The Role of GLP-1 in the Regulation of Metabolism and Immune Responses

by

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DEDICATION

To my family and friends who have ardently supported my scientific endeavors

To my husband, Christopher, who has listened to all my practice presentations

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
CGM	continuous glucose monitoring
CNS	central nervous system
CREB	cAMP response element-binding protein
DAB	3,3'-Diaminobenzidine
DPP4	dipeptidyl-peptidase 4
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
EPAC	exchange protein directly activated by cAMP
ERk1/2	extracellular signal-regulated kinases 1/2
Ex4	exendin-4
Ex9	exendin 9-39
F4/80	EGF-like module-containing mucin-like hormone receptor like 1
FXR	farnesoid x receptor
Gcg	preproglucagon
GcgR	glucagon receptor
GIP	gatric inhibitory polypeptide
GLP-1	glucagon-like peptide-1
GLP-1R	glucagon-like peptide-1 receptor
GLP-2	glucagon-like peptide-2
GLUT2	glucose transporter 2
GPCR	G protein-coupled receptors
GRPP	glicentin-related pancreatic polypeptide
GWAS	genome-wide association study
HFD	high-fat diet
ICV	intracerebroventricular injection
IHC	immunohistochemistry
IL-10	interleukin 10
IL-1β	interleukin 1β
IL-6	interleukin 6
IP	intraperitoneal
Katp	ATP-sensitive potassium channel
LPS	lipopolysaccharide
MCP1/CCL2	chemokine ligand 2
MPGF	major proglucagon fragment
NTS	nucleus tractus solitarius

PBH	post-bariatric hypoglycemia
PC	prohormone convertase
PI3K	phosphoinositide 3-kinase
PKA	cAMP-dependent protein kinase
POMC	proopiomelanocortin
PVN	paraventricular nucleus of hypothalamus
qPCR	real-time quantitative polymerase chain reaction
RER	respiratory exchange ratio
SGLT1	sodium-glucose transport proteins
STZ	streptozotocin
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TCF7L2	transcription factor 7-like 2
TGR5	G protein-coupled bile acid receptor 1
TNF-α	tumour necrosis factor α
Veh	vehicle
VSG	vertical sleeve gastrectomy

ABSTRACT

Glucagon-like peptide-1 (GLP-1) is encoded by the preproglucagon gene (*Gcg*) and expressed in the intestine, pancreas, and central nervous system (CNS). Activation of GLP-1 receptors (GLP-1R) on pancreatic β-cells induces insulin secretion in a glucose-dependent manner while activation of CNS GLP-1Rs suppress feeding. Thus, *Gcg*-derived peptides play an important role in gluco- and body weight regulation, and GLP-1 has been implicated in the success of bariatric surgery. GLP-1 agonists are an effective treatment of type 2 diabetes mellitus (T2DM) and obesity. The predominant source of circulating GLP-1 is the intestine, but the α-cell becomes an important source when the islet is metabolically stressed. Further, plasma GLP-1 is increased in T2DM patients in response to inflammation. Nutrient-stimulated GLP-1 functions as an incretin, however, the function of GLP-1 during inflammation is unknown. My dissertation proposes that during inflammation, GLP-1 plays a metabolic role, functioning to regulate glucose levels and food intake, and an immunologic role, functioning to regulate inflammation.

I examined the metabolic and immunologic role of *Gcg* under inflammatory conditions. Using a combination of high-fat diet (HFD)-induced obesity and a mouse model of tissue-specific *Gcg* expression, I explored the function of GLP-1 in response to inflammation by administering lipopolysaccharide (LPS), a well-established tool for inducing inflammation. LPS is a known anorectic agent that also alters glucose homeostasis; both functions of GLP-1.

I hypothesized that HFD would exacerbate physiological responses to LPS including increased plasma GLP-1, decreased blood glucose levels, and increased sickness-induced anorexia, as well as systemic inflammatory responses including increased plasma cytokines. Indeed, HFD did increase plasma pro-inflammatory cytokines, and GLP-1 levels in response to LPS and this was associated with greater anorexia in HFD-

fed animals. In the next set of studies, I tested whether GLP-1 secreted from either the pancreas or intestine was directly regulating feeding and glucose responses to LPS. I hypothesized that increases in circulating GLP-1, primarily from the pancreas, were necessary for feeding and glucose responses to LPS. I found that while both pancreatic and intestinal *Gcg* contribute to circulating levels of GLP-1 after LPS, the availability of either source of GLP-1 had no impact on glucoregulatory or feeding responses. Because pancreatic GLP-1 is the more novel contributor to circulation, I investigated the impact of *Gcg* on pancreatic inflammation. I found that 24h after LPS, whole-body chow-fed *Gcg* Null animals had increased macrophage accumulation in the pancreas. I saw a similar trend in HFD-fed *Gcg* Null mice. Using a GLP-1R reporter mouse, I found that macrophages isolated from the pancreas, but not the bone marrow, express GLP-1R. These data suggest that pancreatic GLP-1 directly regulates local macrophage responses to inflammation. I conclude that under severe inflammatory conditions, GLP-1 plays an immunologic rather than metabolic role in the pancreatic responses to LPS, through direct macrophage regulation.

This dissertation indicates a new role for GLP-1 signaling to pancreatic macrophages in response to inflammation. Future studies will explore the impact of this increased macrophage accumulation on long-term pancreatic function. In fact, my preliminary data demonstrate that IP glucose tolerance was impaired 2 weeks following LPS. This lasting impact of inflammation on pancreatic function points to a new use of GLP-1 agonists to protect pancreatic tissue during severe inflammation such as sepsis, or more recently, COVID-19.

CHAPTER 1

[1]Glucagon-like peptide-1: Actions and Influence on Pancreatic Hormone Function

Abstract

Glucagon-like petptide-1 (GLP-1) was described as an incretin over 30 years ago. GLP-1 is encoded by the preproglucagon gene (*Gcg*) which is expressed in the intestine, the pancreas, and the central nervous system. GLP-1 activates GLP-1 receptors (GLP-1R) on the β-cell to induce insulin secretion in a glucose-dependent manner. GLP-1 also inhibits α-cell secretion of glucagon. As few, if any, GLP-1R are expressed on α-cells, indirect regulation, via β- or δ-cell products has been thought to be the primary mechanism by which GLP-1 inhibits glucagon secretion. However, recent work suggests that there is sufficient expression of GLP-1R on α-cells for direct regulation as well. Although the predominant source of circulating GLP-1 is the intestine, the α-cell becomes a source of GLP-1 when the islet is metabolically stressed. Recent work suggests the possibility that this source of GLP-1 is also important in regulating nutrient-induced insulin secretion in a paracrine fashion. More work is also accumulating regarding the role of glucagon, another *Gcg*-derived protein produced by the α-cell, in stimulating insulin secretion by acting on GLP-1R. Altogether, these data clearly demonstrate the important role for *Gcg*-derived peptides in regulating insulin secretion. Because of GLP-1's important role in glucose homeostasis, it has been implicated in the success of bariatric surgery and has been successfully targeted for treatment of type 2 diabetes mellitus.

[1] This chapter is a reproduction of the following manuscript:

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Introduction: History of GLP-1 as an incretin

After the discovery of insulin and the development of the radioimmunoassay to assess plasma insulin levels, a debate ensued over whether glucose was the only stimulus for insulin secretion. Spurred on by work demonstrating that glucose removal from the blood was more rapid with oral vs. IV glucose in dogs (1), McIntyre et al. found that glucose was lower but insulin was higher after isocaloric loads of glucose administered into the jejunum vs. intravenously in man (2). Months later, a second study had similar findings with oral vs. IV glucose (3). These data in conjunction with multiple reports in the 1920's and 30's that intestinal mucosa had hypoglycemic properties led the authors of both papers to hypothesize that a gut-derived humoral substance contributed to the regulation of insulin secretion (**Figure 1.1**). Yet, it was not until the 1970's when the first "incretin" was discovered. When gastric inhibitory polypeptide (GIP; aka glucose-dependent insulinotropic peptide) was infused into humans intravenously, insulin immunoreactivity increased and glucose tolerance was improved (4). However, while gut extracts containing GIP increased insulin secretion, removal of GIP from the extracts only blunted the insulin response by 30% suggesting the presence of additional incretins (5).

With the discovery of glucagon and in the pursuit of understanding its form and function, two other peptides were found on the same mRNA (6,7). These peptides were glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-2 (GLP-2). At this time the biological function of these peptides was unknown but later work demonstrated that GLP-1, but not GLP-2, stimulated insulin release in rat islets (8) and when infused in humans (9). This latter study found that GIP was less effective at increasing insulin levels and concluded that GLP-1 was a physiological incretin in man. We now know that GLP-1 has a wide array of physiological functions, yet its role in the pancreas is still the most widely studied. The purpose of this review is to discuss the role and mechanisms associated with GLP-1 and GLP-1 receptor (GLP-1R) signaling in regulation of pancreatic hormone secretion and consequently glucose homeostasis. Consideration is also given to its role in bariatric surgery and the current state of GLP-1 pharmaceuticals.

Regulation of preproglucagon (*Gcg*) expression

We now know that preproglucagon (*Gcg*) is the gene that codes for GLP-1 and it is expressed in a specific population of intestinal enteroendocrine cells called L-cells. *Gcg* is also expressed within pancreatic islet α-cells, and in a distinct set of neurons within the nucleus of the solitary tract (NTS) (10,11). *Gcg* undergoes tissue-specific post-translational modification by prohormone convertases (PC). In intestinal L-cells and neurons of the NTS, a specific isoform of PC, PC1/3, is predominantly expressed and yields GLP-1, oxyntomodulin, and GLP-2 as the physiologically relevant products (12–14). In contrast, the α-cell predominantly expresses another PC isoform, PC2, which yields glucagon (15). *Gcg* codes for other bioactive proteins including the proglucagon fragments glicentin, glicentin-related pancreatic polypeptide (GRPP), and major proglucagon fragment (MPGF) but the functional significance of these peptides is unclear.

Nutrient status is clearly an important factor in regulating *Gcg* expression across all 3 organs for which it is expressed. Re-feeding after fasting (16,17), dietary fibers (17–19), long chain fatty triglycerides (16), and peptones (20,21), all increase intestinal *Gcg* expression, and amino acids stimulate α-cell hyperplasia and glucagon secretion (22). Interestingly, *in vitro* work in cell lines suggest that physiological stimuli such as peptones act via cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) signaling to regulate transcriptional responses of *Gcg* (23,24). In fact, increases in cAMP leads to increases in *Gcg* expression in both the pancreas and intestine (17,25–27). Downstream of cAMP but a pathway that is distinct from protein kinase A (PKA), activation of exchange protein activated by cAMP *2 (*EPAC) also increases *Gcg* transcription in both α- and L-cells (28,29). In contrast, specific to the L-cell, *Gcg* expression has been shown to be downstream of the canonical Wnt signaling pathway (a specific signaling transduction pathway), β-catenin and transcription factor 7 like 2 (TCF7L2) (30). Although the mechanisms are unclear, it is also well established that bowel resection or injury causes a large increase in intestinal *Gcg* expression (31). The function is likely related to the increase in GLP-2, which functions as an intestinal growth factor (see (32) for review). Distinct regulatory mechanisms for *Gcg*

transcriptional control between tissues is in parallel with the distinct patterns of prohormone processing in the major cell types producing *Gcg* peptides.

Regulation of intestinal GLP-1 secretion

Within the intestine, the density of *Gcg*-expressing cells increases from the proximal to distal gut with the expression being highest in the colon (33). L-cells within the intestinal epithelium and have apical processes that extend into the gut lumen allowing direct access to ingested nutrients (34), and all three macronutrients (carbohydrate, fat, protein) individually stimulate GLP-1 secretion (35,36).

