, S., Ortsäter, H., Olverling, A., Nozadze, N., Kappe, C., Iverfeldt, K., Tracy, L.M., Grankvist, N., Sjöholm, Å., Patrone, C. Glucagon-like peptide-1 receptor activation reduces ischaemic brain damage following stroke in type 2 diabetic rats. *Clin Sci (Lond)*. 2012; 122: 473-483.
- II. Kappe, C., Tracy, L.M., Iverfeldt, K., Sjöholm, Å. GLP-1 secretion by microglial cells and decreased CNS expression of proglucagon in obesity. *J. Neuroinflammation*. Conditionally Accepted.
- III. Wu, L., Olverling, A., Fransson, L., Ortsäter, H., Kappe, C., Gao, X, Sjöholm, Å. Early intervention with liraglutide improves glucose tolerance without affecting islet microcirculation in young Goto-Kakizaki rats. *Regul Pept*. 2012; 177: 94-96.

## LIST OF ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
AMPK	AMP-activated protein kinase
ASK-1	Apoptosis signal regulating kinase 1
ATP	Adenosine triphosphate
BMI	Body mass index
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CPT-1	Carnitine palmitoyltransferase-1
CREB	cAMP response element-binding protein
CD	Control diet
DMEM	Dulbecco's modified eagle medium
DPP-4	Dipeptidyl peptidase-4
ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
EPAC	Exchange protein activated by cAMP
FBS	Fetal bovine serum
FFA	Free fatty acid
Foxa2	Forkhead box protein A2
FoxO1	Forkhead box protein O1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide-1
GLP-1R	GLP-1 receptor
GLUT-2	Glucose transporter 2
GLUT-4	Glucose transporter 4
GPR40	G protein-coupled receptor 40
GPR119	G protein-coupled receptor 119
GPR120	G protein-coupled receptor 120
GSIS	Glucose-stimulated insulin secretion
HFD	High fat diet
IGT	Impaired glucose tolerance
IR	Insulin receptor
IRS	Insulin receptor substrate
JNK	c-Jun <i>N</i> -terminal kinase
K <sub>ATP</sub> channels	ATP-sensitive potassium channels
Malonyl-CoA	Malonyl coenzyme A
MAPK	Mitogen activated protein kinase
MAPKK	Mitogen activated protein kinase kinase
mTOR	Mammalian target of rapamycin
NEFA	Non-esterified fatty acid
OCT-1	Organic cation transporter

p53	p53 tumor suppressor protein
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDX-1	Pancreatic duodenal homeobox 1
PI3K	Phosphatidylinositol 3 kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PMAT	Plasma membrane monoamine transporter
PPAR $\gamma$	Peroxisome proliferator activated receptor $\gamma$
PVDF	Polyvinylidene fluoride
qPCR	Quantitative reverse transcriptase PCR
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SGLT	Sodium–glucose transporter
siRNA	Short interfering RNA
T2D	Type 2 diabetes
TCF7L2	Transcription factor 7-like 2
TGR5	G protein-coupled bile acid receptor
TPA	12- <i>O</i> -tetradecanoylphorbol-13-acetate
UCP2	Uncoupling protein 2

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# INTRODUCTION

## 1.1 TYPE 2 DIABETES

T2D is a syndrome characterized by disordered metabolism, resulting in inappropriately high blood sugar (hyperglycemia). According to the World Health Organization (WHO), diabetes is diagnosed by fasting plasma glucose levels  $> 7$  mmol/l, or by a plasma glucose concentration  $> 11.1$  mmol/l 2 h following an oral glucose load. The characteristic feature of the disease results from a combination of impaired insulin production from the pancreatic  $\beta$ -cells and insulin resistance in peripheral tissues. The most common long-term complication is cardiovascular disease which stands for 75-80 % of all deaths related to diabetes [1]. Clinical management of the disease involves a combination of dietary treatment, application of oral anti-diabetic drugs and oftentimes also insulin injections. Diabetes is widespread and it is the fourth leading cause of death in the U.S. [1]. According to the WHO, its incidence is increasing rapidly and is estimated to double by the year 2030 and the expenses to diabetes have been shown to be a major drain on health- and productivity-related resources for healthcare systems and governments. In the U.S. alone, the annual cost for diabetes amounts to the humongous sum of \$ 174 billion, of which  $\sim 97$  % is targeted to T2D. Improved glycemia is still the main focus of T2D therapy and glycated hemoglobin ( $\text{HbA}_{1c}$ ) levels of 5-6 % (DCCT standard; corresponding to 31-42 mmol/mol by IFCC standard) are recommended treatment goals. However, more than 50 % of patients with T2D have a  $\text{HbA}_{1c}$  level of  $>7$  % (53 mmol/mol by IFCC standard) and are thus inadequately controlled [2].

## 1.2 THE INCRETIN SYSTEM AND GLP-1

### 1.2.1 GLP-1 and the GLP-1 receptor

Glucagon-like peptide-1 (GLP-1) is a small peptide hormone, released from enteroendocrine intestinal L-cells in response to hormonal, neural and nutrient stimuli, which exerts numerous pleiotropic effects in various tissues [3]. The most well-known action of GLP-1 is enhanced insulin release in the context of hyperglycemia [4]. The biologically active forms of GLP-1 are GLP-1(7-37) and GLP-1(7-36) $\text{NH}_2$ , the actions of which are exerted through a G protein-coupled receptor. The GLP-1 receptor (GLP-1R) was first cloned from rat pancreatic islets [5] and is widely distributed and expressed in several organs: pancreatic islets, stomach, heart, intestine, adipocytes, lung and kidney, as well as in the CNS [6-10]. Although previously not believed to be expressed in hepatocytes, some recent studies have reported the presence of the GLP-1R on human hepatocytes [11], and GLP-1 has been shown to promote glycogen accumulation in rat hepatocytes [12-13], independent of changes in insulin [14].

Activation of the GLP-1R results in stimulation of adenylate cyclase, increased intracellular cAMP levels [15], and subsequent activation of protein

kinase A (PKA) [16] and exchange protein activated by cAMP 2 (EPAC2) [17].

### 1.2.2 The incretin effect and pancreatic actions of GLP-1

Blood glucose is maintained within normal ranges by sophisticated mechanisms under physiological conditions. In diabetes, insufficient insulin production, glucagon excess and peripheral insulin resistance result in hyperglycemia and other metabolic disturbances. Under physiological conditions, glucose is the major stimulator of insulin secretion. Incretin hormones such as glucose-dependent insulintropic polypeptide (GIP) and GLP-1 serve to augment meal-stimulated insulin secretion from  $\beta$ -cells in a glucose-dependent manner [18]. The incretin effect is described as the ability of gastrointestinal hormones such as GLP-1, released in response to food intake, to stimulate insulin release from the endocrine pancreas [19]. The incretin effect can be quantified by measuring insulin secretion after oral glucose ingestion and after intravenous infusion of glucose resulting in an equivalent rise in plasma glucose concentration, and accounts for 20 to 60 % of the overall postprandial insulin secretion in healthy subjects [20].

Intracellular cAMP levels are critical for normal glucose-stimulated insulin secretion (GSIS) [21]. Consequently, receptors, such as the GLP-1R, that are associated with increased cAMP levels have the potential to play an important part in GSIS. GLP-1-mediated activation of PKA/EPAC results in closure of ATP-sensitive  $K^+$  channels, depolarization of the plasma membrane and opening of voltage-gated  $Ca^{2+}$  channels, influx of  $Ca^{2+}$  and the subsequent triggering of insulin exocytosis [22-25]. Activation of the GLP-1R also increases intracellular  $Ca^{2+}$  through  $Ca^{2+}$  release from the endoplasmic reticulum via inositol 1,4,5 trisphosphate receptors activated by PKA and ryanodine receptors activated by EPAC2 [26-27]. However, contribution of other signaling pathways, including protein kinase C (PKC) through activation of phospholipase C (PLC) and phosphatidylinositol 3-kinase gamma (PI3-K $\gamma$ ), in GSIS has also been reported [20, 28-29]. Previous studies have shown that activation of PI3-K $\gamma$  plays an essential role in the regulation of GLP-1-stimulated insulin secretion, and that PI3-K $\gamma$  deficiency may result in dissociation between cAMP levels and GSIS [28].

*In vitro* and *in vivo* studies show that GLP-1, and its analogs, induce  $\beta$ -cell proliferation in normal rodent islets and insulinoma cell lines [30-32], and that GLP-1R activation also reduces  $\beta$ -cell apoptosis in purified rodent and human islets as well as  $\beta$ -cell lines after exposure to many cytotoxic agents, including reactive oxygen species (ROS), palmitate, and dexamethasone [33]. To stimulate  $\beta$ -cell proliferation and survival, GLP-1 acts through PI3K/protein kinase B (PKB) [34]. Specifically, promotion of forkhead box protein O1 (FoxO1) nuclear exclusion and subsequent up-regulation of the transcription factors pancreatic duodenal homeobox 1 (PDX-1) and forkhead box protein A2 (Foxa2) expression via PI3K-dependent activation of PKB and cAMP/PKA-dependent activation of cAMP response element-binding protein (CREB), lead to up-regulation of insulin receptor substrate 2 (IRS2) protein expression and activation of PKB [35]. In addition, a role for EPAC

in GLP-1R-mediated reductions of induced ROS production has also been demonstrated [36].

GLP-1 also decreases glucagon secretion from the pancreas in the presence of hyperglycemia, an effect that ceases at glucose levels  $< 4$  mmol/l, thus also involving low risk of hypoglycemic side effects [4]. However, whether this inhibition of glucagon secretion is a direct effect on the glucagon-secreting  $\alpha$ -cells or an indirect effect through insulin is still debated. However, there are reports on low expression of the GLP-1R on  $\alpha$ -cells. Further, it has been shown that in  $\alpha$ -cells elevation of intracellular cAMP levels exerts both inhibitory and stimulatory effects via activation of PKA and EPAC, respectively [37]. Specifically, the very low GLP-1R expression results in small increases in cAMP as compared to activation of G protein-coupled adrenalin receptors, which are abundant on the  $\alpha$ -cells. Consequently, GLP-1R activation induces inhibitory PKA activity, but does not induce the stimulatory EPAC activity seen in response to adrenalin [37].

### 1.2.3 Extrapaneatic actions of GLP-1

A portal signal is of importance for the glucose lowering effects of GLP-1 [38] and intraportal GLP-1 increases insulin secretion and glucose clearance [39]. Further, a role for GLP-1 in the portal glucose sensor has been reported [40]. As the GLP-1 produced in the L-cells is drained into the portal circulation prior to hepatic passage and extraction, this particular route may not be targeted by the therapeutic injection of GLP-1 analogs used in contemporary T2D therapy. Conversely, enhancing short- or long-term endogenous GLP-1 production may thus offer treatment benefits as compared to exogenous GLP-1 replacement therapy.

GLP-1 regulates gastrointestinal motility and gastric secretion by mechanisms dependent on vagal innervations [41-44]. In addition, pharmacological levels of GLP-1 inhibit both gastric emptying and gastric acid secretion in human subjects [45]. This inhibitory effect of GLP-1 on gastric emptying may account for the glucose-lowering effects observed not only in T2D but also in type 1 diabetic patients [46].

The GLP-1R is expressed throughout the CNS, where GLP-1 exerts actions regulating satiety and food intake [20, 47]. Further, GLP-1R activation in the CNS has been shown to exert neuroprotection, with beneficial effects in neurological disorders, including Alzheimer's and Parkinson's disease [48-50].

GLP-1 has also been shown to exert effects on fat and muscle cells *in vitro*, including increased fatty acid synthesis [51] and increased glucose uptake [52] in adipocytes, as well as stimulated glycogen synthesis and glucose utilization in cultured myotubes [53]. However, the nature and physiological relevance of these actions remain to be confirmed.

### **1.3 GLP-1 IN OBESITY, INSULIN RESISTANCE, TCF7L2 POLYMORPHISMS AND TYPE 2 DIABETES**

Attempts to identify genes predisposing for T2D have shown a strong association between the transcription factor 7-like 2 (TCF7L2) rs7903146T allele and T2D, independent of ethnicity [54-55]. Further studies have revealed defective insulin secretion following oral glucose tolerance test (OGTT), but not following i.v. glucose challenge, in carriers of this allele [56]. The differential effects of orally and i.v. administered glucose provoked assessment of incretin-induced insulin secretion in patients with variants in the TCF7L2 gene. Resulting data support impaired GLP-1-induced insulin secretion with variants of TCF7L2, resulting from a functional defect in the GLP-1 signaling in  $\beta$ -cells - rather than defect GLP-1 secretion [56].

However, defects in the response to GLP-1 are not generally associated with T2D [57], and further studies are necessary to rule out involvement of other stimuli -- such as GIP [58] -- and confirm a potential specific defect in  $\beta$ -cell GLP-1 signaling with TCF7L2 variants and the central role of GLP-1 in the pathophysiology of T2D that this would infer.

In addition, defective GLP-1 secretion has been reported in T2D [59-61], indicated to result from defective secretion of the hormone and not from a transcriptional defect, as increased intestinal proglucagon mRNA has been observed [62] [63]. However, some studies have failed to show defective GLP-1 secretion in T2D, and a generalized association of defective GLP-1 secretion and T2D as such cannot be made [64].

The strong co-morbidity of obesity and T2D has led to studies on effects of TCF7L2 polymorphisms on obesity. Such studies reveal that TCF7L2 is not a risk factor for obesity, but obesity modifies the risk that TCF7L2 variants confer [65]. Although different variants of TCF7L2 display differential patterns of association with BMI, rs7903146T was associated with a higher risk for T2D in non-obese subjects [65-66], suggesting that the mechanisms for glucose intolerance are different in severe obesity.

Decreased levels of circulating GLP-1 in response to meal ingestion have been linked to impaired glucose tolerance (IGT) [67-68] and increasing BMI [67]. Further, decreased GLP-1 levels have been observed with increased BMI and obesity in patients with or without T2D [69], where defective GLP-1 secretion has also been correlated to insulin resistance [70]. These data are further supported by the observation of defective GLP-1 secretion in T2D but not in type 1 diabetes [71].

Whether defective GLP-1 secretion in obesity overshadows indicated TCF7L2-associated defective GLP-1 signaling, and differences in BMI contribute to discrepancies between different studies of GLP-1 secretion/signaling, TCF7L2 polymorphisms and T2D is not known.

GLP-1 administration to patients with T2D may completely normalize hyperglycemia with the avoidance of hypoglycemia regardless of the GLP-1 dose administered due to the dual glucose-dependency of GLP-1 action on both insulin and glucagon secretion [20].

### **1.4 GLP-1 SYNTHESIS, SECRETION AND DEGRADATION**

GLP-1 is produced by posttranslational cleavage of proglucagon. Available evidence suggests that only one proglucagon gene is expressed in mammals

giving rise to an identical mRNA in brain, pancreas, and intestine [72]. Tissue-specific posttranslational cleavage of proglucagon gives rise to five distinct peptides: Glucagon in the pancreatic  $\alpha$ -cells -- although there are reports indicating a low expression of prohormone convertase 1 (PC1) and GLP-1 secretion also from pancreatic  $\alpha$ -cells [73] --, glicentin, oxyntomodulin, GLP-1 and GLP-2 in the intestine, while GLP-1 also is secreted in parts of the CNS. GLP-1 in the enteroendocrine L-cells results from proglucagon processing by the prohormone convertase PC1/3. Further, there is evidence for cell type-specific regulation of proglucagon gene expression mediated by the Wnt signaling pathway. Specifically, Wnt signaling regulates proglucagon expression in intestinal GLP-1-secreting cells, but not in pancreatic glucagon-producing cells. Interestingly, the T2D risk gene TCF7L2 is an effector of the Wnt signaling pathway and thus controls transcription of the proglucagon gene in L-cell lines [74-75]. In addition, knockdown of TCF7L2 in cells expressing the proglucagon gene reduced intestinal glucagon gene expression and glucose-induced insulin secretion in mice [74]. These mice also displayed defective glucose homeostasis; however, no effect on pancreatic glucagon gene expression was detected [74].