Greater numbers of enteroendocrine cells that express *Gcg* within the lower intestine suggests that postprandial GLP-1 secretion is derived from the ileum and colon. Indeed, nutrient infusions directly into the ileum causes significantly greater increases in portal vein (the major blood vessel that collects intestinal secretions) concentrations of GLP-1 (37). Interestingly, in *ex vivo* studies from human tissue, the duodenum and ileum but not the colon were found to be glucose responsive (38) which is different than what is reported in mouse colon (39,40) but are consistent with data where patients that have had colon resection have normal GLP-1 responses to glucose (41). An argument has been made that duodenal secretion of GLP-1 is responsible for early phase GLP-1 secretion (42) but no direct link has been found. However, the anatomic distribution of the L-cells (highest number in distal gut) and the rapid increase in postprandial circulating GLP-1 (43) that occurs before nutrients reach the distal gut (44,45) is evidence supportive of neural, endocrine and/or paracrine mechanisms being more critical to nutrient-induced increases in circulating GLP-1 (46).

One possibility is that there are feed forward neural or paracrine signals from the upper gut to the ileum that stimulates the release of GLP-1 (47,48). Transection of the gut below the duodenum resulted in a delayed GLP-1 response to nutrients compared to when the gut is simply ligated, the latter being a procedure which preserves neural innervation (49). Further support for neural regulation of GLP-1, bilateral subdiaphragmatic vagotomy in conjunction with intestinal transection completely abolishes nutrient induced GLP-1 secretion (49). In rodents, human enteroendocrine

cells, and in perfused porcine ileum, cholinergic agonists, which bind to receptors in both the enteric and parasympathetic nervous system, stimulate GLP-1 secretion (26,48,50,51). Conversely, norepinephrine and α-adrenergic agonists inhibit (50) and β-adrenergic agonists stimulate (52,53) GLP-1 secretion suggesting that sympathetic neural innervation of the gut is also important in the regulation of GLP-1 secretion. In humans, atropine, which is a parasympathetic nervous system agonist, blunts glucose-induced increases plasma GLP-1 (45). Together these data lend support to the idea that neuronal influences are at play in regulation of postprandial GLP-1 secretion.

Despite the anatomic limitations, there are several potentially overlapping nutrient-sensing mechanisms that have been found to regulate intestinal GLP-1 secretion. Both passive glucose transport through the sodium glucose cotransporter 1 (SGLT1) (39,54) and active glucose transport through glucose transporter 2 (GLUT2) have been found to regulate intestinal GLP-1 secretion as inhibitors for each transporter blocks GLP-1 release (38). Similar to pancreatic β-cells that secrete insulin, potassium adenosine tri-phosphate (K_{ATP}) channels and its associated sulfonylurea receptor are also expressed in L-cells (55,56). Data suggest that increased glucose metabolism within L-cells leads to an increase in ATP and consequently depolarization of K_{ATP} channels triggering GLP-1 secretion (39,55,57). Interestingly, GLUT2 is linked to K_{ATP} channels (58) while SGLT1 is linked to voltage gated sodium and calcium channels in GLP-1 secretion (38). However, in animal models pharmacological (59) or genetic (60) blockade of SGLT1 had a bigger impact on blunting GLP-1 secretion compared to the same manipulations towards GLUT2. In contrast, *ex vivo* studies in human tissue suggests GLUT2 may be more important (38). It is unknown whether these differences are simply due to the species or the experimental conditions; in particular for the *ex vivo* experiments.

Early work demonstrating that PKA activators could stimulate GLP-1 secretion from cell lines suggested that G-protein coupled receptors (GPCR) were involved in the regulation of GLP-1 secretion (51,61,62). $G_{\alpha s}$ and $G_{\alpha q}$-coupled receptors responding to luminal bile acids and long-chain fatty acids have demonstrated potent effects on GLP-1 secretion (63). Bile acids signaling through the $G_{\alpha s}$ protein-coupled bile acid receptor 1, or TGR5, stimulate GLP-1 secretion (63,64). Bile acids also signal through farnesoid X

receptor, a nuclear transcription factor, but TGR5 is the primary bile acid receptor that can drive GLP-1 secretion (65). Offering clinical promise, *in vitro* data using primary enteroendocrine cells and an intestinal cell line indicated that pharmacologic TGR5 agonists are more potent GLP-1 secretagogues and are better at enhancing L-cell responses to calcium and glucose-induced GLP-1 secretion compared to naturally occurring bile acids (64).

Long-chain fatty acids and their derivatives specifically activate multiple types of GPCRs including, GPR119, GPR120, and GPR40 (66), and this activation also stimulates GLP-1 secretion. GPR119 is a $G_{\alpha s}$-coupled protein receptor and it is highly co-localized to L-cells within the GI tract. GPR119 agonists increase GLP-1 secretion (67) and GPR119 knockout mice have a reduced GLP-1 response to an oral glucose load (68). However, a randomized double-blind placebo controlled trial revealed that GPR119 agonism was not as effective at increasing total and active GLP-1 responses to glucose or a mixed liquid meal nor was it as effective at improving glucose control over a 2-week study period (69). In contrast to GPR119, a $G_{\alpha s}$-coupled receptor, GPR120 and GPR40 are $G_{\alpha q}$-coupled receptors that signal through protein kinase C. Activation of both GPR40 and GPR120 result in increased GLP-1 secretion (70–73), however, their role may be more pharmacological than physiological. While members of these GPCR families were under active investigation as drug targets for the treatment of type 2 diabetes mellitus (T2DM), due to lack of efficacy the number of drugs in the pipeline have been drastically reduced.

Another type of GPCR that is expressed in the GI tract and has been found to be linked to GLP-1 secretion are “sweet taste receptors” (74). These same receptors are expressed in the tongue and are integral in sweet taste perceptions. Their function in the GI tract is less clearly understood. Non-nutritive agonists of sweet taste receptors do increase GLP-1 levels and mice null for one such sweet taste receptor, α-gustducin, have reduced GLP-1 responses to an oral glucose load (74). Some data suggest that pharmacological inhibition of sweet taste receptors blunt GLP-1 responses to nutrients (75), but other studies found no impact of sweet taste receptor agonists on GLP-1 secretion in humans (40,76) or in *in vivo* or *ex vivo* studies in rats (77).

Altogether it seems that many molecular signaling pathways have been linked to GLP-1 secretion (**Figure 1.2**), and it is possible that all of these pathways are involved in order to provide an integrated response that also allows for fine tuning of GLP-1 secretion. One example of this is recent work that found a synergistic action of pharmacological TGR5 and GPR40 activation on the electrophysiological properties of L-cells (78). However, while data in mouse models are promising, there are currently no therapies currently in clinical trials focused on GPCR signaling to increase plasma GLP-1. Whether this is because these GPCRs are not important or whether increasing intestinal GLP-1 secretion, alone, is sufficient for obesity and/or T2DM treatment remains to be determined.

GLP-1 Receptor distribution and signaling

Direct regulation of insulin secretion

The GLP-1R is a GPCR that is expressed within the pancreas, lung, adipose tissue, kidney, heart, vascular smooth muscle, and in a number of specific nuclei within the CNS (25,79). Most of what we know about GLP-1R signaling is derived from studying the β-cell population of GLP-1R. When glucose is transported into the β-cell, its metabolism generates ATP which provides energy for closure of K_{ATP} channels and consequently an increase in intracellular calcium; the latter being necessary for exocytosis of insulin. Binding of the GLP-1R produces cAMP and consequently activation of PKA and EPAC2 (80) which potentiates glucose-stimulated insulin release (80–82). With fasting and when intracellular ADP is increased, PKA activation hyperpolarizes the β-cell membrane by increasing K_{ATP} channel conductance. When ADP levels are reduced with glucose metabolism, PKA phosphorylates a subunit of the K_{ATP} channel leading to channel closure and depolarization (83). This extends to pharmaceutical activation of the GLP-1R in that GLP-1R agonists are weak insulin secretagogues at basal glucose levels. Further, the ability of GLP-1 to suppress feeding, an action dependent upon CNS receptors, is blunted in fasted conditions (84,85) and dependent upon nutrient-dependent intracellular pathways (86) indicating that CNS GLP-1R signaling is also dependent upon nutrient availability.

Although pharmacological data make it very clear that GLP-1 regulates insulin secretion, genetic data suggest that the system is more complex than a simple endocrine model of insulin regulation. Genetic models demonstrate that although GLP-1 regulates insulin secretion through the β-cell GLP-1R (87), the necessity of these receptors depend on the route of delivery of nutrients. Mice with an inducible knockout of the β-cell GLP-1R have normal oral, but impaired intraperitoneal (IP) glucose tolerance (88). This is interesting as even whole body GLP-1R KO mice have greater impairments in IP vs. oral glucose tolerance. Importantly, these responses could also be explained by the redundancy of insulinotropic signals from the gut or nervous system during oral vs. IP glucose loads, but in the end still demonstrate that β-cell GLP-1R, in and of themselves, are not necessary for oral glucose tolerance.

GLP-1R regulation of insulin secretion independent of the β-cell GLP-1R

If GLP-1 has a role for regulating insulin secretion independent of its β-cell receptor, what would the population of receptors be? Given the rapid postprandial increase in insulin, one possibility is nervous system GLP-1R. Indeed, direct administration of GLP-1 into the 3rd cerebral ventricle (ICV) increases insulin secretion (89,90); an effect that is maintained in mice fed a high fat diet (91). In both mice and rats, administration of ICV exendin-4(9-39) (Ex9), a potent GLP-1R antagonist, during oral glucose impairs glucose tolerance and reduces insulin levels (89,92,93).

There are no detectable GLP-1R on the liver or on skeletal muscle and yet GLP-1 has repeatedly been shown to not just increase insulin secretion but also to improve insulin sensitivity. Despite the lack of hepatic GLP-1R expression, intravenous GLP-1 inhibits hepatic glucose production independent of islet hormones in humans (94). CNS GLP-1R activation may also explain this finding. When administered directly into the arcuate nucleus of rats, GLP-1 decreases hepatic glucose production under clamped conditions where glucose and insulin were held constant (89). The specific population of neurons responsible for this effect is not clear as deletion of GLP-1R within the hypothalamus or even more specifically on pro-opiomelanocortin neurons with the arcuate nucleus of the hypothalamus also do not impact normal glucose regulation (95). GLP-1 activates vagal afferent neuronal activity and administration of GLP-1R antagonists into the portal vein

impairs glucose tolerance (96) suggesting that the peripheral nervous system is also important in mediating GLP-1 effects on glucose homeostasis. However, neither genetic deletion of CNS nor vagal neuronal GLP-1R is not necessary for normal glucose regulation (97), but CNS (97) and specifically glutamatergic excitatory (vs. GABAergic inhibitory) neurons (98) that express GLP-1R are necessary for the weight-reducing effects of liraglutide, a long-acting GLP-1 agonist. Lastly, in obese mice and in humans, administration of GLP-1 agonists reduces hepatic steatosis (99–102). Whether these effects are also due to CNS activation is unclear but they do seem to be independent of the effect of chronic GLP-1 administration to reduce body weight. It remains to be seen whether the discrepancy between the genetic vs. pharmacological manipulation of CNS GLP-1R signaling and the impact on glucose regulation is due to a species difference or a development compensation in the mice. The CNS distribution of the GLP-1R is disperse and different populations of receptors have sometimes very different functions (see (103) for review). As neuroscience technology becomes more sophisticated, the capability to tease this system apart becomes more promising.

GLP-1R regulation of β-cell mass

In addition to stimulating insulin secretion, GLP-1R activation benefits β-cell survival and importantly does so in the presence of multiple apoptotic conditions including hyperglycemia, hyperlipidemia, inflammatory cytokines, and oxidative stress (104). The exact signaling mechanisms that drive β-cell growth and differentiation are still being resolved. However, phosphatidylinositol 3-kinase (PI3K) rather than PKA activation seems to be the principle signaling mechanism by which the GLP-1R controls β-cell growth and apoptosis. GLP-1 also induces a rapid cAMP-dependent activation of extracellular signaling kinase, ERK1/2, and a delayed β-arrestin (an adaptor protein necessary for GLP-1R signaling (105))-dependent increase of ERK1/2 signaling (106). Providing a direct link, β-arrestin has been found to be necessary for the anti-apoptotic effects of GLP-1 (106) and GLP-1 administration to a β-cell line reduces H_2O_2-induced apoptosis through both cAMP and PI3K (but not ERK1/2) signaling with independent but additive effects (107). Liraglutide also protects against apoptosis via PI3K signaling and the consequent phosphorylation of AKT (108). Recent work has demonstrated that

GLP-1-induced activation of PI3K is through activation of epidermal growth factor receptor 1 (EGFR), a tyrosine kinase receptor. EGFR has been found to be necessary for the ability of exendin-4 (Ex4), another long-acting GLP-1R agonist, to regulate β-cell mass and proliferation (109). Because EGFR directly activates PI3K, these data provide a link between GLP-1R signaling, PI3K, and β-cell proliferation. Downstream of AKT phosphorylation in the activation of β-cell proliferation is Wnt signaling, a pathway established in cancer biology to be critical for cell proliferation and survival. Activation AKT leads to accumulation of cytosolic β-catenin and subsequent translocation to the nucleus where it forms a complex with TCF7L2 (110), a transcription factor that activates expression of Wnt target genes (111). siRNA silencing of β-catenin and a dominant negative insertion of TCF7L2 in INS-1 cells (a β-cell line) blunted the ability of Ex4 to stimulate β-cell proliferation (110), indicating that Wnt signaling is necessary for GLP-1-induced β-cell proliferation.

These data illustrate the wide-ranging signaling pathways induced by GLP-1R activation to regulate β-cell mass and function. There is still much debate about the relevance of this impact of GLP-1 signaling in cell lines and in mice vs. in humans. While it was found that short-term incretin therapies do not expand β-cell mass in young male mice (112), in a model that enables assessment of human β-cell replication *in vivo*, it was found that Ex-4 induced proliferation occurred only in juvenile, but not adult islets (113). This work provides an important advance in our understanding of the decline in β-cell proliferation that occurs with aging and indicates that even pharmacological GLP-1 signaling may not be critical in driving proliferation in humans.

Impact of GLP-1 on glucagon secretion

GLP-1-induced inhibition of glucagon secretion has been demonstrated in a variety of species including humans (114,115). Activation of the GLP-1R also inhibits glucagon release from isolated islets or in perfused pancreas studies (116). While the data are clear that GLP-1 inhibits glucagon, the mechanisms are debated as some report low (117,118), if any (99,119), expression of the GLP-1R on α-cells. One possibility is that the impact of GLP-1 on glucagon release is indirect through release of glucagon-

regulating hormones from nearby β- and/or δ-cells (116,120). While somatostatin secreted from δ-cells inhibited glucagon secretion, a somatostatin antagonist only partially blunted the GLP-1-induced decrease in glucagon (121) suggesting additional mechanisms at play.

GLP-1 also acts on β-cells to secrete insulin which is known to suppress glucagon (122). However, co-secreted with insulin are amylin, zinc (Zn^{2+}) and GABA; all of which have also been shown to individually suppress the release of glucagon. For example, GABA released from β-cells enhances glucose inhibition of glucagon secretion by acting via an Akt kinase-dependent pathway (123). Co-secreted with insulin in hyperglycemic conditions, Zn^{2+} has been found to have inhibitory action on glucagon release from α-cells (124–126) and α-TC6 cells (an α-cell line) (126). The potential role of Zn^{2+} has found increasing interest due to the fact that genome-wide association studies (GWAS) have revealed that rare variants of a Zn^{2+} transporter gene are associated with improved glucose homeostasis and protection from T2DM (127). To determine whether the impact of Zn^{2+} was independent of insulin, streptozotocin-treated (to kill β-cells) rats where studied during pancreatic perfusion studies (125). In these rats, disruption of the intrapancreatic infusion of insulin bound to Zn^{2+}, but not of insulin unbound to Zn^{2+}, accelerated glucagon secretion, indicating that Zn^{2+} but not insulin inhibits glucagon secretion. The thinking is that Zn^{2+} inhibits pyruvate-induced glucagon secretion via opening of K_{ATP} channels and subsequent inhibition of α-cell electrical activity (128). It is important to note that contrasting data exist suggesting that Zn^{2+} does not regulate glucagon (129). However, given the connection to the GWAS data, at a minimum Zn^{2+} is important for glucose control and a logical link for that is through inhibiting release of glucagon.

In addition to Zn^{2+}, insulin is co-secreted with amylin, and amylin has also been found to dose-dependently suppress arginine-mediated glucagon secretion in rats (130) while pharmacological inhibition of amylin signaling enhances glucagon secretion (131). Pramlintide, an amylin receptor agonist, improves glycemic control in T2DM patients and at least part of that effect is via inhibition of postprandial glucagon secretion (132). A caveat to all of these studies demonstrating that β-cell products could play an indirect

role in GLP-1-mediated regulation of glucagon is that GLP-1 maintains the ability to inhibit glucagon secretion in type 1 diabetic patients who have little to no endogenous insulin (133,134) demonstrating that GLP-1 inhibition of glucagon secretion does not fully depend on β-cell products.

Besides the low expression levels of the GLP-1R on the α-cell, another caveat to the potential direct role of GLP-1 on glucagon regulation is that generally, GLP-1R activation generates cAMP and increases in cAMP are associated with increased, rather than decreased, glucagon release. Recently, an α-cell GLP-1R KO mouse was generated by crossing a loxP flanked humanized GLP-1R mouse with a *Gcg*-Cre mouse (135). Theoretically, this will eliminate the GLP-1R only from α-cells as neither L-cells nor *Gcg*-expressing neurons express the GLP-1R. In these mice, the glucagon response to increasing glucose loads was increased rather than decreased in the α-cell GLP-1R KO mice. While ad lib fed glucagon levels were higher in the α-cell GLP-1R KO mice, a curious finding was that these mice had impaired IP glucose tolerance and increased glucagon response to an IP glucose load, a condition that does not stimulate gut-derived GLP-1 secretion. Regardless, a couple of studies have provided a basis for an evolving story on how GLP-1 might mechanistically inhibit glucagon levels. The idea is that there are low numbers of GLP-1R on α-cells but these receptors have enough capacity to generate proportionately small amounts of cAMP (117). This small amount of cAMP mediates suppressive effects glucagon secretion through discrete inhibition of high voltage N-type calcium channels in mice (117) and via P/Q-type voltage-gated calcium channels in humans (136). In their hands, GLP-1 retained this inhibitory effect with either insulin or somatostatin antagonists onboard (117,136). However, it would be interesting to know if somatostatin and insulin signaling are synergistic in the paracrine effect; ie. if both antagonists were given, would GLP-1 still inhibit glucagon release. Regardless, this model explains how low and high intra-cellular cAMP concentrations with the α-cell could have opposing actions on glucagon secretion. Thus, while there is much to be learned about the signaling that drives GLP-1-induced inhibition of glucagon secretion, there are multiple indirect and direct mechanisms at play. The inhibitor action of GLP-1 on glucagon levels is often overlooked in favor of its role as an insulin

secretagogue, but it is clear that pharmacologically this is one mechanism by which GLP-1R agonists improve glycemia in T2DM patients (137).

Role of α-cell produced *Gcg* in insulin secretion

α-cell GLP-1 during metabolic stress

PC1/3, which processes GLP-1 (and GLP-2 and oxyntomodulin) from *Gcg*, is more predominantly expressed in the gut and CNS, but α-cell PC1/3 activity and/or expression is found in embryonic and neonatal mice, with pregnancy, and in models of prediabetes and diabetes (138–141). Along with this, α-cell GLP-1 clearly increases when the pancreas is under metabolic stress (142–144). Although the incretin model that intestinally-secreted GLP-1 is the functional source of GLP-1, these data do suggest that the α-cell pool of GLP-1 also has a functional role in the pancreas.

Streptozotocin (STZ), a β-cell toxin, is used to model diabetes in animals. In rats administered STZ, there is an acute increase in islet *Gcg* and PC1/3 expression that leads to an increased processing of α-cell *Gcg* to GLP-1 (141). While the function of α-cell GLP-1 under metabolic stress conditions is unknown, glucagon receptor (GcgR) KO animals have a developmentally driven increase in pancreatic GLP-1 production and are also, interestingly, resistant to STZ-induced diabetes (145). Further, blockade of the GLP-1R in GcgR deficient mice prevented the improved glucose tolerance seen in the mice (146). Additionally, mice with a cre-inducible α-cell KO of PC1/3 (although the extent to which intestinal and CNS PC1/3 expression was intact is unknown) had reduced levels of GLP-1 in the islet and greater impairments of glucose and insulin in response to STZ (144). Altogether these data suggest that α-cell GLP-1 production provides a protective effect on β-cell function during times of stress. Further examples of this are that in cultured α-cell lines or isolated islets, high media glucose concentrations increase PC1/3 expression and cellular GLP-1 content (147,148). α-cell hyperplasia also occurs with high fat diet and this precedes β-cell mass expansion (149), and in both human and mouse islets there is positive correlation between islet levels of GLP-1 and adiposity (150). Together these data suggest a role for α-cell GLP-1 production in the adaptation to metabolic disease. Whether this increase is as a protective factor that eventually fails or a part of the pathophysiology is unknown.

Although our focus here is on pancreatic GLP-1, GLP-1 in the circulation, presumably due to intestinally-secreted GLP-1, also increases with inflammation and the mechanism by which this occurs has been linked to one particular cytokine, interleukin 6 (IL-6). IL-6 is increased in inflammatory conditions including exercise, and obesity (143,149). Under severe inflammatory conditions such as sepsis, or when induced by exogenous lipopolysaccharide (LPS) administration, there is a systemic increase in GLP-1 that is dependent on IL-6 (151,152). In response to LPS, the increase in GLP-1 is dose-dependent, and GLP-1 levels remain elevated for 8-hours (151). Whereas in IL-6 knockout mice, there is no increase in GLP-1 after LPS administration (151). With 90 min of exercise in mice, there is an acute increase in IL-6 and GLP-1 and again this increase in GLP-1 was not seen in IL-6 knockout mice (143). To look at α-cell production specifically, IL-6 injections were given twice daily for over a week and the protein content of pancreatic GLP-1 and insulin were both increased (143). Despite the evidence showing that IL-6 increases GLP-1 secretion from the α-cell, the function, and whether it relates to glucose regulation or not, during this kind of inflammatory state is unknown. After several hours, LPS treatment causes elevated IL-6 levels and hypoglycemia to develop and Ex4 administration blunts this hypoglycemic effect in rats suggesting that the effect of GLP-1 is related to glucose control (153) although the direction goes opposite of what we normally think of as GLP-1-mediated glucose control. Another leading hypothesis is that IL-6 induced GLP-1 is a part of a negative-feedback loop to inhibit or restrain inflammatory responses. Rat islets treated with liraglutide showed both decreased pro-inflammatory cytokine levels (IL-6 and TNF-α) and the islets had improved function (154). Both the glucose and anti-inflammatory effect of GLP-1 point to α-cell GLP-1 acting locally rather than centrally. However, whether these pharmacological agonists have the same function as endogenously secreted GLP-1 from the α-cell remains an important unresolved question.

Thus, both metabolic (hyperglycemia, STZ-induced, diabetes) and physiological (exercise, inflammation) stress conditions influence IL-6 circulating levels which may be the factor that triggers GLP-1 secretion from the α-cell (**Figure 1.3**). However, the role of increased GLP-1 secreted from the α-cell under inflammatory conditions, how it impacts

overall glucose homeostasis, and how this may be targeted pharmacologically is unknown.