Plasma GLP-1 levels rise within 10–15 min of food ingestion and reach peak levels of 15–50 pmol/l by 40 min [76]. Further, GLP-1 -- like insulin -- is secreted in a pulsatile manner with a frequency comparable to that of pancreatic hormones [77].

#### 1.4.1 Nutrient-stimulated secretion

GLP-1 is secreted in a nutrient-dependent manner [78], where GLP-1 secretion is responsive not only to metabolizable sugars (glucose or fructose) and nonmetabolizable monosaccharides [79-80], but also to proteins and fats. The mechanisms of GLP-1 secretion has been investigated using different cell lines, and is indicated to involve membrane depolarization resulting in the opening of voltage-gated  $\text{Ca}^{2+}$  channels, where the resulting elevated  $\text{Ca}^{2+}$  concentrations stimulate secretion of GLP-1.

Glucose stimulates GLP-1 secretion in rodents and human subjects when given orally but systemic hyperglycemia does not activate L-cell secretion. It is indicated that L-cell glucose sensing is complex and involves several glucose-sensing mechanisms that work synergistically to define the glucose response, as reviewed in [81].

The intestinal L-cells express the  $\text{K}_{\text{ATP}}$  channel subunits and glucokinase is expressed in murine L-cells [82-84]. Further, the facilitative glucose transporter GLUT-2 plays a role in basolateral glucose flux in small intestinal epithelial cells, and if similarly expressed in L-cells could provide both a glucose efflux pathway and a potential means of responding to changes in glycemia [85]. However, the  $\text{EC}_{50}$  of glucose-induced GLP-1 secretion in models of the L-cells (0.2–0.5 mmol/l) differ substantially from the half-maximally effective substrate concentration of glucokinase ( $S_{0.5} \sim 8$  mmol/l) [86]. Another possible mechanism of glucose response is through sodium–glucose transporter (SGLT) activity [87]. SGLTs are expressed in L-cells and use an inwardly directed sodium gradient to facilitate glucose uptake into the cells, even at low luminal glucose concentrations. Using cell line models of L-cells, it has been shown that the electrogenic activity of

SGLT-1 is sufficient for membrane depolarization and GLP-1 release. There is, however, also evidence for the expression of sweet taste receptors [88] in intestinal endocrine cells. The effects of ligands for sweet taste receptors, such as artificial sweeteners, on GLP-1 secretion are still debated. However, there is data in support of a glucose responsiveness regulated by sweet taste receptors, where the activation of sweet taste receptors may alter SGLT expression [81, 89].

The mechanism of protein-triggered GLP-1 release remains unclear, but it has been reproduced by peptones [90-92], as well as by amino acids [93-94].

Fatty acids stimulate GLP-1 release in human subjects by enteral stimulation, whereas increased plasma levels of free fatty acids do not alter secretory response from GLP-1-secreting cells [95-96]. However, different types of fat differ in their ability to stimulate GLP-1 release, where saturated fatty acids induce a higher increase in plasma triacylglycerol but lower GLP-1 secretory response [97]. The molecular mechanisms linking fatty acids to GLP-1 secretion are not fully understood. However, short chain fatty acids (SCFAs) and SCFA receptors as well as G protein-coupled receptors (GPCR) are involved in mediating fatty acid-induced GLP-1 secretion [98-100].

GPR40, GPR119 and GPR120 are GPCRs that have fatty acids as ligands and are indicated to be involved in the fatty acid-induced GLP-1 secretion [100-101].

GPR40 and GPR119 are abundantly expressed in the human pancreas and gastrointestinal tract, and have attracted considerable interest as T2D drug targets in the past few years, as specific agonists for GPR40 and GPR119 have been demonstrated to mediate dual elevation of both insulin and GLP-1. Specifically, activation of GPR119 causes intracellular accumulation of cAMP, and enhanced glucose-dependent secretion of both insulin and GLP-1, and GPR40 is coupled to  $G_q$  and results in PKC- and inositol-1,4,5-trisphosphate-dependent  $Ca^{2+}$  release inducing insulin and GLP-1 secretion [102-117]. Interestingly, GPR40 and GPR119 agonists show promising antidiabetic effects in clinical trials [118-119].

GPR120 is expressed in L-cells and has been shown to mediate GLP-1 release *in vitro* [99].

However, there may be additional fatty acid-responsive pathways. Specifically, uncoupling protein 2 (UCP2), regulating the production of reactive oxygen species (ROS), is indicated to mediate GLP-1 secretion following acute exposure to fatty acids [120]. Interestingly, however, UCP2 expression exerts opposing actions on glucose-induced GLP-1 secretion, where an inhibitory role has been suggested [121].

#### **1.4.2 Neural and hormonal regulation of GLP-1 secretion**

Hormonal and neuronal signals enhance secretion downstream of membrane depolarization by increasing cAMP production or triggering  $Ca^{2+}$  release from intracellular stores [122-124]. There is also evidence that direct cholinergic muscarinic control of the L-cells regulates the pulsatility of GLP-1 secretion [77].

Whether the L-cell density is high enough in the upper intestine for direct nutrient stimulation to account for the acute phase of GLP-1 secretion is

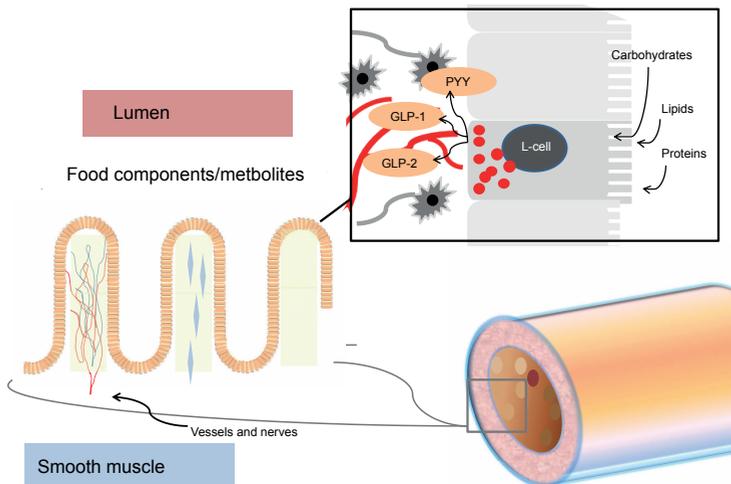
debated. However, there is clearly -- in addition to direct nutrient stimulation - - also a neural and hormonal regulation of GLP-1 secretion, as illustrated by the presence of a proximal–distal loop, involving nutrient detectors located in upper regions of the GI tract controlling release of GLP-1 from distal L-cells, as well as by the importance of the vagus nerve for GLP-1 secretion [125].

In addition, there is evidence for cephalic GLP-1 secretion with subsequent insulin secretion resulting from meal anticipation [126]. However, other studies demonstrate that insulin levels increase rapidly after meal ingestion, and before an increase in GLP-1 levels can be detected [127].

### 1.4.3 Additional regulators of GLP-1 secretion

Studies using GLP-1-secreting cell lines have demonstrated that bile acids promote GLP-1 secretion through a G protein-coupled bile acid receptor (TGR5), where the stimulation of GLP-1 secretion is cAMP-dependent [128-129]. Subsequent studies have also demonstrated that TGR5 agonists increase GLP-1 secretion and improve glucose homeostasis in mice [130-132]. Further, peroxisome proliferator-activated receptor (PPAR)  $\beta/\delta$  activation increases proglucagon expression and enhances bile acid-induced GLP-1 secretion by L-cells *in vitro* and *ex vivo* in human jejunum [133].

Progesterone was very recently identified as yet another regulator of GLP-1 secretion. Progesterone increased GLP-1 secretion from an L-cell line and enteral progesterone administration increased plasma levels of GLP-1, GIP, and insulin, while improving oral glucose tolerance independent of the classical progesterone receptor. Further, this effect of enteral progesterone was indicated to be independent of classical incretin receptor signaling and preserved in *glp1r*<sup>-/-</sup> and *glp1r*<sup>-/-</sup>:*gipr*<sup>-/-</sup> mice [134].



**Figure 1:** Simplified illustration of the intestinal L-cells' unique environment, allowing for direct nutrient sensing as well as neuronal and hormonal stimulation. See text for details.

## 1.4.4 Degradation

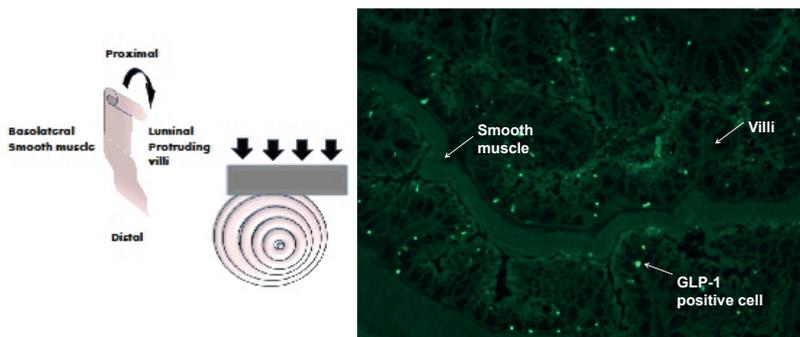
Native GLP-1 has a half life of less than 2 minutes, due to rapid degradation by dipeptidyl peptidase-4 (DPP-4, a.k.a. CD26) ubiquitously present in plasma, and this complicates its application as an antidiabetic drug. In fact, > 80 % of GLP-1 is degraded between secretion and post-hepatic passage. DPP-4 is a peptidase existing both as a soluble form present in plasma and as a membrane-associated form expressed on the cell surface of many cell types, including intestinal cells. DPP-4 cleaves proteins with an *N*-terminal proline or alanine at position 2, rendering GLP-1 with alanine at this position an ideal substrate. The cleavage of GLP-1 generates an NH<sub>2</sub>-terminally truncated (9-36) metabolite in addition to renal excretion. Stable GLP-1 analogs and DPP-4 inhibitors are available as diabetes therapy. However, enhancing endogenous GLP-1 production may be more physiological as it allows also for portal signaling of GLP-1.

## 1.5 THE INTESTINAL EPITHELIUM

### 1.5.1 Morphology

The gastrointestinal (GI) tract is divided into the small and large intestine, where the cecum is considered to identify the beginning of the large intestine. The small intestine is further divided into three structural parts: the duodenum, jejunum and ileum. The majority of GLP-1-secreting L-cells are located in the distal gut, predominantly in the ileum and colon.

Digested food passes through and is absorbed from the intestinal cavity or the so called lumen. Going from the lumen and radially outward, the epithelium containing myofibroblasts, blood vessels, and nerves is followed by the muscularis mucosa and the muscular external, which are two layers of smooth muscle. Lastly there is the serosa which is made up of loose connective tissue and coated in mucus to prevent friction damage from the intestine rubbing against other tissue.



**Figure 2.** Image showing GLP-1-positive cells migrating from the intestinal crypts up the intestinal villi. Illustrated to the left is how the mouse intestinal sample was prepared to generate the morphology of the section presented to the right. The sample was cut open from the distal to the proximal end and rolled to have the luminal side with protruding villi facing the center of the roll. The samples were then sectioned as illustrated.

### 1.5.2 The L-cells

Along the whole length of the GI tract in the epithelium are specialized cells, including goblet cells, but also specialized enteroendocrine cells such as the GLP-1-secreting L-cells and the GIP-secreting K-cells. The mucosa and epithelium of the intestinal wall forms finger-like protrusions called villi that increase the surface area. The adult mouse intestinal epithelium undergoes continuous renewal, which takes place in these villi, and the crypts that surround the base of each villus. L-cells originate from progenitor cells in the crypts – data support the presence of specific progenitors for the L-cell lineage in the intestinal crypts and the involvement of GLP-1 signaling in the proliferation of these precursor cells [135]. The L-cells differentiate as they migrate up the villus, before eventually being shed off the villus tip [136-137]. The origin of enteroendocrine cell lineages is not fully understood, but studies support the idea of a multipotent endocrine stem cell giving rise to multiple lineages [137]. Situated on the villi lining the intestine the L-cells have apical microvilli facing the gut lumen and secretory vesicles located adjacent to the basolateral membrane. This morphology also makes it possible for the L-cells to be regulated by neuronal factors and hormones, as well as by direct nutrient stimulation [79, 81].

### 1.6 GLUTAG CELLS AND GLP-1 SECRETION

Preparations of adult L-cells enriched by elutriation or derived by fetal rat intestinal culture might theoretically be superior to cell lines for studies of GLP-1 release, because they are not immortalized. However, because they contain, at best, only ~ 10 % L-cells, they are inappropriate for single-cell studies. GLUTag cells are a stable immortalized murine enteroendocrine cell line that expresses the proglucagon gene and secretes GLP-1. This cell line was isolated from a glucagon-producing enteroendocrine cell tumor that arose in glucagon gene-SV40 T antigen transgenic mice [138] and provides a homogenous population of enteroendocrine cells. They are electrically excitable cells that depolarize and fire action potentials in response to a range of nutrients, including monosaccharides and amino acids. Nutrients trigger membrane depolarization and the opening of voltage-gated  $Ca^{2+}$  channels and an elevation of intracellular  $Ca^{2+}$ , necessary for the stimulation of GLP-1 release [124]. Further, peptides or pharmacological agents that increase cyclic AMP also increase levels of intracellular  $Ca^{2+}$  in GLUTag cells and thereby stimulate GLP-1 secretion [124, 139]. GLUTag cells have been propagated since 1991, and early passage cells have retained a well differentiated phenotype indicated by a responsiveness to physiological and pharmacological secretagogues that is very similar to that of primary rat intestinal cell cultures [123, 140]. GLUTag cells display a pattern of receptor-, ion channel- and glucose sensor-expression that is very similar to primary murine L-cells [84]. In addition, signal transduction pathways that operate in GLUTag cells are very similar to those operating in primary murine L-cells, and GLUTag cells even display a very similar  $EC_{50}$  for glucose-stimulated GLP-1 secretion [84].

However, one major general drawback of *in vitro* studies of L-cells is the inability to assess effects of apical vs. basolateral stimulation with agents.

## 1.7 DIABETIC HYPERLIPIDEMIA AND LIPOTOXICITY

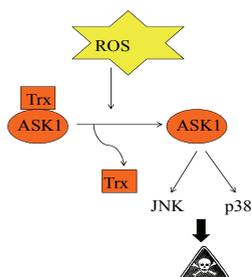
T2D, characterized by chronic insulin resistance and a progressive decline in  $\beta$ -cell function [141], may be considered a lipid disorder as well as a disease of dysregulated glycemia [142], as obesity (defined as body mass index [BMI] > 30) is a major risk factor for the development of T2D [143], and obesity and T2D are associated with hyperlipidemia or increased circulating concentrations of free fatty acids (FFA) [144-146]. The elevated FFA concentrations have been shown to result from increased release of FFAs from adipose tissue, where this increased lipolysis is linked to adipocyte size. As insulin decreases lipolysis and increases glycerol and fatty acid synthesis, insulin resistance will lead to increased lipolysis. The high levels of FFAs induce insulin resistance and are toxic to many cell types, including the insulin-secreting  $\beta$ -cells - where impaired  $\beta$ -cell function and decreased viability is observed following long term FFA exposure [147]. Normal circulating FFA levels are approximately 0.5 mmol/l [148].