α-cell GLP-1 in normal glucose regulation

The accepted dogma of GLP-1 secreted from the intestine and acting on the pancreas in an endocrine manner is difficult to reconcile given the observations that GLP-1 is rapidly degraded by dipeptidylpeptidase 4 (DPP4) and very little, in fact only ~10% of intestinally-secreted GLP-1, reaches the circulation (155–157). While the role of α-cell secreted GLP-1 became established during states of metabolic stress, the question remains whether or not it has a role in normal glucose control. Although controversial, pancreatic GLP-1 has been found in normal islets and its expression increases with increasing glucose concentrations (144,147,148). In isolated human islets the amount of GLP-1 was low under basal conditions and was only present in the cell lysates, not the culture medium in one study (148). However, others have found pancreatic GLP-1 to be higher in human vs. mouse islets (144). In addition, PC1/3 activity can also be up-regulated by activating a bile acid receptor (TGR5) known to regulate GLP-1 secretion (147). These data suggest that the conditions by which α-cell GLP-1 is assessed may be important in the ability to detect GLP-1 levels.

Recently, the role of pancreatic *Gcg,* the gene that encodes GLP-1, was explored using a Cre lox-P mouse model that selectively reactivated the endogenous *Gcg* gene in the pancreas vs. the intestine while the remaining tissues remained devoid of *Gcg* (142). To understand the role of GLP-1R activation specifically, glucose responses to Ex9, a GLP-1 receptor antagonist, was examined. Ex9 had no impact on *Gcg* deficient animals indicating that Ex9 was a true GLP-1R antagonist *in vivo.* This indicates that the presence of GLP-1 is necessary for the ability of Ex9 to impair glucose. Interestingly, animals that only expressed pancreatic, but not animals that expressed only intestinal *Gcg* had impaired glucose tolerance (whether oral or IP) in response to Ex9. Thus, the source of the GLP-1 ligand necessary for the ability of Ex9 to impair glucose tolerance was pancreatic and not intestinal GLP-1.

However, there is an important caveat to this work. It has been known for quite some time that glucagon increases insulin secretion (122) and this was presumed to be through glucagon receptors. However, three independent islet perfusion studies demonstrated that glucagon increases insulin not by acting on glucagon receptors but by acting on GLP-1R (158–160). At a first pass, these data suggest the possibility that it was pancreatic glucagon rather than pancreatic GLP-1 that was responsible for the ability of Ex9 to impair glucose tolerance in the previous study (142). However, the ability of glucagon to bind to the GLP-1R is extraordinarily less potent than GLP-1 (159). In addition, it would mean that the entirety of Ex9's action is by impairing glucagon action on β-cell GLP-1R since Ex9 had no impact on glucose tolerance in animals that had fully restored intestinal *Gcg* expression and postprandial circulating GLP-1 levels (142). Lastly, experimental conditions that lead to the increase in glucagon and glucagon action on the GLP-1R was specific to having both elevations in glucose and amino acids. The *in vivo* experiments described above (142) were only done with oral glucose.

Other mouse models have been derived in attempt to separate the role of α-cell glucagon and GLP-1. One used a diphtheria toxin-inducible α-cell KO of *Gcg* and this mouse had a small impairment of age-induced IP glucose tolerance (144). Administration of DPP4 inhibitor, but not glucagon, restored glucose tolerance in these mice. The authors suggest this provides evidence that intestinally-derived GLP-1 can compensate for the lack of α-cell GLP-1. However, DPP4 inhibitors increase bioactive GLP-1 *AND* GIP and previous work demonstrates that these drugs can fully improve glucose tolerance even if only one of the incretin's have intact signaling (161). Isolated islets from another mouse model with α-cell KO of PC1/3 had reduced levels of GLP-1 in the islet and reduced glucose-stimulated insulin secretion (144). These mice also had impaired intraperitoneal, but not oral glucose tolerance (144). Similarly, β-cell GLP-1R KO mice have normal oral but not intraperitoneal, glucose tolerance (87,162). Thus, a model where paracrine, rather than endocrine action of preproglucagon peptides in regulating insulin emerges. Because of the nature of preproglucagon processing, it will be difficult to distinguish between the impact of α-cell derived glucagon vs. GLP-1 on local GLP-1R. However, this work clearly demonstrates that the conventionally accepted

role of GLP-1 biology is inadequate. The combined impact factors driving an increase in α-cell GLP-1 and glucagon are summarized in **Figure 1.4**.

If we ignore the intestinal vs. pancreatic source of GLP-1 topic and just focus on the fact that GLP-1 regulates insulin secretion by acting directly on β-cell GLP-1 receptors and either directly or indirectly suppresses glucagon then under what circumstance is glucagon important for β-cell GLP-1R signaling? Of the components of a mixed meal (lipids, carbohydrates, and proteins), free fatty acids, if anything decrease glucagon levels in man (163), carbohydrates potently suppress glucagon, and proteins (amino acids) potently stimulate glucagon secretion (164). Interestingly, free fatty acids suppress the ability of arginine to stimulate glucagon secretion in man (163) suggesting that even in a mixed meal situation, increases in glucagon levels are restrained. In addition to GLP-1 suppressing glucagon, insulin and somatostatin, which also increase during a meal, also suppress glucagon levels. Many questions arise from these observations. Is the system set-up to suppress redundant signals? Is the increase in glucagon during a high protein meal necessary to increase insulin? Is GLP-1 vs. glucagon necessary for different phases of insulin secretion? Are both GLP-1 and glucagon synergistic or additive in insulin control? All of these are possibilities. However, in animals devoid of both GLP-1 and glucagon, insulin response to an intravenous and oral glucose load appear to be normal suggesting that GLP-1 and glucagon, together, are not necessary for insulin secretion and/or that redundant *in vivo* mechanism are able to compensate (142). As has been suggested before, it could be that glucagon offers an additional redundant signal that allows for fine-tuning of glucose control in the face of metabolic stress whether it is exercise, hypoglycemia, or postprandial glucose control.

Targeting α-cell production of GLP-1, specifically, in T2DM therapeutics is an idea that is being explored. One study used adenovirus-mediated expression of PC1/3 in α-cells to increase islet production of GLP-1 and was able to improve glucose-stimulated insulin secretion in a mouse model of type 1 diabetes mellitus (T1DM) (165). In addition, pharmaceutical activation of GPR142, a GPCR that is expressed in pancreatic islets and that has previously been shown to enhance glucose-dependent insulin secretion

(166,167), also increases GLP-1 secretion from the α-cell (168). Moreover, using isolated mouse islets treated with Ex9, the researchers showed that insulin secretion induced by GPR142 activation is dependent on GLP-1 (168). Thus, regardless of our understanding of the physiology or pathophysiology of islet produced GLP-1, these data suggest that this pool of GLP-1 could be targeted to treat T2DM and avoid some of the CNS side-affects (ie. nausea) associated with long-acting GLP-1 agonists.

Bariatric surgery and GLP-1

Why does GLP-1 increase with surgery?

Bariatric surgery is currently the most effective strategy at treatment of obesity and its co-morbidities. There are many types of bariatric surgeries. Roux-en-Y gastric bypass (RYGB; a small gastric pouch is formed and the jejunum is connected directly to the small pouch) used to be the most widely performed bariatric surgeries but its utilization has been reduced to about 20% of the procedures in the last couple of years (169). Currently the most common surgery in the US is vertical sleeve gastrectomy (VSG; 80% of the stomach along the greater curvature is removed) which comprises about 60% of performed bariatric procedures. The switch is likely due to the fact that VSG is surgically more simplistic, leads to fewer long-term malabsorptive issues, and although dogma persists that it is less effective, randomized clinical trials demonstrate similar efficacy between VSG and RYGB (see (170) for meta-analysis).

Among the many similar physiological effects between these two surgeries is about a 10-fold increase in postprandial levels of GLP-1; something observed in both patients and rodent models of surgery (171–174). A long-standing hypothesis for why GLP-1 (and other gut peptides for that matter) are increased after RYGB is that the shorter length of small bowel leads to more rapid nutrient delivery further down into the GI tract where the majority of L-cells are located (175). VSG also increases nutrient delivery to the distal gut thanks to a restricted stomach size that increases gastric pressure (176) and consequently gastric emptying rate (176,177). Indeed, speed of nutrient delivery may be important after RYGB as the increase in nutrient-induced GLP-1 was eliminated if nutrients were delivered to the bypassed limb (178). However, in rats after VSG, glucose infused slowly and directly into the duodenum caused similar increases in GLP-

1 as when the same glucose load was delivered orally (176). These data suggest that intestinal L-cells are either increased in number or in nutrient-sensitivity after VSG. While one study demonstrated that VSG in rats increased L-cell number (179) another did not (180). Differences in diet as well as the control groups utilized (pair-fed vs. ad lib fed sham groups) could lead to differential structural and functional changes in the gut nutrient-sensing pathways (181). On the other hand, RYGB is more consistently associated with intestinal hypertrophy regardless of dietary exposure (179,182). The intestine is considered a major site of glucose disposal after RYGB and this may provide energy for intestinal metabolic pathways to support tissue growth (183). In humans, this increase in glucose absorption after RYGB was associated with the exaggerated release of insulin and GLP-1 (184). Thus, the anatomical differences between the surgeries may lead to different adaptations in either the morphological or mechanical function of the GI tract and either of these adaptations can regulate prandial GLP-1 responses.

Another common physiological response to both RYGB and VSG is the significant increase in total and various subspecies of plasma bile acids (185–187). Bile acids have demonstrated effects on stimulating GLP-1 secretion from L-cells by acting through a specific G protein-coupled receptor (TGR5) vs. their other common receptor a nuclear transcription factor, farnesoid X receptor (FXR) (188). However, surgery-induced increases in bile acids have been demonstrated to be important for the increase in postprandial GLP-1 in one (189), but not another study (190). Further, these two studies had divergent results on the necessity of TGR5 for surgery-induced weight loss and improvements in glucose homeostasis (189,190). Conversely, FXR seems to be necessary for the full effects of VSG, independent of GLP-1 (191). Thus, although this will require future validation, it seems that the surgery-induced increase in GLP-1 is due to intestinal responses to nutrient delivery. With RYGB, this is more acute, but with VSG, chronic adaptations are more critical.

GLP-1 as mechanism for the metabolic success of surgery

Although T2DM is thought to be chronic and progressive, bariatric surgery leads to large improvements in insulin secretion and sensitivity which results in a remission of T2DM

for many patients. The consistency of the finding that GLP-1 increases with surgery in addition to this increase being associated with greater prandial insulin release (174) and greater weight loss (192) after surgery has led to the suggestion that GLP-1 is an integral mechanism for the success of surgery. While the postprandial GLP-1 response may be required for the insulin and glucose responses to a meal after bariatric surgery, whether GLP-1 is responsible for resolution of T2DM is less clear. One study found that postprandial GLP-1 responses were a significant predictor of T2DM remission after RYGB (193), yet another found similar postprandial GLP-1 responses 2-years after VSG whether the patients had postoperative remission, relapse, or lack or remission of T2DM (172).

In support of a role for GLP-1, multiple studies have demonstrated that administration of the GLP-1 receptor antagonist, Ex9, reduces the insulin response to a glucose load in both humans and rodents (194–197). However, these data come with an interpretative problem (198). There is no dispute that GLP-1 regulates postprandial insulin. The same dose of Ex9 impairs glucose and reduces insulin in control subjects (172) and rats (197). The degree of this impairment is similar between surgery and control conditions. If GLP-1 was more important in glucose control after surgery, the degree of impairment should be greater. Thus, the question is whether what we are seeing after surgery is reflective of the normal response or is reflective of greater importance of GLP-1 during surgery.

The incretin effect is credited to both GLP-1 and GIP (199,200) with each thought to contribute equally to insulin secretion in non-obese, non-T2DM subjects (199). Both T2DM and to a lesser extent, obesity, reduce (201,202), while bariatric surgery enhances, the incretin effect (203). However, there is little agreement as to whether bariatric surgery leads to an increase in GIP suggesting that the extent to which GIP contributes to the enhanced incretin effect is debatable. In an effort to determine the importance of GIP after RYGB, RYGB patients were given a DPP4 inhibitor to increase bioavailability of both GIP and GLP-1 and then combined this with Ex9 to block GLP-1R signaling; an experimental condition that would isolate the impact of GIP signaling on glucose tolerance (204). The DPP4 inhibitor failed to improve glucose tolerance or β-cell

function while GLP-1R signaling was blocked in RYGB patients. In contrast, T2DM patients that had not undergone bariatric procedures fully responded to the DPP4 inhibitor with improved glucose tolerance and insulin secretion even when combined with Ex9 (205). Together these data suggest that RYGB shifts the balance of the incretin effect toward GLP-1 and away from GIP.