Differential pathological processes have been suggested to underlie the toxic effects of sustained hyperlipidemia. FFAs enter cells mainly through fatty acid transporters, such as CD36/FAT, and FATPs [149-150]. However, fatty acids can also diffuse across or acts as ligands for G protein-coupled receptors on the cell surface. Once inside the cell, fatty acids can be esterified, metabolized to lipid second messengers, or  $\beta$ -oxidized in the mitochondria. The mechanisms behind the metabolic fate of FFAs taken up into the cells are incompletely understood. However, the type of FFA, exposure time and energy demand of the tissue are of importance [151-153]. AMP-dependent protein kinase (AMPK) activation plays a major role in fatty acid  $\beta$ -oxidation and fatty acid synthesis by phosphorylating and inhibiting the enzyme acetyl-CoA carboxylase (ACC). ACC catalyzes carboxylation of acetyl-CoA producing malonyl-CoA, which is used as a substrate for fatty acid biosynthesis, and a potent inhibitor of mitochondrial fatty acid uptake secondary to inhibition of carnitine palmitoyltransferase 1 (CPT1) [153]. The accumulation of fatty acids as triglycerides in nonadipose tissue (steatosis), seen in hyperlipidemia, is associated with impaired insulin signaling and lipotoxicity [154]. However, although non-adipose tissue accumulation of triglycerides is an indicator of lipid overload and hyperlipidemia in humans and animal models, triglyceride accumulation in tissue and triglycerides themselves are most likely inert in terms of lipotoxicity [152, 155]. Rather, it is FFAs channeled to other metabolic fates, such as production of ROS or ceramide, which mediate the lipotoxic effects.

### 1.7.1 ROS production

*De novo* ceramide production, as well as increased production of ROS resulting from increased  $\beta$ -oxidation, has been implicated in lipotoxic effects in insulin-secreting  $\beta$ -cells [156-160]. ROS are chemically reactive molecules containing oxygen, such as oxygen ions and peroxides. ROS are natural byproducts of the normal metabolism of oxygen. However, during times of environmental/metabolic stress, ROS levels can increase

dramatically, which may result in significant damage to cell structures. Apoptosis signal-regulating kinase 1 (ASK1) is a member of the MAP kinase kinase kinase family and a part of the MAP kinase signaling pathway. When cells are exposed to oxidative stress, such as ROS, the ASK1-interacting molecule thioredoxin dissociates from ASK1. This converts oxidative stress to a phosphorylation-dependent signal where ASK1 phosphorylates downstream substrates, such as c-Jun *N*-terminal kinase (JNK) and p38 mitogen-activated protein kinases, mediating cellular dysfunction and apoptosis [161-162].



**Figure 3.** *Simplified scheme of ROS-mediated phosphorylation signaling in induced cytotoxicity. See text for details.*

## 1.8 DIABETES THERAPY

### 1.8.1 Lifestyle intervention

Body weight loss and modifications in food intake result in decreased lipid levels and inflammatory activity. Regular exercise and/or dietary modification -- even without weight loss -- can improve insulin sensitivity, and dietary caloric restriction alone normalizes  $\beta$ -cell function and hepatic insulin sensitivity. In addition, the progression into overt diabetes can be prevented by moderate lifestyle changes in individuals with IGT [163].

### 1.8.2 Sulfonylureas

Sulfonylureas are a class of drugs that act by increasing insulin release from the  $\beta$ -cells and do so irrespective of ambient glycemia. The main target of action of sulfonylureas is the SUR/Kir 6.2 subunit of the ATP-dependent potassium channel ( $K_{ATP}$ ) found in many cell types, including the insulin secreting  $\beta$ -cells, cardiomyocytes and GLP-1-secreting L-cells. The binding of sulfonylureas to the  $K_{ATP}$  channel on the  $\beta$ -cells mediates closure of the channel, inhibited efflux of potassium and membrane depolarization with the subsequent opening of voltage-gated  $Ca^{2+}$  channels and insulin secretion. However, despite the presence of  $K_{ATP}$  channels on GLP-1-secreting cells, sulfonylureas have not been shown to stimulate GLP-1 release in humans [79].

### 1.8.3 Metformin

Metformin is an oral, anti-diabetic, cationic drug of the biguanide class and the most widely prescribed anti-diabetic agent in the world. Intestinal absorption of metformin involves an active, saturable uptake process, where metformin has been shown to be transported by the human organic cation transporters 1 (OCT-1), and the proton-activated organic cation transporter, plasma membrane monoamine transporter (PMAT).

Metformin is a first-line drug in the management of T2D and frequently prescribed if set glycemic goals for lifestyle changes are not reached within 1-3 months. Metformin reduces blood glucose by direct effects on the liver and reduced gluconeogenesis as well as reduced insulin resistance. The mechanisms behind the anti-diabetic actions of metformin are still unclear, although the best known target for metformin is the 5' AMP-activated protein kinase (AMPK) - a fuel and stress-sensing enzyme that is considered a master switch of glucose and lipid metabolism in various organs [164]. Metformin has been shown to protect isolated islets from lipotoxicity [165]. Metformin treatment of non-diabetic patients is associated with increased levels of circulating GLP-1 following an oral glucose load [166], as well as additive glucose-lowering benefits in diabetic subjects following GLP-1-based therapy [167]. Recent reports also indicate increased GLP-1R expression in pancreatic islets in response to metformin treatment [168]. The mechanisms behind metformin-induced increases in plasma GLP-1 levels are largely unknown, but have been suggested to result from inhibited enzymatic degradation of GLP-1 [166], although several studies show no effect of metformin on GLP-1 degradation [169-170] as well as additive effects on plasma GLP-1 in response to metformin and a DPP-4 inhibitor [171]. The acute stimulation of GLP-1 secretion by metformin in rats has been suggested to be indirect, requiring M3 muscarinic receptors and gastrin-releasing peptide (GRP) [172].

### 1.8.4 Insulin

As T2D progresses, insulin replacement therapy oftentimes eventually becomes necessary. Insulin has been shown to acutely stimulate GLP-1 secretion, through PI3K activity, in L-cell models and fetal rat intestinal cells [173].

### 1.8.5 Exendin-4 and incretin agonists

Exendin-4, a 39-amino acid peptide hormone, was found in the venom of the Gila monster (*Heloderma suspectum*) and it is a high-affinity agonist of mammalian GLP-1R. It shares 53 % of its amino acid sequence with that of native GLP-1. Exendin-4 displays biological properties similar to GLP-1 as a regulator of glucose metabolism and insulin secretion. Unlike GLP-1, exendin-4 has a much longer half-life (60-90 min) and therefore is currently used in the management of T2D. Since 2007 it is registered in EU against T2D and is available in European pharmacies under the brand name Byetta. Long-acting exendin-4 (Bydureon) has also recently been made available for

once weekly dosing. Liraglutide (brand name Victoza) is another GLP-1 analog with 97 % homology to the native peptide, which contains an arginine 35 lysine substitution, a glutamine substitution and a FFA addition to lysine. The FFA addition greatly increases liraglutide binding to albumin, resulting in a plasma half-life of 10-14 hours after s.c. injection [174]. Liraglutide can be administered once daily and reduces both fasting and postprandial glycemia.

Lixisenatide (brand name Lyxumia) is another GLP-1R agonist in late-stage development. The half-life of lixisenatide is 2-4 hours. Despite its short half-life, lixisenatide is intended for once daily dosing due to its strong affinity for the GLP-1R. Lixisenatide has been shown to have beneficial effects on HbA<sub>1c</sub> and good prandial coverage in combination with other commonly used oral agents for T2D, with no increased risk of hypoglycemia [175].

### **1.8.6 DPP-4 inhibitors**

Another approach to increase/normalize serum levels of GLP-1 is to prevent its breakdown by employing inhibitors of DPP-4. One such drug, sitagliptin, was approved in EU in 2007 against T2D and is available in pharmacies under the name Januvia. However, recent reports indicate that sitagliptin may also exert direct -- DPP-4-independent -- effects on intestinal L-cells, activating cAMP and ERK1/2 signaling and stimulating total GLP-1 secretion [176]. Other recently launched or approved DPP-4 inhibitors include vildagliptin (Galvus), saxagliptin (Onglyza), alogliptin (Nesina) and linagliptin (Trajenta). Most of the DPP-4 inhibitors are structurally distinct. Alogliptin is a quinazolinone-based compound, linagliptin is a xanthine derivative, saxagliptin is a hydroxyadamantyl compound, sitagliptin is a triazole-pyrazine compound, and vildagliptin is a pyrrolidine-carbo-nitrile compound.

### **1.8.7 $\alpha$ -glucosidase inhibitors**

$\alpha$ -glucosidase inhibitors, such as acarbose, miglitol and voglibose, are saccharides that act as competitive inhibitors of pancreatic  $\alpha$ -amylase and intestinal brush border  $\alpha$ -glucosidases producing a delayed hydrolysis of ingested polysaccharides, oligosaccharides and disaccharides to monosaccharide. Consequently, less glucose is absorbed and the postprandial rise in plasma glucose is blunted. Studies have shown that  $\alpha$ -glucosidase inhibitors increase postprandial levels of GLP-1 in normal and diabetic subjects - most likely due to reduced carbohydrate absorption in the proximal part of the intestine, which increases the load of these nutrients in the distal intestine where the secretion of GLP-1 is greater [177-179].

## 2 AIMS

The over-arching aim of this work was to investigate effects of diabetic lipotoxicity and commonly prescribed anti-diabetic agents on growth, viability and function of GLP-1-secreting cells, in an attempt to also unravel some of the mechanisms regulating these enteroendocrine cells. The ultimate aims were to gain pathogenic understanding of the impaired incretin response characterizing T2D patients and to identify potentially druggable targets and pathways for therapeutic efforts to enhance GLP-1 production in a physiologic way.

More specifically the aims were:

- To determine whether metformin exerts direct long-term effects on the regulation of GLP-1-secreting cells in terms of apoptosis and secretion, the nature of such effects, and the possible involvement of AMPK.
- To determine the molecular mechanisms mediating lipotoxicity, and metformin-induced lipoprotection, in GLP-1-secreting cells.
- To investigate the effect of insulin signaling as well as possible autocrine action of GLP-1 on apoptosis and function of the GLP-1-secreting cells.
- To determine the effects of a high fat diet on the number of enteroendocrine GLP-1-secreting cells and GLP-1 plasma levels in a rodent model, and possible effects of metformin treatment on these parameters.

### 3 MATERIALS AND METHODS

This study was initiated as a scanning study encompassing a large number of anti-diabetic agents and hormones that were scanned for direct effects on the proliferation, apoptosis and function of GLP-1-secreting cells using *in vitro* incubations of GLUTag cells with or without the different agents. In addition, these agents were evaluated for effects also in the presence of simulated diabetic hyperglycemia/hyperlipidemia. This scanning study remains to be published as such.

The present thesis focuses on the effects observed in response to metformin, exendin-4, and insulin.

#### 3.1 STUDY PROTOCOLS

##### 3.1.1 Cell culture and incubation (study I-III)

The GLP-1-secreting GLUTag cell line (source: glucagon-producing enteroendocrine cell tumor that arose in transgenic mice generated on an out-bred CD-1 background) [138], graciously donated by Dr. Neil Portwood at Karolinska Institutet, Solna, Sweden, and originally from Dr. Daniel J. Drucker, Mount Sinai Hospital, Samuel Lunenfeld Research Institute, University of Toronto, Canada, was cultured in DMEM supplemented with 10 % fetal bovine serum (FBS), 5.5 mmol/l glucose, 10,000 U/ml penicillin and 10 mg/ml streptomycin sulfate under 5 % CO<sub>2</sub>. Palmitate exposure media were supplemented with 2 % FBS and 0.5 % bovine serum albumin (BSA). Palmitate was dissolved in 12.5 % ethanol during heating to 60 °C. Control cells were given vehicle with equal amounts of ethanol as the palmitate exposed cells (final concentration of ethanol: 0.03 %).

##### 3.1.2 Western blot (study I-II)

Western blotting was applied to quantify total and phosphorylated ACC, AMPK, JNK (Study I), and p38 (Study II) following culture w/w/o agents in the presence or absence of palmitate-albumin complex at 2 % serum DMEM. GLUTag cellular protein was extracted, using RIPA lysis buffer, for 30 min on ice. Cells were sonicated and lysates were cleared by centrifugation. Protein concentration was determined and cell extracts were stored at -80 °C. Equal amounts of protein were then mixed with reducing SDS-PAGE sample buffer, boiled for 5 min and proteins separated by SDS-PAGE. Samples containing 25-30 µg of protein were electrophoresed against a pre-stained protein ladder on a 10-12 % polyacrylamide gel under denaturing conditions, followed by transfer to PVDF membrane. Membranes were blocked with 5 % milk solids in PBST; primary (overnight) and secondary (1 h) antibody incubations were performed in the same buffer, with three 10-min washes in PBS-T intervening.

Horseradish peroxidase-conjugated secondary antibodies (1:5,000) and ECL (enhanced chemiluminescence) reagents were used to detect proteins. Images and quantifications were obtained using Molecular Imager ChemiDoc XRS with Quantity One Software (v. 4.6.5). After imaging, the PVDF membranes were stained with Coomassie Brilliant Blue for total protein normalization. Phosphorylation was determined after normalization with total

(phosphorylated and non-phosphorylated) forms of the protein or  $\alpha$ -tubulin/Coomassie staining.

### **3.1.3 MTT assay (study I)**

The MTT assay was used to determine the number of viable cells after treatment with/without palmitate. GLUTag cells were plated and cultured in 96-well plates at a density of 136,000 cells/ml for 24 h. Cells were then washed and treated with or without 0.125 mmol/l palmitate for 48 h in the presence of low serum medium (2 % FBS). Viable cell densities were determined by metabolic conversion of the dye MTT. 15  $\mu$ l of the supplied MTT solution was added to each well and the plates were then incubated for an additional 4 h. The MTT reaction was terminated by the addition of 100  $\mu$ l acidified isopropanol, dissolving the formazon product formed. After 1-2 h at 4 °C, MTT assay results were read by measuring absorbance at 540 nm.

### **3.1.4 Protein assay (study I-III)**

GLUTag cells were washed twice with phosphate buffered saline (PBS) and lysed on ice in a RIPA lysis buffer containing 150 mmol/l NaCl, 20 mmol/l Tris, 0.1 % SDS, 1 % Triton X-100, 0.25 % Na-deoxycholate, 1 mmol/l Na<sub>3</sub>VO<sub>4</sub>, 50 mmol/l NaF, 2 mmol/l EDTA and Protease inhibitory cocktail (Sigma-Aldrich) for 30 min. Samples were clarified by centrifugation, supernatants were transferred to new tubes and the total protein concentration was determined with Bio-Rad DC protein assay (method of Lowry [180]), using BSA as a standard (Bio-Rad Laboratories, Hercules, CA).

### **3.1.5 Apoptosis assays (study I-III)**

#### *3.1.5.1 DNA fragmentation ELISA (study I)*

GLUTag cells were plated at a density of 180,000 cells/ml and grown in 24-well plates for 24 h. Cells were then washed twice with low serum medium (2 % FBS, 5.5 mmol/l glucose) and then treated with metformin at indicated doses in 2 % FBS and 5.5 mmol/l glucose medium for an additional 48 h. DNA fragmentation ELISA (Roche Diagnostics GmbH, Mannheim, Germany) was used according to the manufacturer's instructions. Briefly, the cells were lysed and lysates and reagents were added as described by the manufacturer and the ELISA plate was read by measuring absorbance at 450 nm. All experiments were performed in triplicates and repeated twice to assess consistency of response.

#### *3.1.5.2 Caspase-3 activity assay (study I-III)*

GLUTag cells were plated at a density of 250,000 cells/ml and grown in Ø60 mm Petri dishes for 24 h. Cells were then washed twice with low serum medium (2 % FBS, 5.5 mmol/l glucose) and then treated with metformin (study I), Trolox (study II), exendin-4/insulin (study III) at indicated doses in 2 % FBS and 5.5 mmol/l glucose with 0.125 mmol/l palmitate/vehicle for an additional 48 h. Caspase-3 activity assay kit (Cell Signaling Technology, Inc., Danvers, MA) was used according to the manufacturer's instructions. Briefly, the caspase-3 colorimetric assay is based on the hydrolysis of a substrate by caspase-3, resulting in the release of fluorescent product, which can be measured at 405 nm.

### 3.1.6 GLP-1 secretion and ELISA (study I and III)

Long term: GLUTag cells were plated at a density of 180,000 cells/ml and grown in 24-well plates for 24-48 h. Cells were then treated with metformin (study I) or exendin-4/insulin (study III) at indicated doses for an additional 48 h. Immediately after the 48 h incubation, medium was collected and DPP-4 inhibitor added (10 µl/ml) (Millipore Corporation, Billerica, MA).

Acute: GLUTag cells were plated at a density of 180,000 cells/ml and grown in 24-well plates for 24-48 h. The medium was discarded and the cells were washed with pre-warmed KRBH buffer/0.2 % BSA/0 mmol/l glucose, followed by a 30 min pre-incubation with the same buffer. Cells were then treated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (PKC activator), forskolin (adenylate cyclase activator) and metformin (study I) or exendin-4/insulin (study III) for 2 h in the presence or absence of 20 mmol/l glucose. Immediately thereafter, DPP-4 inhibitor (Millipore Corporation) was added and the buffer was collected. GLP-1 Active ELISA (Millipore Corporation) was used according to the manufacturer's instructions.

### 3.1.7 Quantitative RT-PCR (study I, III)

Quantitative RT-PCR was used to determine the expression of proglucagon mRNA in GLUTag cells (study I) and proglucagon/GLP-1R mRNA in intestinal tissue (study IV). GLUTag cells/intestinal tissue samples were lysed and RNA extracted using Aurum total RNA mini kit (BioRad Laboratories) according to the manufacturer's instructions. cDNA was synthesized for qPCR using iScript™ cDNA synthesis kit (BioRad Laboratories) according to the manufacturer's instructions.

A one-step RT-PCR kit with SYBR Green (iScript™) (BioRad Laboratories) was used for real-time quantitative RT-PCR. This kit utilizes iScript RNase H<sup>+</sup> reverse transcriptase and hot-start iTaq DNA polymerase. GAPDH was used as housekeeping gene for normalization.

### 3.1.8 Animals and diet (study IV)

C57/Bl6 mice (Nova/Scanbur, Sollentuna, Sweden) arrived 9 weeks old and were housed in our animal department for 1 week prior to initiation of the study. Animals were housed in groups of four in a total of eight cages under standard laboratory conditions of light, temperature and humidity, and received food and water *ad libitum*. At the initiation of the study, the animals were divided into two groups. One group received normal chow (Nova/Scanbur) and the other group received the fat-enriched D12492 diet consisting of 60% kcal% fat (Research Diets, New Brunswick, NJ). After 10 weeks on each respective diet, the two groups were each divided into two groups of animals where eight animals received oral gavage administration of 300 mg/day metformin and the remaining eight animals received oral administration of saline. 12 weeks after the initiation of the study, animals were euthanized using CO<sub>2</sub>. Throughout the study, weight and food consumption for all animals/cages were recorded once a week.

### 3.1.9 Blood glucose and HbA<sub>1c</sub> levels (study IV)

Blood samples for glucose measurements were obtained from each mouse by needle puncture of the tail tip vein. Both fasting and prandial glycemia were

measured at three time points (before initiation of metformin/saline treatment [time 0], 1 week after initiation [time 1] and 2 weeks after initiation before the end of the study [time 2]). Blood glucose concentrations were determined by means of Bayer's Elite<sup>®</sup> Glucometer and compatible blood glucose test strips. Fasting blood samples for HbA<sub>1c</sub> measurements were obtained at time 0 and time 2 from the same puncture of the tail tip vein. HbA<sub>1c</sub> was determined using a DCA Vantage<sup>®</sup> Analyzer (Siemens Healthcare Diagnostics, Tarrytown, NY) and compatible sample cassettes.

### **3.1.10 Serum insulin, FFA and GLP-1 determinations (study IV)**

Blood samples were collected from the saphena vein after 6 h fasting at time 0, 1 and 2 (see above) and transferred to centrifuge tubes at room temperature. At time 2, immediately after euthanization, a heart puncture was also performed and a larger volume of prandial blood collected. The blood samples were spun to obtain serum. The serum was stored in a freezer at -70 °C for later analysis of insulin, total GLP-1 (7-36 and 9-36), active GLP-1 (7-36) and FFAs. Serum insulin concentrations were determined using a mouse ultrasensitive ELISA (Mercodia, Uppsala, Sweden), according to the manufacturer's instructions. Serum GLP-1 (7-36 and 9-36) and GLP-1 (7-36) levels were determined using specific ELISAs (Millipore, Billerica, MA) according to the manufacturer's instructions. Serum FFAs were determined using the NEFA-HR(2) assay kit (Wako Chemicals, Richmond, VA) according to its instructions.

### **3.1.11 Oral glucose tolerance test (OGTT) (study IV)**

Food was removed 6 h prior to oral gavage administration of 1.5 g/kg glucose at the end of the last week of treatment. At 15, 30 and 60 min following administration, blood samples for glucose measurements were obtained from each mouse by needle puncture of the tail tip vein. Blood glucose concentrations were determined by means of Bayer's Elite<sup>®</sup> Glucometer and compatible blood glucose test strips.

### **3.1.12 Immunohistochemistry and quantification of L-cells (study IV)**

After euthanization, intestinal tissue was collected from the animals. The intestine was extracted and carefully removed of all fat. A 4-cm section proximal/distal to the appendix was removed and carefully cut open to have the lumen exposed and facing upward. The intestinal tissue section was then rolled up, starting with the most proximal end and with the lumen facing the center of the roll (technique illustrated in Figure 2). The rolls were attached to plastic caps and immediately submerged in a freeze bath of 99 % ethanol and dry ice. The rolls were stored in a freezer at -70 °C pending sectioning of the intestinal tissue using a cryostat. The intestinal tissue was sectioned using a cryostat in 12 µm thick sections from the top of the roll towards the bottom, leaving each section to expose the lumen and villi of the full length of the intestinal tissue section. These sections were placed in consecutive order on six glass slides starting with position 1 of all slides, followed by position 2 of all slides, *etc.*, leaving each and all slides with 10 sections representing the full cross section of the lumen. Sections were stored at -70 °C.

Prior to IHC, sections were washed in PBS and fixed using a 10 min incubation with acetone at -20 °C. Sections were then washed 3 x 10 min in

PBS-T and incubated overnight with a rabbit antibody specific for GLP-1 (Phoenix Europe GmbH, Karlsruhe, Germany) at 1:500 dilution in PBS-T containing 5 % donkey serum. Tissue sections were washed in PBS-T 3 x 10 min and incubated for 2 h at room temperature in the dark with the secondary antibody (ALEXA Fluor 488 conjugated donkey anti-rabbit) (Invitrogen) at 1:200 dilution in PBS-T. Sections were washed in PBS 3 x 10 min and allowed to dry before a cover glass was mounted using Polyvinyl Alcohol mounting medium with DABCO antifading (Fluka Biochemica, Ronkonkoma, NY). For the purpose of quantifying cells after IHC, an advanced stereology platform, which allows unbiased quantification of cell number/volume and structural measurements within the intestinal structure [181], was used.

### **3.1.13 ROS measurement (study II-III)**

GLUtag cells were plated at a density of 300,000 cells/ml and grown in 6-well plates for 24 h. Cells were then washed twice with low serum medium (2 % FBS, 5.5 mmol/l glucose) prior to treatment with 0.125 mmol/l palmitate and/or 2 mmol/l metformin (study II) or exendin-4 (study III) in 2 % FBS and 5.5 mmol/l glucose for an additional 24 h. The Image-iT<sup>TM</sup> LIVE Green ROS Detection Kit (Invitrogen, Inc.) used provides the key reagents necessary for the detection of ROS in live cells. The assay is based on 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H<sub>2</sub>DCFDA), a reliable fluorogenic marker for ROS in live cells.

### **3.1.14 Statistical analysis (study I-IV)**

Comparisons between groups, treatments and time were made by a one-way ANOVA for repeated measures. Comparisons between control and single treatment groups were done using two-tailed Student's *t* test. Correlations were evaluated by determining the Pearson correlation coefficients.  $P < 0.05$  was deemed statistically significant. Power analysis was performed and taken into consideration for all experiments.

## 4 RESULTS

### 4.1 *IN VITRO* EXPERIMENTS (STUDY I-III)

#### 4.1.1 *Study I*

In study I, we demonstrate that long term exposure to the saturated fatty acid palmitate induced lipopoptosis of the GLP-1-secreting GLUTag cell line. Consistent with increased caspase-3 activity in response to palmitate, DNA fragmentation increased, while the number of viable cells was significantly reduced. We could also demonstrate reduced palmitate-induced caspase-3 activity and DNA fragmentation in response to co-incubation with metformin – indicative of a lipoprotective effect of the drug. Palmitate induced phosphorylation of JNK, while metformin phosphorylated AMPK in conjunction with a decrease in the palmitate-induced phosphorylation of JNK. Further, reduction of palmitate induced phosphorylation of JNK -- using the JNK inhibitor SP600125 -- significantly reduced palmitate-induced caspase-3 activity. The AMPK phosphorylation induced by 2 mmol/l metformin was rapidly detectable and sustained for at least 24 h. However, co-incubation with the AMPK activator AICAR, or 500  $\mu$ mol/l metformin, resulted in a very rapid and transient phosphorylation of AMPK, while also failing to reproduce the lipoprotective effect of metformin. Co-incubation with the AMPK inhibitor compound C significantly attenuated the metformin-induced reduction of palmitate-induced caspase-3 activity. In study I, we also demonstrate reduced expression of proglucagon mRNA, but increased GLP-1 secretion, following long term exposure to 500  $\mu$ mol/l metformin.

#### 4.1.2 *Study II*

Our results from study II show that palmitate significantly increased ROS production, while co-incubation of palmitate with the vitamin E derived antioxidant Trolox inhibited the induction of caspase-3 activity. Further, palmitate led to phosphorylation of the ROS-sensitive kinase p38, the inhibition of which -- using the p38 inhibitor SB203580 -- significantly reduced the palmitate-induced caspase-3 activity. We could also demonstrate that, although metformin significantly increased ROS production, there was a strong down regulation of p38 expression in response to metformin.

#### 4.1.3 *Study III*

In this study, we demonstrate expression of the GLP-1R in GLP-1-secreting cells, and that GLP-1 secretion was increased in response to treatment with exendin-4, an effect that can be detected acutely as well as after more long term exposure. Further, the increased GLP-1 secretion following exposure to exendin-4 was effectively and significantly blocked by co-incubation with the GLP-1R antagonist exendin 9-39. Moreover, we provide evidence for a lipoprotective effect of exendin-4 treatment where palmitate-induced ROS production and caspase-3 activity were significantly reduced by exendin-4. However, co-incubation of palmitate with the PKA activator Sp-cAMP[S] did

not reduce caspase-3 activity in response to palmitate. Nor did co-incubation with the PKA inhibitor Rp-cAMP[S] significantly alter the reduction of palmitate-induced caspase-3 activity seen in response to exendin-4. In this study, we also demonstrate that insulin increases secretion of GLP-1 from GLUTag cells, again an effect seen acutely as well as after prolonged exposure. We also show a reduction of palmitate-induced caspase-3 activity in response to co-incubation with insulin.

#### **4.2 *IN VIVO* EXPERIMENTS (STUDY IV)**

In study IV, we demonstrate that a high fat diet rapidly induced insulin resistance and diabetes, with a significant improvement in response to metformin, which also was indicated to improve the prandial incretin response of HFD-fed mice. However, no significant effect on fasting or prandial levels of GLP-1 in response to HFD feeding was detected. Further, we provide evidence for a reduced number of intestinal L-cells and lipotoxicity in HFD-fed mice, although no significant effect on the intestinal proglucagon mRNA expression was detected. We also present data in support of increased GLP-1R mRNA expression in HFD-fed mice, an effect not observed in HFD-fed mice receiving metformin treatment.

## 5 GENERAL DISCUSSION

Defective GLP-1 secretion has been observed in T2D patients, and administration of GLP-1 can normalize fasting and prandial glycemia. The insulin-potentiating actions of GLP-1 have made stable GLP-1 analogs and DPP-4 inhibitors among the best anti-diabetic drugs available. However, increased endogenous GLP-1 secretion may offer a better and more physiological approach for T2D therapy, as endogenous GLP-1 -- unlike stable GLP-1 analogs and DPP-4 inhibitors -- is released directly into the portal vein prior to hepatic passage. The portal effects of GLP-1 are indicated to be of considerable importance, in line with the relatively small portion of GLP-1 (7-36) remaining post-hepatic passage, including reports of a GLP-1-regulated glucose sensor in the portal vein wall that (via nervous signals) controls insulin secretion [40]. Further, the liver receives about 75 % of its blood through the portal vein, and direct effects of GLP-1 on hepatocytes are reported. Further, and in support of the benefits of enhancing the endogenous incretin response, the insulinotropic effect of GLP-1 is poorly replicated by short-acting GLP-1R agonists, whereas the effect on gastric emptying is lost with long-acting GLP-1R agonists -- probably due to continuous activation and de-sensitization of the GLP-1R [182]. It should also be considered that much like other GPCRs, on binding to its ligand, the GLP-1R internalizes [183]. This receptor internalization upon ligand binding is seen also with the insulin receptor, and much like pulsatile insulin secretion is indicated to prevent insulin resistance [184-185], the pulsatile nature of endogenous GLP-1 secretion [77] may prevent GLP-1R de-sensitization and GLP-1 resistance. Consequently, enhancing endogenous GLP-1 secretion provides yet another advantage to current incretin therapy. Enhanced endogenous GLP-1 secretion can be obtained either acutely by directly stimulating GLP-1 secretion -- ideally in a nutrient-dependent manner -- or chronically through preserving/protecting the native L-cells and thereby obtaining an increased secretory capacity through expansion of the L-cell mass. The importance of the intestinal L-cell mass for glucose homeostasis has previously been demonstrated [186].

Understanding what regulates the GLP-1-secreting cells may make it possible to modulate the endogenous secretion. In addition, diabetic patients represent a selected group that is chronically exposed to certain drugs, the long-term effects of which on the intestinal L-cells are unknown. Consequently, the aim of this study was to investigate effects of commonly prescribed anti-diabetic agents on growth, viability and function of GLP-1-secreting cells, in an attempt to also unravel some of the mechanisms regulating these enteroendocrine cells. T2D patients often have elevated levels of plasma FFAs [144-145], and high levels of FFAs induce insulin resistance and are toxic to many cell types. In study I, we demonstrate that palmitate, used to simulate hyperlipidemia, induces massive cell death of GLP-1-secreting cells *in vitro*. In this study, we also show -- for the first time -- that the anti-diabetic drug metformin has direct long-term effects on the regulation of GLP-1-secreting cells *in vitro*, where GLP-1 secretion is induced after long term exposure and metformin protects GLP-1-secreting cells from lipotoxicity. Study I also attempts to determine some of the molecular mechanisms underlying palmitate induced lipotoxicity. JNKs, which are downstream components of the mitochondrial death signal [162] and reported to mediate

palmitate-induced apoptosis in other cell systems [187], were indicated to play a role in mediating lipotoxicity in GLP-1-secreting cells as well. Palmitate treatment induced phosphorylation of JNK2, an effect significantly attenuated by co-treatment with metformin. Further, the JNK inhibitor SP600125 significantly attenuated palmitate-induced caspase-3 activity. However, the fact that SP600125 could not, like metformin, completely block palmitate-induced caspase-3 activity indicates the involvement of additional signaling pathways. The metformin-induced attenuation of JNK activation occurred in conjunction with a significant AMPK activation by metformin. Differential activation of AMPK in response to 2 mmol/l metformin and AICAR/500  $\mu$ mol/l metformin, where all treatments will induce rapid and transient phosphorylation of AMPK but only 2 mmol/l metformin will induce a later sustained activation of AMPK, may explain why neither AICAR nor 500  $\mu$ mol/l metformin could reproduce the lipoprotective effect of 2 mmol/l metformin. The lipoprotective effect of metformin may require a sustained activation of AMPK, as also previously reported in other cell systems [188]. The finding in study I, that co-incubation with the AMPK inhibitor compound C resulted in an attenuation of the lipoprotective effect of metformin by approximately 50 %, agrees well with the partial (50 %) inhibition of AMPK activation achieved by the co-incubation with compound C, and an AMPK dependent effect.