Qualitatively, rodents and humans respond similarly to bariatric surgery. Both have substantial weight loss, elevated gastric emptying rate, and increased postprandial insulin and GLP-1 levels. While human work is limited to acute pharmacological intervention, preclinical work offers the additional ability to genetically manipulate the GLP-1 system and test its role in the metabolic success of surgery. One would hope that this would lead to less interpretive issues. Whole-body GLP-1R KO mice have similar weight loss and improvements in glucose tolerance compared to littermate controls after both VSG (206,207) and RYGB (208) suggesting a limited role of GLP-1R in surgical success. Central nervous system GLP-1R have been shown to be important for regulating body weight and glucose homeostasis in rodents (89,90,95) and may be a target population for the impact of surgically-induced GLP-1 to act in regulating body mass and glucose. However, Ex9 infused directly into the CNS of rats during RYGB or sham surgeries (208) had no significant impact on the surgery-induced reductions in body mass. Thus far, the data would seem to be in agreement that GLP-1R signaling is not necessary for the metabolic success of surgery. However, in the last few years, conflicting reports have been published. To examine the specific role of β-cell GLP-1R, two slightly different versions of an inducible Cre-loxP strategy was used to knock out these receptors and VSG was performed. One study found that β-cell GLP-1R were necessary for surgery-induced improvements in glucose tolerance and glucose-stimulated insulin secretion, but not weight loss (209); while the other found not only that these receptors were not necessary for VSG-induced improvements in glucose tolerance but that glucose responses were essentially normalized to the WT levels (210). Differences in diet, mouse models, and/or some other factor may contribute either independently or in combination to the differences in these experimental findings. Regardless, we are still left with no solid conclusion as to whether GLP-1 is necessary for the success of surgery.

The increase in GLP-1 after surgery may also have a trophic effect on β-cell mass. GLP-1 increases β-cell mass in rodents, and has also been suggested to increase β-cell function in humans following bariatric surgery (211). In isolated islets, VSG mice had changes in their genetic and functional signature favoring calcium signaling and insulin secretion (212). In a pooled group of VSG and RYGB patients, pancreatic fat deposition as assessed by PET imaging was found to be reduced alongside improvements in β-cell function (213). These data suggest the possibility that the impact of surgery on the pancreas could be due to the weight loss itself and is independent of GLP-1's trophic effect.

With both human and rodent work, the clear finding is that the increase in postprandial GLP-1 drives acute glucose responses to a meal after bariatric surgery but whether or not they are required for long-term improvements in glucose control, T2DM remission, and/or for weight loss are debatable. However, this may be difficult to determine as the degree of β-cell destruction prior to surgery may be more critical in determining whether those β-cells can recover sufficiently to resolve T2DM (214).

GLP-1 in post-bariatric hypoglycemia

One increasingly recognized surgery complication is post-bariatric hypoglycemia (PBH) (215). This is reflected in a subset of bariatric patients and is associated with symptoms of postprandial "dumping syndrome" characterized not only by hypoglycemia, but also hyperinsulinemia, sweating, nausea or vomiting, and heart palpitations. Given that adrenergic and cholinergic symptoms in the postprandial state can be nonspecific, PBH has recently been re-defined as the presence of neuroglycopenic symptoms (difficulty thinking, weakness, fatigue) with concomitant hypoglycemia (<54 mg/dL) (215) that is relieved within minutes of carbohydrate ingestion. This condition threatens the safety of affected patients as hypoglycemia impairs cognition and increases the risk for syncope, cardiac arrhythmias, seizures, coma, and even death. Moreover, many patients may be rendered unable to perform job-related tasks or to safely operate a motor vehicle.

Initial reports indicated a prevalence of <1% for hypoglycemia requiring hospitalization, but 10% for clinically recognized hypoglycemia (216,217). However, the use of

continuous glucose monitoring (CGM) has highlighted that hypoglycemia occurs much more frequently (closer to 30%), and is observed with similar frequency in both RYGB and VSG (218–220). Using CGM, one study found that 75% of post-RYGB patients had glucose levels of <55 mg/dl compared to no hypoglycemia in nonsurgical controls (221). Given the increasing numbers of bariatric procedures, and that hypoglycemia typically emerges only after 2-3 years postoperatively, the prevalence of PBH is likely to further increase over the next decade.

Hypoglycemia is a complication for T1DM and T2DM, but for those patients medications can be adjusted to minimize occurrence. The mechanism for PBH is unknown and creates a difficult therapeutic challenge. The dominance of postprandial timing indicates that hypoglycemia is partly due to exaggerated systemic appearance of ingested glucose secondary to altered anatomy and subsequent disproportionate insulin response to a meal. In healthy individuals, regulation of postprandial glucose excursions is tightly controlled by regulating the rate of nutrient entry and hormonal responses that coordinate nutrient assimilation. Both RYGB and VSG alter many steps in this assimilation process including more rapid pouch or gastric emptying, increased glucose absorption, increased postprandial GLP-1, and finally markedly increased insulin secretion and sensitivity (**Figure 1.5**). Thus, most initial treatment strategies focus on reducing simple carbohydrate intake or using medication to slow carbohydrate absorption in order to minimize glucose and consequently insulin “spikes” (222). Strategies to reduce insulin itself have also been used including somatostatin analogues (e.g. octreotide), diazoxide (reduces insulin secretion), partial pancreatectomy, and reversal of the surgical procedure toward normal anatomy if possible. Unfortunately, all of these therapies are limited by side effects or incomplete efficacy, even pancreatectomy and surgical revision.

One hypothesis is that this phenomenon is caused by the exaggerated postprandial GLP-1 and consequently insulin levels. Postprandial glucose, GLP-1, and insulin have been found to be even higher in patients susceptible to PBH (223). Multiple studies have now demonstrated that administration of Ex9 can lower postprandial insulin and prevent hypoglycemia in RYGB patients (224–227). However, patients with PBH still

have higher peak glucose levels after GLP-1R antagonist treatment compared to asymptomatic patients. In addition, the rapid time course of the post-surgical increase in postprandial GLP-1 and insulin secretion (days) does not mirror the delayed development of PBH (years). Importantly, although GLP-1 is likely not a mechanism for PBH, Ex9 therapies may still be a way to treat PBH.

In conclusion, GLP-1 increases with bariatric surgery are likely due to acute and/or chronic responses to rapid nutrient entry seen with RYGB and VSG, respectively. This increase in GLP-1 likely plays an important role in the acute glucose and insulin responses to a given meal. However, whether these increases are responsible for the overall improvements in glucose homeostasis, body mass, or in the onset of PBH is something that is still debated.

Targeting GLP-1 in Pharmacology

GLP-1 agonists and DPP4 inhibitors

The development of GLP-1-based drugs has been one of the major advances in diabetes medicine in recent years. The currently approved pharmaceutical strategies targeted to the GLP-1 system are aimed at either increasing endogenous GLP-1 levels with inhibitors for the protease that inactivates GLP-1 or long-acting GLP-1R agonists resistant to DPP4 cleavage (228,229). DPP4 inhibitors are effective at stimulating insulin and reducing glucagon actions; attributes that are credited to GLP-1R signaling (230). However, DPP4 acts on GIP as well 40 additional substrates (231). GIP is the only additional substrate of note for glucose regulation and work in both humans (205,232) and mice (161,233) suggest that both GIP and GLP-1 signaling are targets for the improved glucose control with these drugs.

There are now multiple GLP-1R agonists currently available for the management T2DM (229,234,235). Various strategies are used to extend the half-lives of these agonists compared to native GLP-1 including using a synthetic analogue of Ex4 (Byetta) and the addition of a fatty acid side chain to native GLP-1 to facilitate albumin binding (Liraglutide/Victoza). In an effort to improve convenience and compliance (236), modifications of these drugs are also being made to extend the half-life to allow for

once-weekly injections (Bydureon and semaglutide/Ozembic). Besides being more convenient for the patient, there seem to be added benefits of creating a more stable pharmacodynamics profile (ie. reducing the peaks and troughs of drug action) as these drugs induce less nausea, a common side-effect of rapid acting compounds (237–240). An added benefit of these drugs compared to DPP4 inhibitors is the added weight loss (241) and for some specific GLP-1R agonists (liraglutide and semaglutide) there is a reduction in cardiovascular events (242). Because of the impact of these drugs on weight loss, there are now multiple formulations approved to specifically treat obesity independent of T2DM. Interestingly, although T2DM patients do see improvements in body mass, there is a larger benefit for the obese non-diabetic patient for weight loss.

The neural pathways leading to the weight loss effects of these drugs are being actively pursued in preclinical work. Part of the reason for this effort is to determine whether the neural circuitry that drives the weight loss effect is distinct from the circuitry that drives the nausea effect of these drugs. In animal work, we know that some neuronal regions regulating the impact of GLP-1 on food intake are distinct from those regions that regulate nausea (243,244). As discussed above, previous preclinical work in mice and rats established that the CNS (97,245), but not the peripheral nervous system (97) is critical in mediating the impact of long-acting GLP-1R agonists on body mass. With newer advances in neuroscience techniques, we now know that glutamatergic rather than GABAergic neurons (98) are the specific type of neurons necessary for the ability of liraglutide to induce weight loss. However, given that neither hypothalamic (Nkx2.1 neurons), PVN (Sim1 neurons), nor POMC neurons were not necessary for the ability of long-acting agonists to reduced body mass (95), the hunt is still on for the specific population of neurons responsible for the pharmaceutical reduction in body mass. Better understanding of the neural mechanisms of these processes would benefit the therapeutic utility of these agents.

Poly-agonists

An exciting recent pharmaceutical strategy to the treatment of obesity and T2DM has been the development of hybrid peptides that activate more than one receptor to generate an effect (246). Given that obesity and T2DM are diseases with integrated

pathology, a multi-faceted approach is likely necessary for more effective treatment. These agents are touted as mimicking the broad range of peptide increases seen after bariatric surgery. Some of the first compounds developed using this strategy were glucagon/GLP-1 co-agonists, peptides engineered to activate the cognate receptors of both peptides in different relative potencies (247,248). The rationale behind this line of drug development is that both glucagon and GLP-1 bind specific and distinct receptor populations in the brain to cause satiety (249,250), and activating both receptors could lead to synergistic effects. Peptides with equal agonism for each of the target drugs (eg. GLP-1/glucagon) seem to have the most therapeutic promise (247,248). In the case of glucagon, greater glucagon potency would lead to greater energy expenditure and suppression of food intake, but there seems to be a threshold beyond which glucose control worsens despite weight loss. The results for combined agonism in mice and rats are promising with improved glucose tolerance but also greater reductions body weight and fat with the dual agonists compared to GLP-1R agonism alone (247,251). Although there may be subtleties in formulation that lead to species differences, the results in humans have not been as exciting as in mice. Specifically, a recent phase 2a clinical trial found reductions in body weight and improvements in glucose control but the degree of the improvements seemed to be within the range of what is seen with long-acting GLP-1R agonists alone (252,253). Interestingly, a GIP receptor/GLP-1R dual agonist in phase 2 trials caused greater improvements in both HbA1c% and weight loss compared to the GLP-1 agonist alone (254). Given that GIP is thought to be important for lipogenesis, the mechanism for this effect of the dual agonist is unclear. Regardless, this drug shows great promise in improving glucose control and weight loss in T2DM patients.