The stimulatory effect of metformin on GLP-1 secretion observed in study I indicates differential effects at the transcriptional level and the level of translation/secretion, as the same concentration of metformin significantly reduced the expression of proglucagon mRNA. Interestingly, PKC is a known target of metformin action [189] and activators of PKC have been shown to stimulate secretion, but not biosynthesis, of the proglucagon derived peptides in GLUTag cell cultures [140]. It may also be that treatment with metformin sensitizes the GLUTag cells to GLP-1 secretagogues present in the cell culture medium, such as glucose. These are nevertheless pure speculations, and further studies are needed to determine the underlying mechanisms. However, a stimulatory secretory effect/enhanced nutrient-stimulated secretion by metformin would be of potential therapeutic importance since the reduced GLP-1 levels seen in T2D patients [60] have been reported to result from defective secretion of the hormone and not from a transcriptional defect [62] [63].

In study II, we continue to investigate the mechanisms inducing lipotoxicity in the GLP-1-secreting cells *in vitro*. We first studied ROS production in the presence/absence of simulated hyperlipidemia, as increased ROS production in response to palmitate has been reported to mediate cell damage and apoptosis in insulin-producing  $\beta$ -cells [152, 157]. Study II demonstrates that simulated hyperlipidemia increases ROS production and phosphorylates p38, where addition of antioxidants or inhibition of p38 can effectively reduce lipotoxicity. With an increased ROS production, it is expected to see the observed activation of ROS-sensitive pathways (such as ASK1) and downstream MKK (such as JNK) -- as demonstrated in study I -- and p38 phosphorylation demonstrated in study II. It appears that JNK and p38 may together be the mediators of lipoapoptosis downstream of increased ROS production as the JNK inhibitor SP600125 significantly attenuated palmitate-induced caspase-3 activity by ~ 20 % and p38 inhibition resulted in an ~ 80 % attenuation of caspase-3 activity. However, pre-incubation of palmitate with both inhibitors would be necessary before concluding that these are

additive effects, and that inhibiting both kinases will completely block palmitate-induced lipotoxicity. In addition, a future goal is to inhibit fatty acid oxidation and to determine ROS production and the effect on JNK and p38 phosphorylation after addition of ROS scavengers, such as SOD and/or Trolox.

Further, we continue to investigate the mechanisms of metformin-conferred lipoprotection in study II. Surprisingly, we did not find a decrease in ROS production by metformin as observed in rat pancreatic islets and  $\beta$ -cells [190], but metformin did significantly reduce the expression of p38 under these lipotoxic conditions. The lipoprotective effects of metformin have, in other *in vitro* cell studies, been reported to result from protection against oxidative cell injury by induction of a metabolic stress response with stabilization of mitochondria whose oxidative capacity is increased [188]. The metformin-induced increase in ROS production, in conjunction with reduced expression of the ROS-sensitive MAPKK p38 shown in study II, may demonstrate a metformin lipoprotective effect dependent on increased oxidative capacity also in GLP-1-secreting cells.

In study III, we provide novel data in support of direct effects, modulating function and viability, of exendin-4 and insulin -- suggesting stimulatory paracrine/autocrine regulation -- on GLP-1-secreting cells. Specifically, our data show the expression of the GLP-1R on GLP-1-secreting GLUTag cells and also indicate that the GLP-1 mimetic exendin-4 increased GLP-1 secretion by direct effects on GLP-1-secreting cells. The difficulty in obtaining antibodies specific for the GLP-1R should not be ignored, wherefore the specificity of the GLP-1R antibody used was confirmed using GLP-1R siRNA-transfected GLUTag cells (study III-supplementary data). However, contradictory to our findings, lack of GLP-1R expression on native murine L-cells was recently reported [135]. The GLP-1R antibody used to detect L-cell GLP-1R expression in that particular study may, despite demonstrated functionality and specificity in islets, have failed to detect intestinal GLP-1R expression due to small tissue specific sequence variations / a comparatively very low intestinal expression of the GLP-1R. In addition, problems with tissue preparation/intestinal immunostaining cannot be excluded, as the same study -- contradictory to known intestinal GLP-1R expression [191-192] -- fails to detect *any* intestinal GLP-1R expression, not only L-cell specific. Further, the presence of autocrine regulation of glucose-induced GLP-1 secretion -- as reported in the present study -- has previously been shown in studies using GLP-1R knockout mice [193].

Consequently, it remains to be determined whether or not GLP-1R expression on GLUTag cells constitutes an important difference from native L-cells, rendering autocrine feedback stimulation an *in vitro* phenomenon. However, a positive autocrine feedback of GLP-1R activation in GLP-1-secreting cells would be consistent with mechanisms operative in other endocrine cell types, e.g. the insulin-producing  $\beta$ -cells [194]. Similar stimulatory effects were observed in response to insulin and both agents protected GLP-1-producing cells against lipoapoptosis.

The exendin-4-mediated GLP-1 secretion observed in this study is, as expected, GLP-1R-dependent, although mechanistic studies are obviously necessary to determine the exact mechanisms behind the observed effects. The observed role for insulin in acute glucose-stimulated GLP-1 secretion has previously been shown [173]. However, the data from the present study

also indicate prolonged stimulatory effects of insulin signaling on GLP-1 secretion. It should be noted that the glucose-dependent stimulation of GLP-1 secretion in response to insulin and exendin-4 demonstrated in study III indicates that the autocrine/paracrine feed-back loop comes into play only when needed. Further, in line with a ROS-induced lipotoxicity as described in study II, and exendin-4-mediated lipoprotection, exendin-4 significantly reduced palmitate-induced ROS production. Further, the GLP-1R-dependent but PKA-independent lipoprotection in response to exendin-4 was expected, as previous studies show that exendin-4 reduces ROS independent of PKA but dependent of EPAC [36]. Interestingly in this context, increased circulating levels of GLP-1 and GLP-1R activation are correlated with an increased L-cell number *in vivo* [135].

However, further studies are necessary to confirm the presence of the GLP-1R on native L-cells and define the intracellular signaling mediating these protective/secretory effects, as these studies may help identify molecular targets for directly enhancing GLP-1 release/increasing L-cell viability and thereby augmenting incretin hormone secretory capacity in T2D. The findings in study IV support lipotoxic effects also *in vivo*, rendering reduced number of GLP-1-positive cells detected in the intestinal tissue from HFD-fed mice as compared to mice receiving control diet. Further, in contrast to numerous reports on enhanced GLP-1 secretion in response to dietary fat [98, 195], no significant increase in fasting or prandial GLP-1 (7-36 and/or 9-36) could be detected in our animals chronically fed a HFD. It can be hypothesized that the difference lies in the FFA exposure time. While most of the studies showing FFA-induced GLP-1 secretion focus on acute effects, chronic hyperlipidemia may become toxic to the L-cells and therefore eventually impair their secretory capacity rendering no significant stimulation as observed in study IV. Such detrimental effects only after persistent and long term exposure to hyperlipidemia could theoretically be explained by an accumulation of FFAs that eventually exceeds the capacity of the L-cell for triglyceride storage and the subsequent increase in  $\beta$ -oxidation and ROS production as previously reported [152] and indicated in study II. The indicated lipotoxicity in study IV is further supported by reports on a negative correlation between GLP-1 plasma levels and BMI [69]. Animals on a HFD develop hyperglycemia in conjunction with hyperlipidemia, as demonstrated in study IV, indicating the manifestation of insulin resistance. In study IV, we also demonstrated improved glycemia, fasting serum insulin and oral glucose tolerance in response to metformin treatment, which was expected and in line with the known anti-diabetic properties of metformin. The oral gavage administration did cause nausea and reduced food intake during the 14 days of treatment, where those mice receiving a HFD displayed significantly reduced appetite. Further, metformin treatment initially induced diarrhea. Starting with a lower dosage and increasing up to desired dosage over 3-4 days may have avoided this side-effect of metformin treatment. To detect if the weight loss during treatment was a confounding factor for the beneficial effects of metformin, we evaluated weight as an independent parameter for the improved metabolic state. The fact that weight alone displayed a significant positive correlation with HbA<sub>1c</sub>, but not fasting serum insulin, indicates that -- although the weight loss most likely contributed to the beneficial effects -- metformin treatment was indeed effective. Further, the tendency towards increased plasma GLP-1 levels in metformin-treated animals on a HFD,

despite large individual differences, is interesting in the context of metformin-induced lipoprotection *in vitro*. However, a statistically significant effect of metformin on the number of intestinal GLP-1-positive cells could not be detected in this study, while reduced intestinal proglucagon expression was found. The reduced expression of proglucagon agrees well with reduced proglucagon expression *in vitro* demonstrated in study I and indicates metformin stimulatory action to be at the level of secretion. However, if metformin treatment also increases the number of viable GLP-1-positive cells after a HFD, an increased intestinal proglucagon expression would be expected despite possible counteracting effects of metformin on proglucagon expression at the level of individual L-cells. It is, at this point, impossible to say if these data result from metformin lipoprotection being an *in vitro* phenomenon or a combination of the adverse GI side-effects induced by the metformin treatment in this study together with the relatively short duration of metformin treatment. The up-regulation of GLP-1R mRNA in response to a HFD -- and normalization thereof by metformin treatment -- provokes further assessment of the intestinal expression of the GLP-1R under these conditions. It is, in light of a defective incretin response in diabetic patients improved by metformin treatment, tempting to hypothesize that compensatory mechanisms underlie increased receptor expression in response to reduced levels of the ligand and/or defective GLP-1R signaling in HFD-induced T2D.

In summary, the present study provides evidence for lipotoxicity resulting from increased ROS production and downstream activation of MAPKK in GLP-1-secreting cells. Further, these studies provide novel and intriguing findings suggesting that metformin, exendin-4 and insulin, by direct effects on GLP-1-secreting cells, promote secretion and confer protection from diabetic lipoapoptosis of these cells. In addition, our findings provide evidence for rapid development of insulin resistance and diabetes in mice receiving a HFD, with a significant improvement in response to metformin, which also was indicated to improve the prandial incretin response of HFD-fed mice. Further, in line with data from the *in vitro* studies, we provide evidence for reduced L-cell mass and lipotoxicity in HFD-fed mice. However, considering the lack of statistically significant effects on fasting or prandial levels of GLP-1 in response to HFD, and in line with previous reports [196], this proposed lipotoxicity of L-cells seemingly does not contribute to the development of oral glucose intolerance, hyperinsulinemia or hyperglycemia in this study. It may rather contribute to the progression of the diabetic state as it may lead to decreased prandial GLP-1 secretion when the reduction of L-cell mass in response to hyperlipidemia overtakes fatty acid-induced potentiation of GLP-1 secretion.

## 5.1 LIMITATIONS OF THE STUDIES

Study I-III: *In vitro* studies obviously have inherent drawbacks, as the cells are isolated preventing paracrine and hormonal interactions, often exposed to an environment of overabundant nutrients *etc.* An additional drawback in culturing and studying models of the L-cells *in vitro* may be non-uniform distribution of receptors and transporters rendering an apical/basolateral surface well adjusted to the existing microenvironment in native L-cells, and the unfeasibility of apical/basolateral exposure to agents *in vitro*. Further, to

investigate lipotoxicity we tested only the saturated fatty acid palmitate. However, many different fatty acids are present in plasma and may have different effects. Also, various fatty acids may interact with each other to coordinate different pathological responses.

Study I: To investigate the role of AMPK in the lipoprotective effect of metformin we used compound C, which is a relatively unspecific inhibitor of AMPK. Another limitation of this study may be that supratherapeutic concentrations of metformin were used to induce the lipoprotective effects. However, metformin has been shown to accumulate in tissues at higher concentrations than in blood [197]. Additionally, as the L-cells directly face the intestinal lumen, they may locally be exposed to very high metformin concentrations.

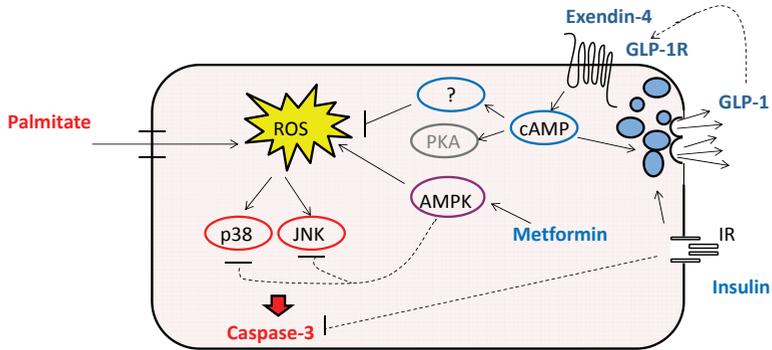
Study II: The role of ROS in p38 phosphorylation was not studied.

Study III: Supratherapeutic concentrations of exendin-4 and insulin were used. Possible effects independent of GLP-1R signaling exerted by exendin (9-39) were not controlled for. Insulin effects on palmitate-induced ROS production was not monitored.

Study IV: An obvious drawback of this study is the side-effects of oral gavage feeding as well as metformin treatment, where nausea and diarrhea were induced. In addition, the study encompassed relatively few animals. Further, metformin treatment could be optimized, perhaps by slowly increasing the dosage to achieve the final dose (as done in clinical practice to improve tolerability) and perhaps the duration of treatment could have been increased.

## 6 CONCLUSIONS

- Palmitate induces lipotoxicity of GLP-1-secreting GLUTag cells through an increased ROS production and mediated by an increased phosphorylation of the kinases p38 and JNK.
- Metformin induces lipoprotection of GLUTag cells through mechanisms independent of ROS production, but likely involving AMPK activation and p38 signaling.
- The GLP-1R is expressed on GLUTag cells and acute as well as prolonged exposure to exendin-4 stimulates GLP-1 secretion through GLP-1R-dependent mechanisms.
- Acute and prolonged exposure to insulin stimulates GLP-1 secretion from GLUTag cells.
- Exendin-4 confers lipoprotection of GLUTag cells, while also reducing ROS production in response to simulated hyperlipidemia. Further, the lipoprotective effect of exendin-4 is mediated through the GLP-1R but independent of PKA.
- Insulin also affords protection of GLUTag cells against lipotoxicity.
- A high fat diet rapidly induces insulin resistance and a diabetic phenotype, while reducing the number of enteroendocrine L-cells and up-regulating the intestinal expression of GLP-1R mRNA. However, this reduction of L-cells and effects on the incretin system are likely to come secondary to development of diet-induced insulin resistance and T2D.
- These findings may be of pathogenic significance in understanding mechanisms of the impaired incretin response characterizing T2D patients.
- The results may also be harnessed to therapeutic advantage in efforts to enhance endogenous GLP-1 production, an approach that has hitherto received little attention but that may be superior to contemporary incretin-based antidiabetic therapy which does not faithfully mimic physiologic GLP-1 release in for instance terms of secretory pattern (*e.g.* pulsatility) and actions on topographically adjacent hormone receptors (*e.g.* in the portal vein).



**Figure 4.** *Simplified scheme of conclusions.* See text for details. ROS; Reactive Oxygen Species, p38; p38 mitogen-activated protein kinase, JNK; c-Jun N-terminal kinase, AMPK; 5' adenosine monophosphate-activated protein kinase, PKA; protein kinase A, cAMP; cyclic adenosine monophosphate, GLP-1R; GLP-1 receptor, IR; Insulin receptor.

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## 7 REFERENCES

1. Meetoo D, McGovern P, Safadi R: **An epidemiological overview of diabetes across the world.** *Br J Nurs* 2007, **16**:1002-1007.
2. Koro CE, Bowlin SJ, Bourgeois N, Fedder DO: **Glycemic control from 1988 to 2000 among U.S. adults diagnosed with type 2 diabetes: a preliminary report.** *Diabetes Care* 2004, **27**:17-20.
3. Gupta V: **Pleiotropic effects of incretins.** *Indian J Endocrinol Metab* 2012, **16**:S47-56.
4. Vilsboll T, Holst JJ.: **Incretins, insulin secretion and Type 2 diabetes mellitus.** *Diabetologia* 2004, **47**:357-366.
5. Thorens B: **Expression cloning of the pancreatic beta cell receptor for the gluco-incretin hormone glucagon-like peptide 1.** *Proc Natl Acad Sci U S A* 1992, **89**:8641-8645.
6. Abu-Hamdah R, Rabiee A, Meneilly GS, Shannon RP, Andersen DK, Elahi D: **Clinical review: The extrapancreatic effects of glucagon-like peptide-1 and related peptides.** *J Clin Endocrinol Metab* 2009, **94**:1843-1852.
7. Alvarez E, Martinez MD, Roncero I, Chowen JA, Garcia-Cuartero B, Gispert JD, Sanz C, Vazquez P, Maldonado A, de Caceres J, et al: **The expression of GLP-1 receptor mRNA and protein allows the effect of GLP-1 on glucose metabolism in the human hypothalamus and brainstem.** *J Neurochem* 2005, **92**:798-806.
8. Drucker DJ: **The biology of incretin hormones.** *Cell Metab* 2006, **3**:153-165.
9. Ussher JR, Drucker DJ: **Cardiovascular biology of the incretin system.** *Endocr Rev* 2012, **33**:187-215.
10. Martin JH, Deacon CF, Gorrell MD, Prins JB: **Incretin-based therapies--review of the physiology, pharmacology and emerging clinical experience.** *Intern Med J* 2011, **41**:299-307.
11. Svegliati-Baroni G, Saccomanno S, Rychlicki C, Agostinelli L, De Minicis S, Candelaresi C, Faraci G, Pacetti D, Vivarelli M, Nicolini D, et al: **Glucagon-like peptide-1 receptor activation stimulates hepatic lipid oxidation and restores hepatic signalling alteration induced by a high-fat diet in nonalcoholic steatohepatitis.** *Liver Int* 2011, **31**:1285-1297.
12. Lopez-Delgado MI, Morales M, Villanueva-Penacarrillo ML, Malaisse WJ, Valverde I: **Effects of glucagon-like peptide 1 on the kinetics of glycogen synthase a in hepatocytes from normal and diabetic rats.** *Endocrinology* 1998, **139**:2811-2817.
13. Valverde I, Morales M, Clemente F, Lopez-Delgado MI, Delgado E, Perea A, Villanueva-Penacarrillo ML: **Glucagon-like peptide 1: a potent glycogenic hormone.** *FEBS Lett* 1994, **349**:313-316.
14. Dardevet D, Moore MC, Neal D, DiCostanzo CA, Snead W, Cherrington AD: **Insulin-independent effects of GLP-1 on canine liver glucose metabolism: duration of infusion and involvement of hepatportal region.** *Am J Physiol Endocrinol Metab* 2004, **287**:E75-81.
15. Drucker DJ, Philippe J, Mojsov S, Chick WL, Habener JF: **Glucagon-like peptide I stimulates insulin gene expression and increases cyclic AMP levels in a rat islet cell line.** *Proc Natl Acad Sci U S A* 1987, **84**:3434-3438.
16. Fehmman 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* 1995, **16**:390-410.

17. 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* 2004, **53**:5-13.
18. Drucker DJ: **The role of gut hormones in glucose homeostasis.** *J Clin Invest* 2007, **117**:24-32.
19. Creutzfeldt W: **The incretin concept today.** *Diabetologia* 1979, **16**:75-85.
20. Meier JJ, Nauck MA: **Glucagon-like peptide 1(GLP-1) in biology and pathology.** *Diabetes Metab Res Rev* 2005, **21**:91-117.
21. Pipeleers DG, Schuit FC, in't Veld PA, Maes E, Hooghe-Peters EL, Van de Winkel M, Gepts W: **Interplay of nutrients and hormones in the regulation of insulin release.** *Endocrinology* 1985, **117**:824-833.
22. Kang G, Chepurny OG, Malester B, Rindler MJ, Rehmann H, Bos JL, Schwede F, Coetzee WA, Holz GG: **cAMP sensor Epac as a determinant of ATP-sensitive potassium channel activity in human pancreatic beta cells and rat INS-1 cells.** *J Physiol* 2006, **573**:595-609.
23. Light PE, Manning Fox JE, Riedel MJ, Wheeler MB: **Glucagon-like peptide-1 inhibits pancreatic ATP-sensitive potassium channels via a protein kinase A- and ADP-dependent mechanism.** *Mol Endocrinol* 2002, **16**:2135-2144.
24. Shibasaki T, Sunaga Y, Seino S: **Integration of ATP, cAMP, and Ca<sup>2+</sup> signals in insulin granule exocytosis.** *Diabetes* 2004, **53 Suppl 3**:S59-62.
25. Yabe D, Seino Y: **Two incretin hormones GLP-1 and GIP: comparison of their actions in insulin secretion and beta cell preservation.** *Prog Biophys Mol Biol* 2011, **107**:248-256.
26. Kang G, Joseph JW, Chepurny OG, Monaco M, Wheeler MB, Bos JL, Schwede F, Genieser HG, Holz GG: **Epac-selective cAMP analog 8-pCPT-2'-O-Me-cAMP as a stimulus for Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release and exocytosis in pancreatic beta-cells.** *J Biol Chem* 2003, **278**:8279-8285.
27. Tsuboi T, da Silva Xavier G, Holz GG, Jouaville LS, Thomas AP, Rutter GA: **Glucagon-like peptide-1 mobilizes intracellular Ca<sup>2+</sup> and stimulates mitochondrial ATP synthesis in pancreatic MIN6 beta-cells.** *Biochem J* 2003, **369**:287-299.
28. Li LX, MacDonald PE, Ahn DS, Oudit GY, Backx PH, Brubaker PL: **Role of phosphatidylinositol 3-kinasegamma in the beta-cell: interactions with glucagon-like peptide-1.** *Endocrinology* 2006, **147**:3318-3325.
29. Suzuki Y, Zhang H, Saito N, Kojima I, Urano T, Mogami H: **Glucagon-like peptide 1 activates protein kinase C through Ca<sup>2+</sup>-dependent activation of phospholipase C in insulin-secreting cells.** *J Biol Chem* 2006, **281**:28499-28507.
30. Buteau J, Foisy S, Joly E, Prentki M: **Glucagon-like peptide 1 induces pancreatic beta-cell proliferation via transactivation of the epidermal growth factor receptor.** *Diabetes* 2003, **52**:124-132.
31. Drucker DJ: **Glucagon-like peptides: regulators of cell proliferation, differentiation, and apoptosis.** *Mol Endocrinol* 2003, **17**:161-171.
32. Li Y, Cao X, Li LX, Brubaker PL, Edlund H, Drucker DJ: **beta-Cell Pdx1 expression is essential for the glucoregulatory, proliferative, and cytoprotective actions of glucagon-like peptide-1.** *Diabetes* 2005, **54**:482-491.
33. Kim W, Egan JM: **The role of incretins in glucose homeostasis and diabetes treatment.** *Pharmacol Rev* 2008, **60**:470-512.
34. Xie T, Chen M, Zhang QH, Ma Z, Weinstein LS: **Beta cell-specific deficiency of the stimulatory G protein alpha-subunit Gsalpha**

- leads to reduced beta cell mass and insulin-deficient diabetes. *Proc Natl Acad Sci U S A* 2007, **104**:19601-19606.
35. Portha B, Tourrel-Cuzin C, Movassat J: **Activation of the GLP-1 receptor signalling pathway: a relevant strategy to repair a deficient beta-cell mass.** *Exp Diabetes Res* 2011, **2011**:376509.
  36. Mukai E, Fujimoto S, Sato H, Oneyama C, Kominato R, Sato Y, Sasaki M, Nishi Y, Okada M, Inagaki N: **Exendin-4 suppresses SRC activation and reactive oxygen species production in diabetic Goto-Kakizaki rat islets in an Epac-dependent manner.** *Diabetes* 2011, **60**:218-226.
  37. De Marinis YZ, Salehi A, Ward CE, Zhang Q, Abdulkader F, Bengtsson M, Braha O, Braun M, Ramracheya R, Amisten S, et al: **GLP-1 inhibits and adrenaline stimulates glucagon release by differential modulation of N- and L-type Ca<sup>2+</sup> channel-dependent exocytosis.** *Cell Metab* 2010, **11**:543-553.
  38. Nakabayashi H, Nishizawa M, Nakagawa A, Takeda R, Nijima A: **Vagal hepatopancreatic reflex effect evoked by intraportal appearance of tGLP-1.** *Am J Physiol* 1996, **271**:E808-813.
  39. Balkan B, Li X: **Portal GLP-1 administration in rats augments the insulin response to glucose via neuronal mechanisms.** *Am J Physiol Regul Integr Comp Physiol* 2000, **279**:R1449-1454.
  40. Burcelin R, Da Costa A, Drucker D, Thorens B: **Glucose competence of the hepatoportal vein sensor requires the presence of an activated glucagon-like peptide-1 receptor.** *Diabetes* 2001, **50**:1720-1728.
  41. Nauck MA, Niedereichholz U, Ettler R, Holst JJ, Orskov C, Ritzel R, Schmiegel WH: **Glucagon-like peptide 1 inhibition of gastric emptying outweighs its insulinotropic effects in healthy humans.** *Am J Physiol* 1997, **273**:E981-988.
  42. Tolessa T, Gutniak M, Holst JJ, Efendic S, Hellstrom PM: **Glucagon-like peptide-1 retards gastric emptying and small bowel transit in the rat: effect mediated through central or enteric nervous mechanisms.** *Dig Dis Sci* 1998, **43**:2284-2290.
  43. Wettergren A, Wojdemann M, Meisner S, Stadil F, Holst JJ: **The inhibitory effect of glucagon-like peptide-1 (GLP-1) 7-36 amide on gastric acid secretion in humans depends on an intact vagal innervation.** *Gut* 1997, **40**:597-601.
  44. Wishart JM, Horowitz M, Morris HA, Jones KL, Nauck MA: **Relation between gastric emptying of glucose and plasma concentrations of glucagon-like peptide-1.** *Peptides* 1998, **19**:1049-1053.
  45. Wettergren A, Schjoldager B, Mortensen PE, Myhre J, Christiansen J, Holst JJ: **Truncated GLP-1 (proglucagon 78-107-amide) inhibits gastric and pancreatic functions in man.** *Dig Dis Sci* 1993, **38**:665-673.
  46. Dupre J, Behme MT, Hramiak IM, McFarlane P, Williamson MP, Zabel P, McDonald TJ: **Glucagon-like peptide I reduces postprandial glycemic excursions in IDDM.** *Diabetes* 1995, **44**:626-630.
  47. Turton MD, O'Shea D, Gunn I, Beak SA, Edwards CM, Meeran K, Choi SJ, Taylor GM, Heath MM, Lambert PD, et al: **A role for glucagon-like peptide-1 in the central regulation of feeding.** *Nature* 1996, **379**:69-72.
  48. Li Y, Perry T, Kindy MS, Harvey BK, Tweedie D, Holloway HW, Powers K, Shen H, Egan JM, Sambamurti K, et al: **GLP-1 receptor stimulation preserves primary cortical and dopaminergic neurons in cellular and rodent models of stroke and Parkinsonism.** *Proc Natl Acad Sci U S A* 2009, **106**:1285-1290.

49. McClean PL, Parthasarathy V, Faivre E, Holscher C: **The diabetes drug liraglutide prevents degenerative processes in a mouse model of Alzheimer's disease.** *J Neurosci* 2011, **31**:6587-6594.
50. Holst JJ, Burcelin R, Nathanson E: **Neuroprotective properties of GLP-1: theoretical and practical applications.** *Curr Med Res Opin* 2011, **27**:547-558.
51. Oben J, Morgan L, Fletcher J, Marks V: **Effect of the entero-pancreatic hormones, gastric inhibitory polypeptide and glucagon-like polypeptide-1(7-36) amide, on fatty acid synthesis in explants of rat adipose tissue.** *J Endocrinol* 1991, **130**:267-272.
52. Sancho V, Nuche B, Arnes L, Cancelas J, Gonzalez N, Diaz-Miguel M, Martin-Duce A, Valverde I, Villanueva-Penacarrillo ML: **The action of GLP-1 and exendins upon glucose transport in normal human adipocytes, and on kinase activity as compared to morbidly obese patients.** *Int J Mol Med* 2007, **19**:961-966.
53. Luque MA, Gonzalez N, Marquez L, Acitores A, Redondo A, Morales M, Valverde I, Villanueva-Penacarrillo ML: **Glucagon-like peptide-1 (GLP-1) and glucose metabolism in human myocytes.** *J Endocrinol* 2002, **173**:465-473.
54. Cauchi S, Froguel P: **TCF7L2 genetic defect and type 2 diabetes.** *Curr Diab Rep* 2008, **8**:149-155.
55. Lyssenko V, Lupi R, Marchetti P, Del Guerra S, Orho-Melander M, Almgren P, Sjogren M, Ling C, Eriksson KF, Lethagen AL, et al: **Mechanisms by which common variants in the TCF7L2 gene increase risk of type 2 diabetes.** *J Clin Invest* 2007, **117**:2155-2163.
56. Schafer SA, Tschrutter O, Machicao F, Thamer C, Stefan N, Gallwitz B, Holst JJ, Dekker JM, Hart LM, Nijpels G, et al: **Impaired glucagon-like peptide-1-induced insulin secretion in carriers of transcription factor 7-like 2 (TCF7L2) gene polymorphisms.** *Diabetologia* 2007, **50**:2443-2450.
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* 1993, **91**:301-307.
58. Faerch K, Pilgaard K, Knop FK, Hansen T, Pedersen O, Jorgensen T, Holst JJ: **Incretin and pancreatic hormone secretion in Caucasian non-diabetic carriers of the TCF7L2 rs7903146 risk T allele.** *Diabetes Obes Metab* 2012.
59. Mannucci E, Ognibene A, Cremasco F, Bardini G, Mencucci A, Pierazzuoli E, Ciani S, Fanelli A, Messeri G, Rotella CM: **Glucagon-like peptide (GLP)-1 and leptin concentrations in obese patients with Type 2 diabetes mellitus.** *Diabet Med* 2000, **17**:713-719.
60. 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* 2001, **50**:609-613.
61. Lugari R, Dei Cas A, Ugolotti D, Finardi L, Barilli AL, Ognibene C, Luciani A, Zandomenoghi R, Gnudi A: **Evidence for early impairment of glucagon-like peptide 1-induced insulin secretion in human type 2 (non insulin-dependent) diabetes.** *Horm Metab Res* 2002, **34**:150-154.
62. Yi F, Sun J, Lim GE, Fantus IG, Brubaker PL, Jin T: **Cross talk between the insulin and Wnt signaling pathways: evidence from intestinal endocrine L cells.** *Endocrinology* 2008, **149**:2341-2351.
63. Jin T: **Why diabetes patients are more prone to the development of colon cancer?** *Med Hypotheses* 2008, **71**:241-244.
64. Ahren B, Carr RD, Deacon CF: **Incretin hormone secretion over the day.** *Vitam Horm* 2010, **84**:203-220.