Conclusions

GLP-1 was suggested to be an incretin over 32 years ago. Indeed, GLP-1 actions in the islet are implicated in the success of surgery and have been exploited for effective glucose control in T2DM patients. However, the incretin model is much too simple for the complexity of the system. GLP-1 has a wide array of physiological effects that go beyond the β-cell. Further, GLP-1 and glucagon released from the α-cell may be

important for β-cell proliferation and function suggesting that paracrine regulation of the β-cell needs to be incorporated into our thinking surrounding GLP-1 function. These interesting interactions and/or overlapping functions of GLP-1 and glucagon and GPCR signaling requires further exploration. What is true, is that GLP-1R agonists are safe and effective therapies for obesity and T2DM and will remain an active area of exploration.

Authorship

Ellen M. Davis, Darleen A. Sandoval

Figure 1.1 The incretin effect: Glucose levels are lower while insulin levels are higher when the same dose of glucose is administered directly into the gut versus when administered intravenously (IV). This difference in insulin between the gut and venous infusion is the "incretin effect" which occurs in response to GLP-1 and GIP secreted from the distal gut. Adapted, with permission, from McIntyre N, et al.

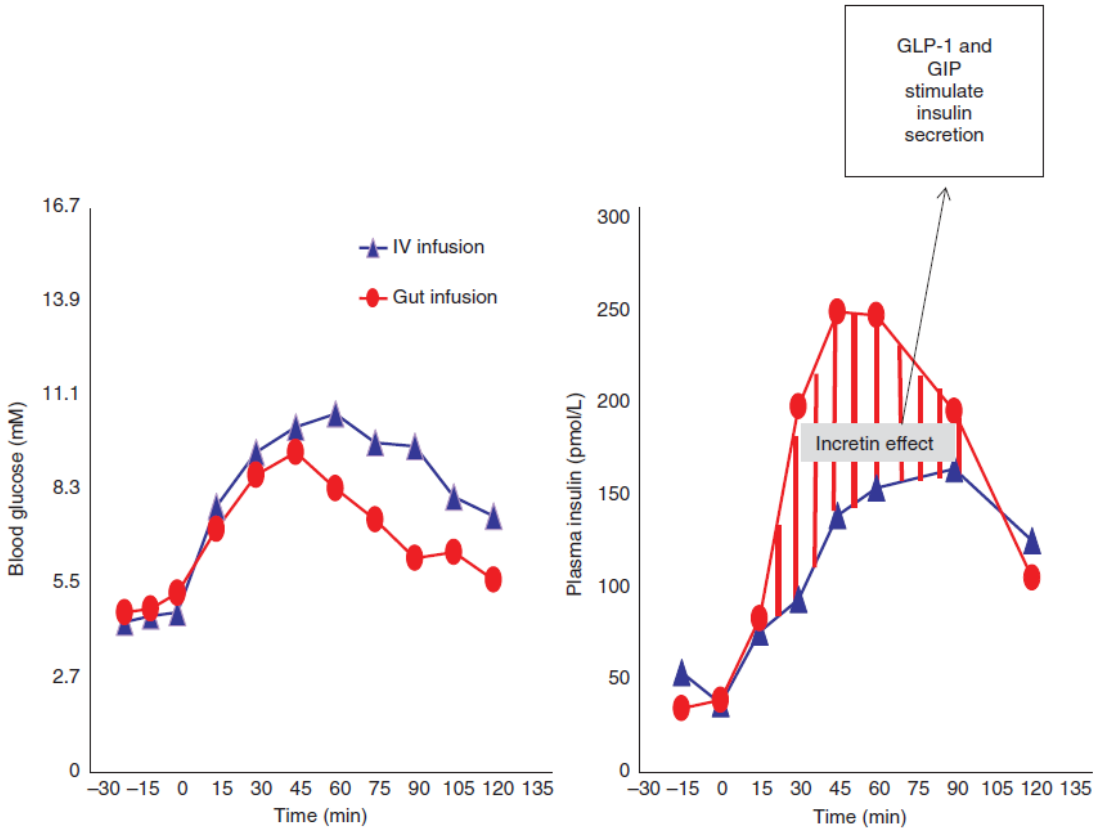


Figure 1.2 Intestinal GLP-1 secretion: Several factors have been linked to GLP-1 secretion. The parasympathetic nervous system (PNS) stimulates GLP-1 secretion via cholinergic muscarinic receptors (MR). Activation of α-adrenergic receptors (AR) stimulates while activation of β-adrenergic receptors inhibits GLP-1 release. Various GPCRs including ones activated by bile acids and various fatty acids stimulate GLP-1 through PKA signaling and increases in calcium-induced exocytosis. Lastly, direct glucose sensing, predominantly via SGLT1 in humans, activates sodium (NA^+), and calcium (Ca^{2+}) voltage-gated channels to lead to the release of GLP-1.

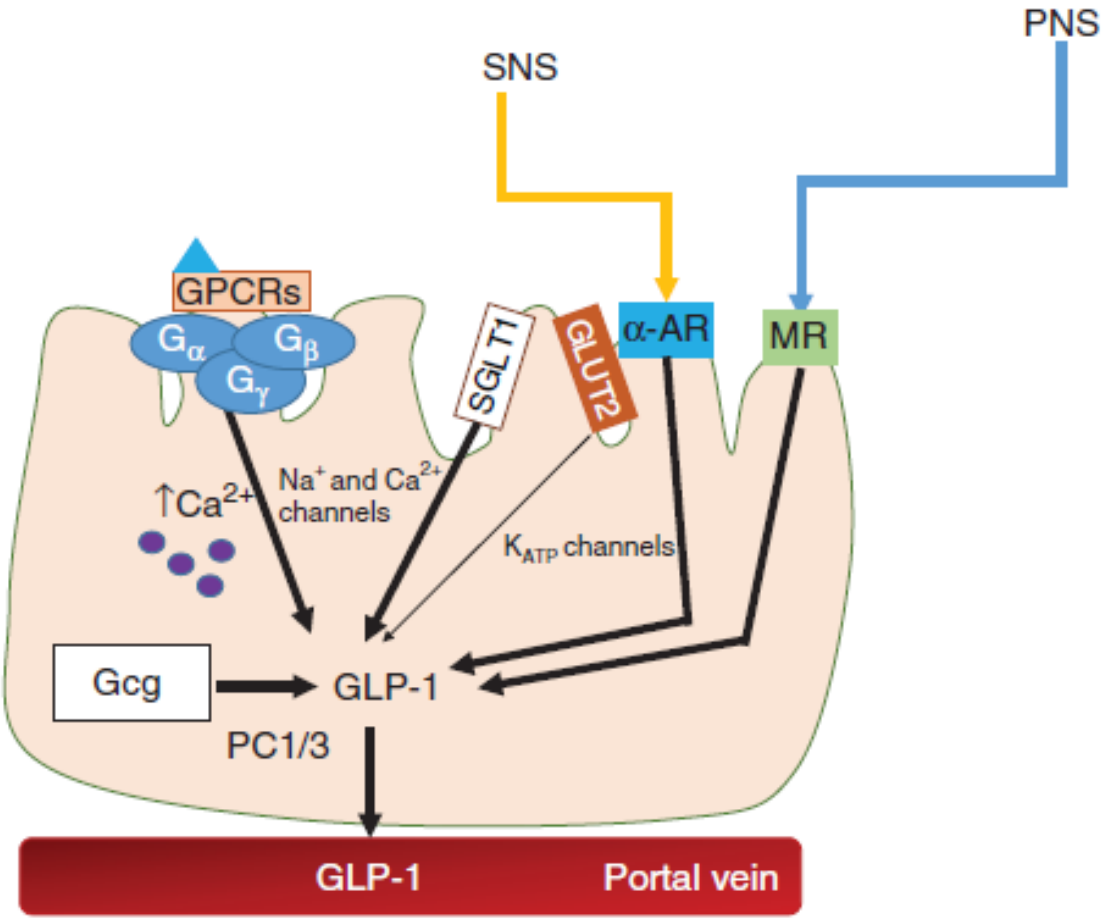


Figure 1.3 Factors that impact α-cell GLP-1 production. Metabolic stress, systemic inflammation, exercise, hyperglycemia, obesity, and diabetes stimulate α-cell GLP-1 production. IL-6 seems to be a primary factory that leads to this increase. The function of this increase is unknown, but regulation of β-cell mass and function is a likely endpoint.

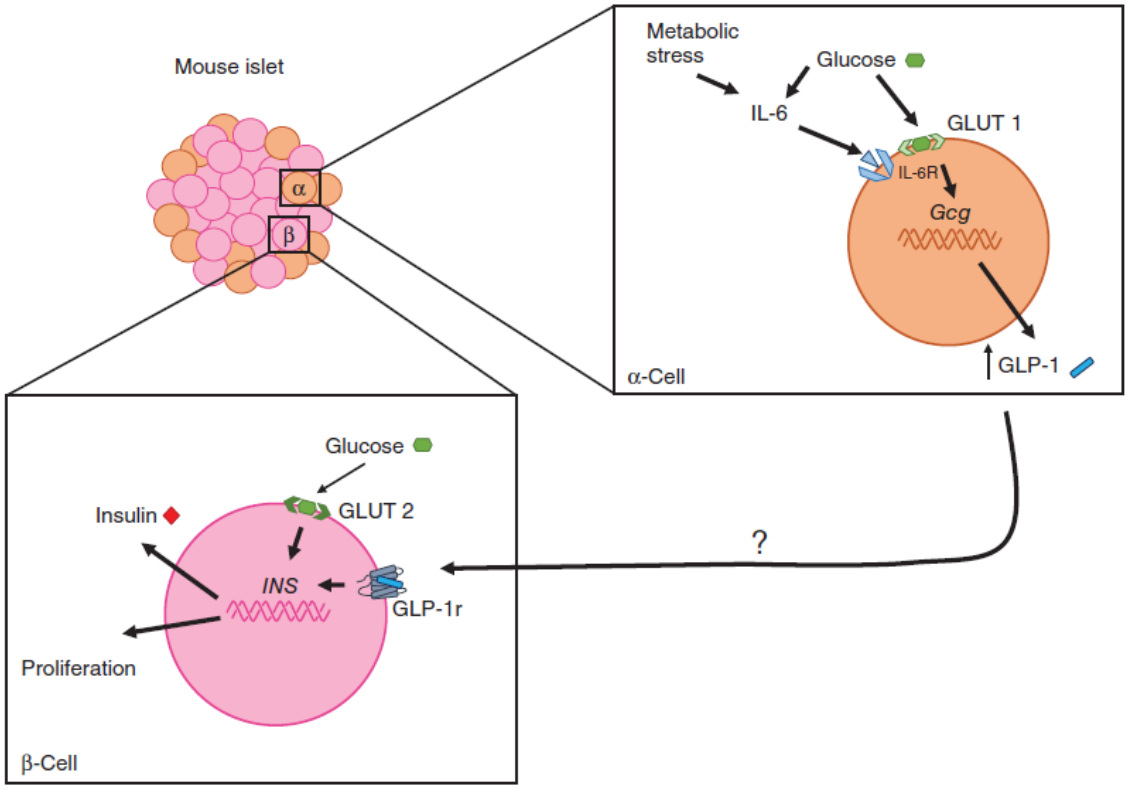


Figure 1.4 Recent work highlights a more complexity to the role of Gcg-derived peptides in the incretin effect. While historical work suggest intestinal GLP-1 is important in regulating glucose homeostasis, there may be a role for α-cell derived GLP-1 as well. In addition, in response to amino acids, glucagon is secreted and acts on local GLP-1R to regulate insulin secretion.