65. Cauchi S, Choquet H, Gutierrez-Aguilar R, Capel F, Grau K, Proenca C, Dina C, Duval A, Balkau B, Marre M, et al: **Effects of TCF7L2 polymorphisms on obesity in European populations.** *Obesity (Silver Spring)* 2008, **16**:476-482.
66. Cauchi S, Meyre D, Dina C, Choquet H, Samson C, Gallina S, Balkau B, Charpentier G, Pattou F, Stetsyuk V, et al: **Transcription factor TCF7L2 genetic study in the French population: expression in human beta-cells and adipose tissue and strong association with type 2 diabetes.** *Diabetes* 2006, **55**:2903-2908.
67. 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* 2001, **86**:3717-3723.
68. Nathanson D, Zethelius B, Berne C, Holst JJ, Sjöholm A, Nystrom T: **Reduced plasma levels of glucagon-like peptide-1 in elderly men are associated with impaired glucose tolerance but not with coronary heart disease.** *Diabetologia* 2010, **53**:277-280.
69. Muscelli E, Mari A, Casolaro A, Camastra S, Seghieri G, Gastaldelli A, Holst JJ, Ferrannini E: **Separate impact of obesity and glucose tolerance on the incretin effect in normal subjects and type 2 diabetic patients.** *Diabetes* 2008, **57**:1340-1348.
70. Rask E, Olsson T, Soderberg S, Johnson O, Seckl J, Holst JJ, Ahren B: **Impaired incretin response after a mixed meal is associated with insulin resistance in nondiabetic men.** *Diabetes Care* 2001, **24**:1640-1645.
71. 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* 2003, **88**:2706-2713.
72. Mojsov S, Heinrich G, Wilson IB, Ravazzola M, Orci L, Habener JF: **Preproglucagon gene expression in pancreas and intestine diversifies at the level of post-translational processing.** *J Biol Chem* 1986, **261**:11880-11889.
73. Whalley NM, Pritchard LE, Smith DM, White A: **Processing of proglucagon to GLP-1 in pancreatic alpha-cells: is this a paracrine mechanism enabling GLP-1 to act on beta-cells?** *J Endocrinol* 2011, **211**:99-106.
74. Shao W, Wang D, Chiang YT, Ip W, Zhu L, Xu F, Columbus J, Belsham DD, Irwin DM, Zhang H, et al: **The Wnt Signaling Pathway Effector TCF7L2 Controls Gut and Brain Proglucagon Gene Expression and Glucose Homeostasis.** *Diabetes* 2012.
75. Yi F, Brubaker PL, Jin T: **TCF-4 mediates cell type-specific regulation of proglucagon gene expression by beta-catenin and glycogen synthase kinase-3beta.** *J Biol Chem* 2005, **280**:1457-1464.
76. Holst JJ: **The physiology of glucagon-like peptide 1.** *Physiol Rev* 2007, **87**:1409-1439.
77. Balks HJ, Holst JJ, von zur Muhlen A, Brabant G: **Rapid oscillations in plasma glucagon-like peptide-1 (GLP-1) in humans: cholinergic control of GLP-1 secretion via muscarinic receptors.** *J Clin Endocrinol Metab* 1997, **82**:786-790.
78. Elliott RM, Morgan LM, Tredger JA, Deacon S, Wright J, Marks V: **Glucagon-like peptide-1 (7-36)amide and glucose-dependent insulinotropic polypeptide secretion in response to nutrient ingestion in man: acute post-prandial and 24-h secretion patterns.** *J Endocrinol* 1993, **138**:159-166.
79. Gribble FM, Williams L, Simpson AK, Reimann F: **A novel glucose-sensing mechanism contributing to glucagon-like peptide-1 secretion from the GLUTag cell line.** *Diabetes* 2003, **52**:1147-1154.

80. Ahren B, Holst JJ, Yu S: **1,5-Anhydro-D-fructose increases glucose tolerance by increasing glucagon-like peptide-1 and insulin in mice.** *Eur J Pharmacol* 2000, **397**:219-225.
81. Tolhurst G, Reimann F, Gribble FM: **Nutritional regulation of glucagon-like peptide-1 secretion.** *J Physiol* 2009, **587**:27-32.
82. Habib AM RF, Parker HE and Gribble FM: **Expression of glucose-sensing machinery in intestinal GLP-1-secreting cells.** *Diabetologia* 2007, **50**:S287.
83. Nielsen LB, Ploug KB, Swift P, Orskov C, Jansen-Olesen I, Chiarelli F, Holst JJ, Hougaard P, Porksen S, Holl R, et al: **Co-localisation of the Kir6.2/SUR1 channel complex with glucagon-like peptide-1 and glucose-dependent insulinotropic polypeptide expression in human ileal cells and implications for glycaemic control in new onset type 1 diabetes.** *Eur J Endocrinol* 2007, **156**:663-671.
84. Reimann F, Habib AM, Tolhurst G, Parker HE, Rogers GJ, Gribble FM: **Glucose Sensing in L Cells: A Primary Cell Study.** *Cell Metab* 2008, **8**:532-539.
85. Mace OJ, Schindler M, Patel S: **The regulation of K- and L-cell activity by GLUT2 and the calcium-sensing receptor CasR in rat small intestine.** *J Physiol* 2012, **590**:2917-2936.
86. Matschinsky FM: **Regulation of pancreatic beta-cell glucokinase: from basics to therapeutics.** *Diabetes* 2002, **51 Suppl 3**:S394-404.
87. Moriya R, Shirakura T, Ito J, Mashiko S, Seo T: **Activation of sodium-glucose cotransporter 1 ameliorates hyperglycemia by mediating incretin secretion in mice.** *Am J Physiol Endocrinol Metab* 2009, **297**:E1358-1365.
88. Rozengurt E, Sternini C: **Taste receptor signaling in the mammalian gut.** *Curr Opin Pharmacol* 2007, **7**:557-562.
89. Margolskee RF, Dyer J, Kokrashvili Z, Salmon KS, Ilegems E, Daly K, Maillet EL, Ninomiya Y, Mosinger B, Shirazi-Beechey SP: **TIR3 and gustducin in gut sense sugars to regulate expression of Na<sup>+</sup>-glucose cotransporter 1.** *Proc Natl Acad Sci U S A* 2007, **104**:15075-15080.
90. Cordier-Bussat M, Bernard C, Levenez F, Klages N, Laser-Ritz B, Philippe J, Chayvialle JA, Cuber JC: **Peptones stimulate both the secretion of the incretin hormone glucagon-like peptide 1 and the transcription of the proglucagon gene.** *Diabetes* 1998, **47**:1038-1045.
91. Dumoulin V, Moro F, Barcelo A, Dakka T, Cuber JC: **Peptide YY, glucagon-like peptide-1, and neurotensin responses to luminal factors in the isolated vascularly perfused rat ileum.** *Endocrinology* 1998, **139**:3780-3786.
92. Layer P, Holst JJ, Grandt D, Goebell H: **Ileal release of glucagon-like peptide-1 (GLP-1). Association with inhibition of gastric acid secretion in humans.** *Dig Dis Sci* 1995, **40**:1074-1082.
93. Herrmann 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.
94. Reimann F, Williams L, da Silva Xavier G, Rutter GA, Gribble FM: **Glutamine potently stimulates glucagon-like peptide-1 secretion from GLUTag cells.** *Diabetologia* 2004, **47**:1592-1601.
95. Thomsen C, Rasmussen O, Lousen T, Holst JJ, Fenselau S, Schrenzenmeir J, Hermansen K: **Differential effects of saturated and monounsaturated fatty acids on postprandial lipemia and incretin responses in healthy subjects.** *Am J Clin Nutr* 1999, **69**:1135-1143.
96. Lindgren O, Carr RD, Deacon CF, Holst JJ, Pacini G, Mari A, Ahren B: **Incretin hormone and insulin responses to oral versus intravenous lipid administration in humans.** *J Clin Endocrinol Metab* 2011, **96**:2519-2524.

97. Thomsen C, Storm H, Holst JJ, Hermansen K: **Differential effects of saturated and monounsaturated fats on postprandial lipemia and glucagon-like peptide 1 responses in patients with type 2 diabetes.** *Am J Clin Nutr* 2003, **77**:605-611.
98. Tolhurst G, Heffron H, Lam YS, Parker HE, Habib AM, Diakogiannaki E, Cameron J, Grosse J, Reimann F, Gribble FM: **Short-chain fatty acids stimulate glucagon-like peptide-1 secretion via the G-protein-coupled receptor FFAR2.** *Diabetes* 2012, **61**:364-371.
99. Hirasawa A, Tsumaya K, Awaji T, Katsuma S, Adachi T, Yamada M, Sugimoto Y, Miyazaki S, Tsujimoto G: **Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120.** *Nat Med* 2005, **11**:90-94.
100. Ahren B: **The future of incretin-based therapy: novel avenues--novel targets.** *Diabetes Obes Metab* 2011, **13 Suppl 1**:158-166.
101. Hansen HS, Rosenkilde MM, Holst JJ, Schwartz TW: **GPR119 as a fat sensor.** *Trends Pharmacol Sci* 2012, **33**:374-381.
102. Parker HE, Reimann F, Gribble FM: **Molecular mechanisms underlying nutrient-stimulated incretin secretion.** *Expert Rev Mol Med* 2010, **12**:e1.
103. Luo J, Swaminath G, Brown SP, Zhang J, Guo Q, Chen M, Nguyen K, Tran T, Miao L, Dransfield PJ, et al: **A Potent Class of GPR40 Full Agonists Engages the EnteroInsular Axis to Promote Glucose Control in Rodents.** *PLoS One* 2012, **7**:e46300.
104. Dhayal S, Morgan NG: **The significance of GPR119 agonists as a future treatment for type 2 diabetes.** *Drug News Perspect* 2010, **23**:418-424.
105. Lauffer L, Iakoubov R, Brubaker PL: **GPR119: "double-dipping" for better glycemic control.** *Endocrinology* 2008, **149**:2035-2037.
106. Lauffer LM, Iakoubov R, Brubaker PL: **GPR119 is essential for oleoylethanolamide-induced glucagon-like peptide-1 secretion from the intestinal enteroendocrine L-cell.** *Diabetes* 2009, **58**:1058-1066.
107. Overton HA, Fyfe MC, Reynet C: **GPR119, a novel G protein-coupled receptor target for the treatment of type 2 diabetes and obesity.** *Br J Pharmacol* 2008, **153 Suppl 1**:S76-81.
108. Rayasam GV, Tulasi VK, Davis JA, Bansal VS: **Fatty acid receptors as new therapeutic targets for diabetes.** *Expert Opin Ther Targets* 2007, **11**:661-671.
109. Shah U: **GPR119 agonists: a promising new approach for the treatment of type 2 diabetes and related metabolic disorders.** *Curr Opin Drug Discov Devel* 2009, **12**:519-532.
110. Shah U, Kowalski TJ: **GPR119 agonists for the potential treatment of type 2 diabetes and related metabolic disorders.** *Vitam Horm* 2010, **84**:415-448.
111. Tan CP, Feng Y, Zhou YP, Eiermann GJ, Petrov A, Zhou C, Lin S, Salituro G, Meinke P, Mosley R, et al: **Selective small-molecule agonists of G protein-coupled receptor 40 promote glucose-dependent insulin secretion and reduce blood glucose in mice.** *Diabetes* 2008, **57**:2211-2219.
112. Winzell MS, Ahren B: **G-protein-coupled receptors and islet function-implications for treatment of type 2 diabetes.** *Pharmacol Ther* 2007, **116**:437-448.
113. Yoshida S, Ohishi T, Matsui T, Shibasaki M: **Identification of a novel GPR119 agonist, AS1269574, with in vitro and in vivo glucose-stimulated insulin secretion.** *Biochem Biophys Res Commun* 2010, **400**:437-441.
114. Yoshida S, Ohishi T, Matsui T, Tanaka H, Oshima H, Yonetoku Y, Shibasaki M: **The role of small molecule GPR119 agonist,**

- AS1535907, in glucose-stimulated insulin secretion and pancreatic beta-cell function.** *Diabetes Obes Metab* 2011, **13**:34-41.
115. Yoshida S, Ohishi T, Matsui T, Tanaka H, Oshima H, Yonetoku Y, Shibasaki M: **Novel GPR119 agonist AS1535907 contributes to first-phase insulin secretion in rat perfused pancreas and diabetic db/db mice.** *Biochem Biophys Res Commun* 2010, **402**:280-285.
116. Yoshida S, Tanaka H, Oshima H, Yamazaki T, Yonetoku Y, Ohishi T, Matsui T, Shibasaki M: **AS1907417, a novel GPR119 agonist, as an insulinotropic and beta-cell preservative agent for the treatment of type 2 diabetes.** *Biochem Biophys Res Commun* 2010, **400**:745-751.
117. Schwartz TW, Holst B: **An enteroendocrine full package solution.** *Cell Metab* 2010, **11**:445-447.
118. Burant CF, Viswanathan P, Marciniak J, Cao C, Vakilynejad M, Xie B, Leifke E: **TAK-875 versus placebo or glimepiride in type 2 diabetes mellitus: a phase 2, randomised, double-blind, placebo-controlled trial.** *Lancet* 2012, **379**:1403-1411.
119. Katz LB, Gambale JJ, Rothenberg PL, Vanapalli SR, Vaccaro N, Xi L, Sarich TC, Stein PP: **Effects of JNJ-38431055, a novel GPR119 receptor agonist, in randomized, double-blind, placebo-controlled studies in subjects with type 2 diabetes.** *Diabetes Obes Metab* 2012, **14**:709-716.
120. Chen Y, Li ZY, Yang Y, Zhang HJ: **Uncoupling protein 2 regulates glucagon-like peptide-1 secretion in L-cells.** *World J Gastroenterol* 2012, **18**:3451-3457.
121. Zhang H, Li J, Liang X, Luo Y, Zen K, Zhang CY: **Uncoupling protein 2 negatively regulates glucose-induced glucagon-like peptide 1 secretion.** *J Mol Endocrinol* 2012, **48**:151-158.
122. Brubaker PL: **Control of glucagon-like immunoreactive peptide secretion from fetal rat intestinal cultures.** *Endocrinology* 1988, **123**:220-226.
123. Brubaker PL, Schloos J, Drucker DJ: **Regulation of glucagon-like peptide-1 synthesis and secretion in the GLUTag enteroendocrine cell line.** *Endocrinology* 1998, **139**:4108-4114.
124. Simpson AK, Ward PS, Wong KY, Collord GJ, Habib AM, Reimann F, Gribble FM: **Cyclic AMP triggers glucagon-like peptide-1 secretion from the GLUTag enteroendocrine cell line.** *Diabetologia* 2007, **50**:2181-2189.
125. Rocca AS, Brubaker PL: **Role of the vagus nerve in mediating proximal nutrient-induced glucagon-like peptide-1 secretion.** *Endocrinology* 1999, **140**:1687-1694.
126. Vahl TP, Drazen DL, Seeley RJ, D'Alessio DA, Woods SC: **Meal-anticipatory glucagon-like peptide-1 secretion in rats.** *Endocrinology* 2010, **151**:569-575.
127. Ahren B, Holst JJ: **The cephalic insulin response to meal ingestion in humans is dependent on both cholinergic and noncholinergic mechanisms and is important for postprandial glycemia.** *Diabetes* 2001, **50**:1030-1038.
128. Katsuma S, Hirasawa A, Tsujimoto G: **Bile acids promote glucagon-like peptide-1 secretion through TGR5 in a murine enteroendocrine cell line STC-1.** *Biochem Biophys Res Commun* 2005, **329**:386-390.
129. Parker HE, Wallis K, le Roux CW, Wong KY, Reimann F, Gribble FM: **Molecular mechanisms underlying bile acid-stimulated glucagon-like peptide-1 secretion.** *Br J Pharmacol* 2012, **165**:414-423.
130. Thomas C, Gioiello A, Noriega L, Strehle A, Oury J, Rizzo G, Macchiarulo A, Yamamoto H, Matak C, Pruzanski M, et al: **TGR5-mediated bile acid sensing controls glucose homeostasis.** *Cell Metab* 2009, **10**:167-177.

131. Pols TW, Auwerx J, Schoonjans K: **Targeting the TGR5-GLP-1 pathway to combat type 2 diabetes and non-alcoholic fatty liver disease.** *Gastroenterol Clin Biol* 2010, **34**:270-273.
132. Adrian TE, Gariballa S, Parekh KA, Thomas SA, Saadi H, Al Kaabi J, Nagelkerke N, Gedulin B, Young AA: **Rectal taurocholate increases L cell and insulin secretion, and decreases blood glucose and food intake in obese type 2 diabetic volunteers.** *Diabetologia* 2012, **55**:2343-2347.
133. Daoudi M, Hennuyer N, Borland MG, Touche V, Duhem C, Gross B, Caiazzo R, Kerr-Conte J, Pattou F, Peters JM, et al: **PPARbeta/delta activation induces enteroendocrine L cell GLP-1 production.** *Gastroenterology* 2011, **140**:1564-1574.
134. Flock GB, Cao X, Maziarz M, Drucker DJ: **Activation of Enteroendocrine Membrane Progesterone Receptors Promotes Incretin Secretion and Improves Glucose Tolerance in Mice.** *Diabetes* 2012.
135. Grigoryan M, Kedeas MH, Charron MJ, Guz Y, Teitelman G: **Regulation of mouse intestinal L cell progenitors proliferation by the glucagon family of peptides.** *Endocrinology* 2012, **153**:3076-3088.
136. Chandrasekaran C, Coopersmith CM, Gordon JI: **Use of normal and transgenic mice to examine the relationship between terminal differentiation of intestinal epithelial cells and accumulation of their cell cycle regulators.** *J Biol Chem* 1996, **271**:28414-28421.
137. Rindi G, Ratineau C, Ronco A, Candusso ME, Tsai M, Leiter AB: **Targeted ablation of secretin-producing cells in transgenic mice reveals a common differentiation pathway with multiple enteroendocrine cell lineages in the small intestine.** *Development* 1999, **126**:4149-4156.
138. Lee YC, Asa SL, Drucker DJ: **Glucagon gene 5'-flanking sequences direct expression of simian virus 40 large T antigen to the intestine, producing carcinoma of the large bowel in transgenic mice.** *J Biol Chem* 1992, **267**:10705-10708.
139. Friedlander RS, Moss CE, Mace J, Parker HE, Tolhurst G, Habib AM, Wachten S, Cooper DM, Gribble FM, Reimann F: **Role of phosphodiesterase and adenylate cyclase isozymes in murine colonic glucagon-like peptide 1 secreting cells.** *Br J Pharmacol* 2011, **163**:261-271.
140. Drucker DJ, Jin T, Asa SL, Young TA, Brubaker PL: **Activation of proglucagon gene transcription by protein kinase-A in a novel mouse enteroendocrine cell line.** *Mol Endocrinol* 1994, **8**:1646-1655.
141. DeFronzo RA: **Lilly lecture 1987. The triumvirate: beta-cell, muscle, liver. A collusion responsible for NIDDM.** *Diabetes* 1988, **37**:667-687.
142. McGarry JD: **What if Minkowski had been ageusic? An alternative angle on diabetes.** *Science* 1992, **258**:766-770.
143. Burke JP, Williams K, Gaskill SP, Hazuda HP, Haffner SM, Stern MP: **Rapid rise in the incidence of type 2 diabetes from 1987 to 1996: results from the San Antonio Heart Study.** *Arch Intern Med* 1999, **159**:1450-1456.
144. Kleinfeld AM, Prothro D, Brown DL, Davis RC, Richieri GV, DeMaria A: **Increases in serum unbound free fatty acid levels following coronary angioplasty.** *Am J Cardiol* 1996, **78**:1350-1354.
145. Shafirir E: **Partition of unesterified fatty acids in normal and nephrotic syndrome serum and its effect on serum electrophoretic pattern.** *J Clin Invest* 1958, **37**:1775-1782.
146. Frazee E, Donner CC, Swislocki AL, Chiou YA, Chen YD, Reaven GM: **Ambient plasma free fatty acid concentrations in noninsulin-**

- dependent diabetes mellitus: evidence for insulin resistance. *J Clin Endocrinol Metab* 1985, **61**:807-811.**
147. Wu P, Yang L, Shen X: **The relationship between GPR40 and lipotoxicity of the pancreatic beta-cells as well as the effect of pioglitazone.** *Biochem Biophys Res Commun* 2010, **403**:36-39.
  148. Sheehan MT, Jensen MD: **Metabolic complications of obesity. Pathophysiologic considerations.** *Med Clin North Am* 2000, **84**:363-385, vi.
  149. Su X, Abumrad NA: **Cellular fatty acid uptake: a pathway under construction.** *Trends Endocrinol Metab* 2009, **20**:72-77.
  150. Glatz JF, Luiken JJ, Bonen A: **Membrane fatty acid transporters as regulators of lipid metabolism: implications for metabolic disease.** *Physiol Rev* 2010, **90**:367-417.
  151. Bell RM, Coleman RA: **Enzymes of glycerolipid synthesis in eukaryotes.** *Annu Rev Biochem* 1980, **49**:459-487.
  152. Listenberger LL, Han X, Lewis SE, Cases S, Farese RV, Jr., Ory DS, Schaffer JE: **Triglyceride accumulation protects against fatty acid-induced lipotoxicity.** *Proc Natl Acad Sci U S A* 2003, **100**:3077-3082.
  153. Lopaschuk GD, Ussher JR, Folmes CD, Jaswal JS, Stanley WC: **Myocardial fatty acid metabolism in health and disease.** *Physiol Rev* 2010, **90**:207-258.
  154. Hagberg CE, Mehlem A, Falkevall A, Muhl L, Fam BC, Ortsater H, Scotney P, Nyqvist D, Samen E, Lu L, et al: **Targeting VEGF-B as a novel treatment for insulin resistance and type 2 diabetes.** *Nature* 2012, **490**:426-430.
  155. Unger RH: **Lipotoxicity in the pathogenesis of obesity-dependent NIDDM. Genetic and clinical implications.** *Diabetes* 1995, **44**:863-870.
  156. Carlsson C, Borg LA, Welsh N: **Sodium palmitate induces partial mitochondrial uncoupling and reactive oxygen species in rat pancreatic islets in vitro.** *Endocrinology* 1999, **140**:3422-3428.
  157. Yuan H, Zhang X, Huang X, Lu Y, Tang W, Man Y, Wang S, Xi J, Li J: **NADPH oxidase 2-derived reactive oxygen species mediate FFAs-induced dysfunction and apoptosis of beta-cells via JNK, p38 MAPK and p53 pathways.** *PLoS One* 2010, **5**:e15726.
  158. Poirout V, Robertson RP: **Minireview: Secondary beta-cell failure in type 2 diabetes--a convergence of glucotoxicity and lipotoxicity.** *Endocrinology* 2002, **143**:339-342.
  159. Shimabukuro M, Zhou YT, Levi M, Unger RH: **Fatty acid-induced beta cell apoptosis: a link between obesity and diabetes.** *Proc Natl Acad Sci U S A* 1998, **95**:2498-2502.
  160. Sjoholm A: **Ceramide inhibits pancreatic beta-cell insulin production and mitogenesis and mimics the actions of interleukin-1 beta.** *FEBS Lett* 1995, **367**:283-286.
  161. Kazuki Hattori IN, Christopher Runchel and Hidenori Ichijo: **The roles of ASK family proteins in stress responses and diseases.** *Cell Communication and Signaling* 2009, **7**.
  162. Tournier C, Hess P, Yang DD, Xu J, Turner TK, Nimnual A, Bar-Sagi D, Jones SN, Flavell RA, Davis RJ: **Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway.** *Science* 2000, **288**:870-874.
  163. Knowler WC, Barrett-Connor E, Fowler SE, Hamman RF, Lachin JM, Walker EA, Nathan DM: **Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin.** *N Engl J Med* 2002, **346**:393-403.
  164. Rutter GA, Leclerc I: **The AMP-regulated kinase family: enigmatic targets for diabetes therapy.** *Mol Cell Endocrinol* 2009, **297**:41-49.
  165. Patane G, Piro S, Rabuazzo AM, Anello M, Vigneri R, Purrello F: **Metformin restores insulin secretion altered by chronic exposure**

- to free fatty acids or high glucose: a direct metformin effect on pancreatic beta-cells.** *Diabetes* 2000, **49**:735-740.
166. Mannucci E, Ognibene A, Cremasco F, Bardini G, Mencucci A, Pierazzo E, Ciani S, Messeri G, Rotella CM: **Effect of metformin on glucagon-like peptide 1 (GLP-1) and leptin levels in obese nondiabetic subjects.** *Diabetes Care* 2001, **24**:489-494.
  167. Zander M, Taskiran M, Toft-Nielsen MB, Madsbad S, Holst JJ: **Additive glucose-lowering effects of glucagon-like peptide-1 and metformin in type 2 diabetes.** *Diabetes Care* 2001, **24**:720-725.
  168. Maida A, Lamont BJ, Cao X, Drucker DJ: **Metformin regulates the incretin receptor axis via a pathway dependent on peroxisome proliferator-activated receptor-alpha in mice.** *Diabetologia* 2011, **54**:339-349.
  169. Hinke SA, Kuhn-Wache K, Hoffmann T, Pederson RA, McIntosh CH, Demuth HU: **Metformin effects on dipeptidylpeptidase IV degradation of glucagon-like peptide-1.** *Biochem Biophys Res Commun* 2002, **291**:1302-1308.
  170. Yasuda N, Inoue T, Nagakura T, Yamazaki K, Kira K, Saeki T, Tanaka I: **Enhanced secretion of glucagon-like peptide 1 by biguanide compounds.** *Biochem Biophys Res Commun* 2002, **298**:779-784.
  171. Yasuda N, Inoue T, Nagakura T, Yamazaki K, Kira K, Saeki T, Tanaka I: **Metformin causes reduction of food intake and body weight gain and improvement of glucose intolerance in combination with dipeptidyl peptidase IV inhibitor in Zucker fa/fa rats.** *J Pharmacol Exp Ther* 2004, **310**:614-619.
  172. Mulherin AJ, Oh AH, Kim H, Grieco A, Lauffer LM, Brubaker PL: **Mechanisms underlying metformin-induced secretion of glucagon-like peptide-1 from the intestinal L cell.** *Endocrinology* 2011, **152**:4610-4619.
  173. Lim GE, Huang GJ, Flora N, LeRoith D, Rhodes CJ, Brubaker PL: **Insulin regulates glucagon-like peptide-1 secretion from the enteroendocrine L cell.** *Endocrinology* 2009, **150**:580-591.
  174. Drucker DJ, Nauck MA: **The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes.** *Lancet* 2006, **368**:1696-1705.
  175. Barnett AH: **Lixisenatide: evidence for its potential use in the treatment of type 2 diabetes.** *Core Evid* 2011, **6**:67-79.
  176. Sangle GV, Lauffer LM, Grieco A, Trivedi S, Iakoubov R, Brubaker PL: **Novel biological action of the dipeptidylpeptidase-IV inhibitor, sitagliptin, as a glucagon-like peptide-1 secretagogue.** *Endocrinology* 2012, **153**:564-573.
  177. Enc FY, Imeryuz N, Akin L, Turoglu T, Dede F, Haklar G, Tekesin N, Bekiroglu N, Yegen BC, Rehfeld JF, et al: **Inhibition of gastric emptying by acarbose is correlated with GLP-1 response and accompanied by CCK release.** *Am J Physiol Gastrointest Liver Physiol* 2001, **281**:G752-763.
  178. Radziuk J, Kemmer F, Morishima T, Berchtold P, Vranic M: **The effects of an alpha-glucosidase inhibitor on glycemia and the absorption of sucrose in man determined using a tracer method.** *Diabetes* 1984, **33**:207-213.
  179. Seifarth C, Bergmann J, Holst JJ, Ritzel R, Schmiegel W, Nauck MA: **Prolonged and enhanced secretion of glucagon-like peptide 1 (7-36 amide) after oral sucrose due to alpha-glucosidase inhibition (acarbose) in Type 2 diabetic patients.** *Diabet Med* 1998, **15**:485-491.
  180. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: **Protein measurement with the Folin phenol reagent.** *J Biol Chem* 1951, **193**:265-275.

181. Gundersen HJ, Bendtsen TF, Korbo L, Marcussen N, Moller A, Nielsen K, Nyengaard JR, Pakkenberg B, Sorensen FB, Vesterby A, et al.: **Some new, simple and efficient stereological methods and their use in pathological research and diagnosis.** *APMIS* 1988, **96**:379-394.
182. Meier JJ: **GLP-1 receptor agonists for individualized treatment of type 2 diabetes mellitus.** *Nat Rev Endocrinol* 2012.
183. Widmann C, Dolci W, Thorens B: **Agonist-induced internalization and recycling of the glucagon-like peptide-1 receptor in transfected fibroblasts and in insulinomas.** *Biochem J* 1995, **310** (Pt 1):203-214.
184. Matthews DR, Naylor BA, Jones RG, Ward GM, Turner RC: **Pulsatile insulin has greater hypoglycemic effect than continuous delivery.** *Diabetes* 1983, **32**:617-621.
185. Goodner CJ, Sweet IR, Harrison HC, Jr.: **Rapid reduction and return of surface insulin receptors after exposure to brief pulses of insulin in perfused rat hepatocytes.** *Diabetes* 1988, **37**:1316-1323.
186. Pedersen J, Ugleholdt RK, Jorgensen SM, Windelov JA, Grunddal KV, Schwartz TW, Fuchtbauer EM, Poulsen SS, Holst PJ, Holst JJ: **Glucose-metabolism is altered after loss of L- and alpha-cells, but not influenced by loss of K-cells.** *Am J Physiol Endocrinol Metab* 2012.
187. Kim DS, Jeong SK, Kim HR, Chae SW, Chae HJ: **Metformin regulates palmitate-induced apoptosis and ER stress response in HepG2 liver cells.** *Immunopharmacol Immunotoxicol*, **32**:251-257.
188. Schulz E, Doppeide J, Schuhmacher S, Thomas SR, Chen K, Daiber A, Wenzel P, Munzel T, Keaney JF, Jr.: **Suppression of the JNK pathway by induction of a metabolic stress response prevents vascular injury and dysfunction.** *Circulation* 2008, **118**:1347-1357.
189. Xie Z, Dong Y, Scholz R, Neumann D, Zou MH: **Phosphorylation of LKB1 at serine 428 by protein kinase C-zeta is required for metformin-enhanced activation of the AMP-activated protein kinase in endothelial cells.** *Circulation* 2008, **117**:952-962.
190. Piro S, Rabuazzo AM, Renis M, Purrello F: **Effects of metformin on oxidative stress, adenine nucleotides balance, and glucose-induced insulin release impaired by chronic free fatty acids exposure in rat pancreatic islets.** *J Endocrinol Invest* 2012, **35**:504-510.
191. Bullock BP, Heller RS, Habener JF: **Tissue distribution of messenger ribonucleic acid encoding the rat glucagon-like peptide-1 receptor.** *Endocrinology* 1996, **137**:2968-2978.
192. Campos RV, Lee YC, Drucker DJ: **Divergent tissue-specific and developmental expression of receptors for glucagon and glucagon-like peptide-1 in the mouse.** *Endocrinology* 1994, **134**:2156-2164.
193. Cani PD, Holst JJ, Drucker DJ, Delzenne NM, Thorens B, Burcelin R, Knauf C: **GLUT2 and the incretin receptors are involved in glucose-induced incretin secretion.** *Mol Cell Endocrinol* 2007, **276**:18-23.
194. Braun M, Ramracheya R, Rorsman P: **Autocrine regulation of insulin secretion.** *Diabetes Obes Metab* 2012, **14** Suppl 3:143-151.
195. Beglinger S, Drewe J, Schirra J, Goke B, D'Amato M, Beglinger C: **Role of fat hydrolysis in regulating glucagon-like Peptide-1 secretion.** *J Clin Endocrinol Metab* 2010, **95**:879-886.
196. Deacon CF, Ahren B: **Physiology of incretins in health and disease.** *Rev Diabet Stud* 2011, **8**:293-306.
197. Proctor WR, Bourdet DL, Thakker DR: **Mechanisms underlying saturable intestinal absorption of metformin.** *Drug Metab Dispos* 2008, **36**:1650-1658.