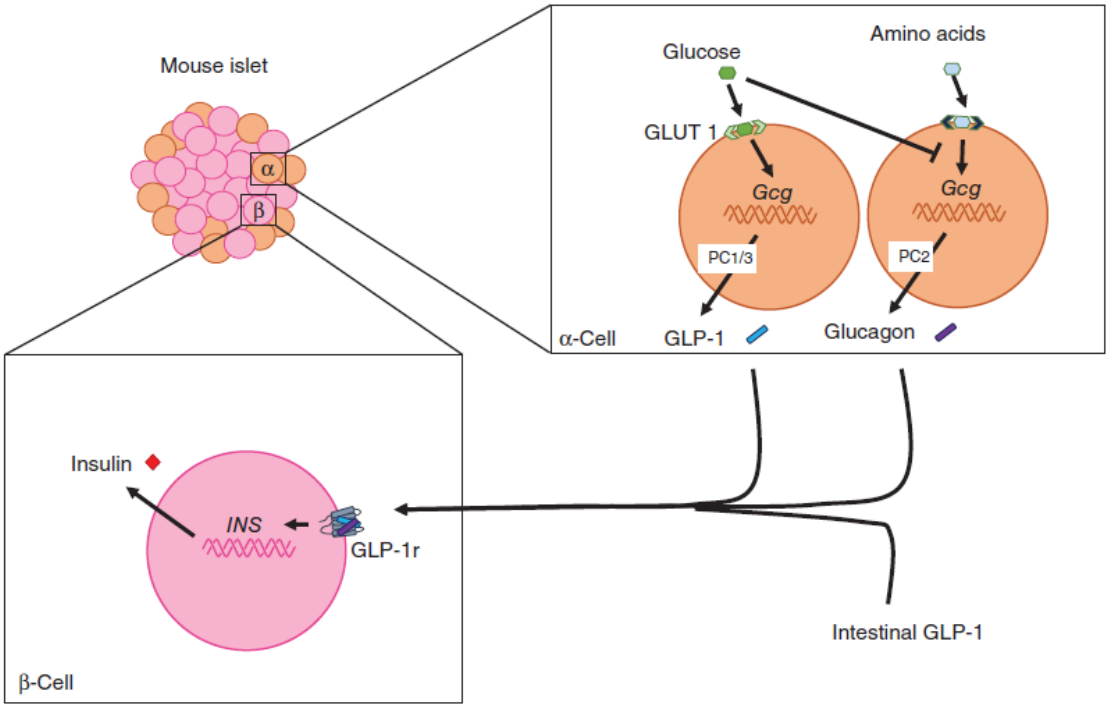
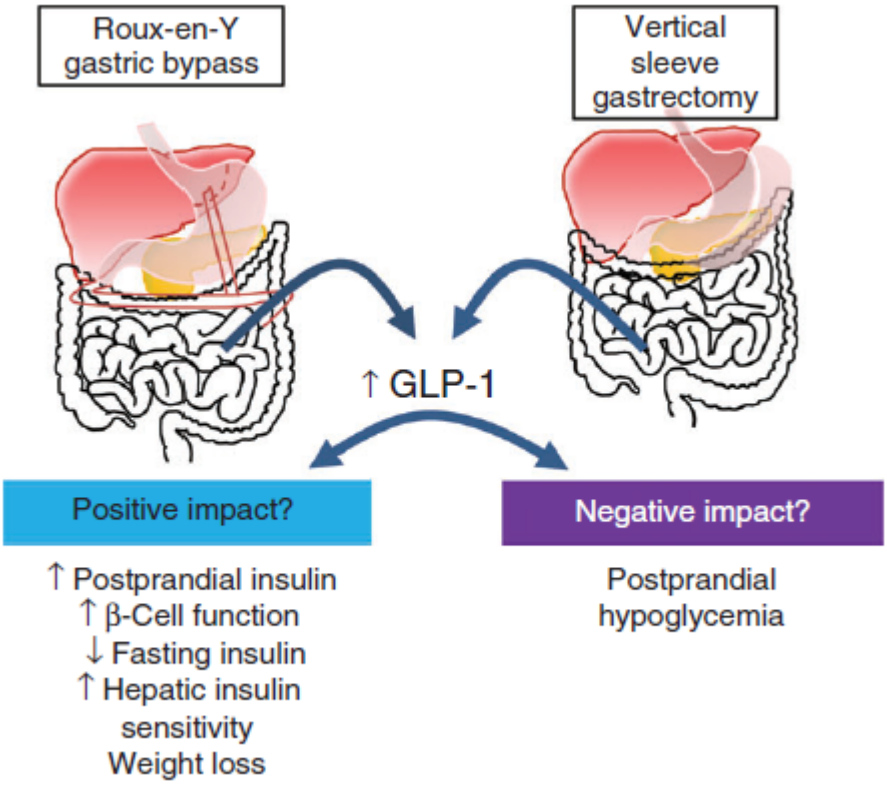


Figure 1.5 Postprandial GLP-1 increases several-fold after bariatric surgery and has been implicated as a mechanism in both positive and negative impacts of bariatric surgery. On the positive side, GLP-1 has been implicated in increasing postprandial insulin levels to restrain postprandial glucose homeostasis improvements in insulin sensitivity lead to reductions in fasting insulin, improved hepatic insulin sensitivity, and overall improved β-cell function. However, on the negative side, the increase in postprandial insulin is thought to contribute to post-bariatric hypoglycemia which occurs in as much as 30% of surgery patients.



CHAPTER 2
Premise and Hypotheses for the Role of GLP-1 in Regulation of Inflammation

Introduction

One predominant theory explaining the link of obesity to type 2 diabetes mellitus (T2DM) involves the obesity-induced chronic low-grade inflammation, or meta-inflammation. This is because inflammation itself has been linked to impaired glucose homeostasis including insulin resistance (255–257). In contrast to the inflammatory response to an infection, where the purpose of immune cells is to identify, locate, and dispose of foreign microorganisms and viruses, the low-grade inflammation associated with obesity is not directed to a specific pathogenic target. Yet, similar to infection-based inflammation, meta-inflammation still results in increased accumulation of immune cells in metabolic tissues and increased systemic and organ-specific levels of cytokines (255,258,259). This includes accumulation of macrophages, phagocytotic cells that are either resident within an organ or that migrate from other tissues and play a large role in organizing immune responses to promote either a pro- or anti-inflammatory state (260). The reverse is also true, that metabolic dysfunction impairs immune responses. For example, T2DM patients have impaired immune responses including neutrophil function and T cell responses to an infection (261–263). T2DM also worsens the mortality prognosis for sepsis, which is hypothesized to be due to innate immune dysregulation, such as macrophages and natural killer cells, and adaptive immune suppression, such as T cells and B cells (264–266). This complex interaction between T2DM and the immune system remains an important inquiry for researchers as there are currently no therapeutics that increase survival to sepsis (267). Thus, the search for those critical signals that link inflammation and metabolism is critical for treating inflammatory and metabolic diseases.

Glucagon-like peptide 1 (GLP-1) is a peptide made within intestinal L-cells, pancreatic α-cells, and a small cluster of neurons in the hindbrain (10–12). GLP-1 is predominantly studied for its role in facilitating postprandial insulin secretion and sensitivity, and consequently regulation of glucose homeostasis (26,27,37,38,57,142). In addition to glucoregulation, activation of the GLP-1R in the CNS decreases food intake (243,268). Although its role in regulating glucose metabolism and food intake has been extensively studied, there is also an emerging connection between GLP-1 and the immune system (151,269). Data demonstrate that in response to nutrients, circulatory GLP-1 comes primarily from the intestine (37,38,42,43). During severe inflammation, the pancreas and intestine increase GLP-1 tissue content in response to IL-6 or lipopolysaccharides (LPS) (151). However, a key unanswered question is whether there is a physiological contribution of the pancreas and/or the intestine to the increase in circulating GLP-1 and, if so, what is the function of that increase. One possibility is that the physiological role for this increase in GLP-1 is similar to postprandial conditions, i.e. to regulate glucose and/or energy homeostasis during inflammation. However, pharmacological data suggest that at least in bone marrow derived macrophages, GLP-1 signaling directly regulates macrophage accumulation and polarization (270). While type 1 diabetes mellitus (T1DM) has long been studied in association with macrophage accumulation in the pancreas, only recently have researchers begun to look at macrophage expansion in the islets in T2DM patients and animal models (271–273). For example, recent studies have demonstrated that macrophage accumulation within the pancreas occurs in response to dietary-induced obesity and that this plays a role in impaired glucose-stimulated insulin secretion (259) and with inflammatory stress (272). Collectively, these data indicate a need to understand the interaction between meta-inflammation in the pancreas and the protective role GLP-1 might be playing. To investigate the physiological role of *Gcg*, the gene that encodes GLP-1, GLP-2, glucagon and other peptides, under inflammatory conditions, I utilized a well-established model to induce severe inflammation, LPS, in combination with the low-grade inflammation seen with high-fat diet (HFD). To distinguish the role of pancreatic and intestinally produced GLP-1, I used our lab's Cre lox-P *Gcg* reactivation model which limits expression to either the pancreas or the intestine (274). I hypothesized that

increased GLP-1 plays a metabolic role, functioning to regulate glucose levels and food intake, and an immunologic role, functioning to decrease inflammation.

Thus, the goal of this proposal is to determine the physiological role of GLP-1 in the regulation of immune cell accumulation and polarization, and downstream metabolic processes during infection-based inflammation and the impact of HFD.

Specific Aims

Aim 1. To test the hypothesis that high-fat diet and impaired glucose tolerance increase inflammatory responses to LPS-induced inflammation.

High-fat diet (HFD) is known to result in an increase in inflammatory cytokines and impaired glucose control. Studies in humans have shown that obesity and impaired glucose control impairs immune responses to severe inflammatory conditions. Using wild-type mice on HFD, I explored how impaired glucose tolerance and chronic low-grade inflammation affect GLP-1 levels, glucose, insulin, cytokine expression, and feeding responses to an inflammatory insult induced by exogenous administration of LPS. I hypothesized that HFD will exacerbate the physiological responses to LPS, including increased GLP-1, sickness-induced anorexia and inflammatory responses compared to chow-fed mice.

Aim 2. To test the hypothesis that pancreatic- and intestinally produced GLP-1 regulates metabolic responses to severe inflammation

Our lab used Cre-loxP technology to limit *Gcg*, the gene that encodes GLP-1, expression to either the pancreatic α-cells or intestinal L-cells. These animals were placed on normal chow or HFD and feeding responses to LPS administration were assessed to determine whether *Gcg* is necessary for the anorectic effect of LPS. Additionally, I determined whether intestinal, pancreatic, or both sources of GLP-1 are necessary for the acute glucose response, systemic and pancreatic inflammatory responses to LPS administration. I hypothesized that pancreatic GLP-1 plays a paracrine role in glucose regulation under LPS-induced inflammation and that both pancreatic and intestinal GLP-1 contribute to the anorectic effect of LPS.

Aim 3. To test the hypothesis that pancreatic GLP-1 regulates local macrophage accumulation in the pancreas during severe inflammation

Our preliminary data suggests that the pancreas contributes to circulatory GLP-1 after LPS administration. Again, I used our chow vs. HFD Cre-loxP *Gcg* model, animals received LPS or vehicle treatment and through a combination of flow cytometry and immunohistochemistry (IHC) techniques, I characterized the immune cells that accumulated within the pancreas after LPS. Then I used a *Glp1r*-GFP reporter mouse to determine whether immune cells in the pancreas expressed *Glp1r*. I hypothesized that pancreatic GLP-1 promotes a local anti-inflammatory state and works to restrain the LPS-induced immune response, thus fewer macrophages will accumulate within the islet when GLP-1 is present.

CHAPTER 3

The Role of GLP-1 in the Regulation of Metabolism and Immune Responses

Abstract

While GLP-1 is classically described as an incretin hormone, it is known to be increased by the pro-inflammatory cytokine, interleukin 6 (IL-6), in both the pancreatic α-cells and intestinal L-cells. Interestingly, the activation of the endogenous GLP-1 system is exaggerated in type 2 diabetes mellitus (T2DM) patients under an inflammatory stimulus. However, the function of GLP-1 during an inflammatory state is unknown. Using a combination of high-fat diet (HFD)-induced obesity and our innovative mouse model of tissue-specific *Gcg* (the gene that encodes GLP-1) expression, I explored the function of GLP-1 in response to inflammation by administering lipopolysaccharide (LPS), a well-established tool for inducing inflammation. LPS is also suppresses feeding and impacts glucose homeostasis; both functions of GLP-1. I hypothesized that HFD would exacerbate physiological responses to LPS including increased plasma GLP-1, decreased blood glucose levels, and increased sickness-induced anorexia, as well as systemic inflammatory responses including increased plasma cytokines. I found that HFD increased LPS-induced suppression of feeding and increased plasma levels of both pro-inflammatory cytokines and GLP-1. I hypothesized that increases in GLP-1 in response to LPS were necessary for the feeding and glucose responses to LPS. To test this hypothesis, I examined the role of tissue-specific GLP-1 in the metabolic and immunologic responses to LPS. I found that while both pancreatic and intestinal *Gcg* expression contribute to LPS-induced increases in GLP-1, *Gcg* was not necessary for the glucoregulatory or feeding responses to LPS. However, whole-body *Gcg* Null animals had increased the macrophage accumulation in the pancreas. Lastly, using a GLP-1R reporter mouse, I found that macrophages that accumulate in the pancreas after LPS express the GLP-1R. Altogether these data suggest that pancreatic production of GLP-1 directly regulates macrophage responses to inflammation. I

conclude that under severe inflammatory conditions, GLP-1 plays an immunologic, rather than metabolic role, through direct macrophage regulation.

Introduction

Within a few decades, obesity has risen in prevalence at an alarming rate and now affects more than a third of US adults (275). Obesity and its concomitant comorbidities are an urgent public health concern and burden on the healthcare system. Patients with obesity are more likely to develop hypertension, cardiovascular disease, osteoarthritis, and Type 2 Diabetes Mellitus (T2DM). T2DM is characterized by insulin resistance, dysregulated glucose control, and impaired islet function. Prevalence of T2DM is rising in parallel to obesity worldwide (276,277). In addition to developing co-morbidities, T2DM patients are at an increased risk for complications associated with inflammation. For example, T2DM patients are at an increased risk for both developing infections that lead to sepsis and mortality associated with sepsis (255,257,262). In 2020, the COVID-19 pandemic highlighted the need to understand the link between T2DM and severe inflammatory responses (278).

One predominant theory explaining the link of obesity to type 2 diabetes mellitus (T2DM) involves the obesity-induced chronic low-grade inflammation. In contrast to the inflammatory response to an infection, where the purpose of immune cells is to identify, locate, and dispose of foreign microorganisms and viruses, the low-grade inflammatory state associated with obesity is not directed to a specific pathogenic target. Yet, similar to infection-based inflammation, meta-inflammation results in increased accumulation of immune cells in metabolic tissues and increased systemic and organ-specific levels of cytokines (255,258,259). The reverse is also true, that metabolic dysfunction impairs immune responses. Macrophages are essential for detecting, engulfing, and destroying apoptotic cells and inflammatory stimuli, typically a pathogen such as a virus or bacteria. During severe inflammation, tissue damage and hyperinflammation can result in an accumulation of neutrophils and other immune cells to remove damaged cells and regulate the immune response within an organ (279,280). While macrophage function and accumulation in the pancreas has been well studied in type 1 diabetes mellitus

(T1DM), recently, researchers have seen that macrophages also accumulate in the islets of T2DM patients and obese animal models (271–273). Under steady state conditions, macrophages within the islets are myeloid derived and express IL1-β and TNF-α (281). These tissue-resident macrophages are established prenatally and self-maintained, and distinct from circulating monocytes (282). Severe inflammation (modeled by exogenous administration of lipopolysaccharide; LPS) will increase inflammatory transcripts from islet-resident macrophages (283). Recent research has shown that macrophages accumulate within the islet during diet-induced obesity leading to elevated levels of pro-inflammatory cytokines (259). Similarly, recent work demonstrates that during obesity, islet-resident macrophages proliferate contributing to a local inflammatory state and this results in impaired β-cell function (259,283). Collectively, these data indicate growing evidence that pancreatic inflammation/macrophage accumulation occurs with obesity and this contributes to impaired β-cell function. The impact of COVID-19 on islet function supports the importance of studying pancreatic inflammatory responses (284).

One islet-derived factor that increases with both inflammation and metabolic stress is glucagon-like peptide-1 (GLP-1). GLP-1 is classically described as an incretin hormone, coded by the preproglucagon gene (*Gcg*). *Gcg* is expressed in the α-cells of the pancreas, the L-cells of the intestinal tract, and in the nucleus of the solitary tract (NTS). As a metabolic hormone, GLP-1 is increased after a meal, secreted predominantly from the intestinal L-cells and functions to decrease blood glucose in an insulin-dependent manner (37,38,42,43). GLP-1 has one known receptor (GLP-1R), a G-protein coupled receptor (GPCR), that is primarily found on the cellular membrane of pancreatic β-cells, and in nerve terminals in the wall of the hepatic portal vein, the lung, and within the brain (25). Activation of these GLP-1R in the CNS decreases food intake (243,268). The primary product of *Gcg* within the pancreas is glucagon. However, with metabolic and inflammatory stress, there is increased pancreatic production of GLP-1 (139,143,285,286). While the physiological function of this increase in pancreatic GLP-1 during an immune response remains unknown, pharmacological GLP-1 agonists have been found to have an anti-inflammatory effect (287,288) and *in vitro* work has shown that GLP-1 agonist treatment decreases TNF-α mRNA expression from macrophages

under LPS-induced inflammation (270). Previously, our lab discriminated between the roles of pancreatic and intestinal GLP-1 and have found that pancreatic GLP-1 is critical in regulating glucose homeostasis using our validated innovative mouse models (142,162,289,290). Using a combination of high-fat diet (HFD)-induced obesity and our tissue-specific *Gcg* expression mouse model, I explored the function of *Gcg*-derived peptides in response to inflammation. A stated above, LPS is a well-established tool for inducing inflammation and is a known anorectic agent that alters glucose homeostasis; both functions of GLP-1. I hypothesized that GLP-1 contributes to the anorectic and glucoregulatory responses to inflammation. I also predict that pancreatic-produced GLP-1 functions to regulate the local pancreatic endocrine cell inflammatory state by organizing macrophage accumulation as part of a complex cross-talk system between immune and endocrine cells. I hypothesized that HFD would exacerbate all these responses.

Methods

Animal Care

8-week old C57BL6/J male mice (The Jackson Laboratory) or genetically crossed mice (discussed below) were used for all studies and housed in the University of Michigan North Campus Research Complex animal facilities under controlled conditions (12:12 light-dark cycle, 50–60% humidity, and 25°C) with ad libitum access to water, and normal chow diet (Chow; *Lab Diet*, 5L0D) or high-fat diet (HFD; Research Diets D12492; 20% Kcal protein, 60% kcal fat, 20% kcal carbohydrate; 5.21 kcal/g) as indicated by each study. Intestinal or pancreatic reactivation of the endogenous *Gcg* gene was accomplished as described previously (274). Briefly, *Gcg* Null mice were crossed with villin 1-*Cre* (Jax Laboratories, stock number 004586) and PDX1-*Cre* (Jax Laboratories, stock number 014647) mice, respectively resulting in offspring with Cre-specific reactivation of the *Gcg* gene, *Gcg* Null, and Cre littermate controls (Con) (274). *Glp1r*-GFP reporter mice were generated as done previously (98,291). Briefly, *Glp1r*$^{Cre/+}$ mice (Jax Laboratory; 029283) were crossed to EGFP-L10a

mice (292) to generate offspring with a GFP labelled *Glp1r*. The University of Michigan Institutional Animal Care and Use Committee approved all procedures.

Lipopolysaccharide (LPS) Dosing

At 8 weeks of age, male mice either remained on chow or were switched to HFD for 9-16 weeks depending on the endpoint of interest to assess the interaction between diet-induced obesity and metabolic response to severe inflammation. To determine whether HFD alters the GLP-1 response to LPS compared to chow, I analyzed plasma levels of GLP-1 and glucose before and after LPS administration. To do this, mice were fasted 4 hours after the onset of light phase. Mice were then administered LPS at a dose (100ng/g BW) previously demonstrated to increase plasma levels of GLP-1 (151,269) (n=4/diet) or vehicle (n=4/diet) via IP injection. Blood glucose was measured at baseline and plasma GLP-1 and blood glucose were measured 2 and 4 hours after LPS via tail blood collected in EDTA-coated tubes. Due to the increase in body weight, the HFD-fed mice received a higher absolute dose of LPS, the above experiment was repeated under the same dietary and experimental conditions except with a flat dose of LPS (0.32µg delivered in 0.22mL) (Chow-LPS, n=8; HFD-LPS, n=9) or vehicle treatment (Chow-Veh, n=6; HFD-Veh, n=9). The flat dose was calculated based on the average dose a chow-fed mouse would receive at 100ng/g LPS dose.

Food Intake

On the experiment day, chow or HFD-fed mice were fasted for then 4 hours prior to lights out. Immediately before lights out, LPS (100ng/g) or vehicle (0.9% NaCl) was administered via IP injection. In one cohort of HFD-fed *Gcg* Null vs. Cre animals the dose was lowered to 50ng/g. Food intake was measured 2, 4, 15, and 24 hours after injections.

Energy Homeostasis

In order to understand the impact of HFD on changes in energy expenditure during severe inflammatory conditions, I utilized indirect calorimetry cages. A cohort of animals was generated under the same chow vs. HFD dietary conditions as described above.

Mice were housed in PhenoMaster chambers (TSE Systems, Bad Homburg, Germany) for 5 days to assess food intake, energy expenditure, and respiratory exchange ratio. Mice acclimated to the chambers and 22°C for 2 days prior to the experiment day. Prior to lights out, mice received a flat LPS dose (4µg delivered in 0.13mL) (n=6/diet) or vehicle (n=6/diet) via IP injection. Mice remained in the metabolic chambers for 3 days to monitor recovery from LPS.

Time Course of Glucose, Glucoregulatory Hormones, and Cytokine Responses to LPS

Because previous experiments were limited by time points and blood volume that could be taken via tail nick, I designed a study to examine the time course responses of plasma levels of metabolic hormones and cytokines in response to LPS. A cohort of male C57Bl/6J animals was generated under the same chow vs. HFD dietary conditions described above. 3-5 days before the experiment, under general anesthesia (isoflurane), mice had catheters placed in the carotid artery (MJC-02, SAI Infusion Technologies) and jugular vein (BTPU-014, Instech Laboratories Inc.; MRE037, Braintree Scientific Inc.). After recovery from surgery, mice regained at least 90% of their pre-surgery body weight by study day. On the experiment day mice were fasted 4 hours after the onset of light phase. To minimize handling of the mice, LPS (4µg delivered in 0.13mL) or vehicle was administered via the jugular vein. Blood samples, about 200 µL, were collected from the carotid artery in EDTA-coated tubes containing aprotinin and dipeptidyl peptidase-4 (DPP-4) inhibitor at baseline, 30, 60, 120 and 180 minutes post-LPS infusion for assessment of plasma GLP-1, insulin, glucagon, IL-6, TNF-alpha, and IL-1β. Additional measures of blood glucose were taken every 30 minutes throughout the experiment. To maintain blood volume due to the frequent sampling, heparin washed red blood cells from donor mice were administered via the jugular vein throughout the three-hour experiment at a rate of 4.4µL/minute for a total of 800µL. During the experiment, I observed stroke-like symptoms in 5 mice and excluded these mice from analysis. Following the experiment, animals (Chow-Veh, n=6; Chow-LPS, n=9; HFD-Veh, n=8; HFD-LPS, n=8) were sacrificed with pentobarbital sodium (Fatal-plus solution) administered via the jugular vein. Tissues were collected for qPCR analysis.

Glucose Tolerance Tests

To determine if LPS administration has a long-term impact on glucose tolerance and whether diet impacted these results, we conducted glucose tolerance tests on male mice (n=12/diet) that were generated under the same chow vs. HFD dietary conditions described above. Glucose tolerance was assessed 2-3 weeks after an IP injection of a flat dose of LPS or vehicle treatment as above. For the IP and oral glucose tolerance tests, mice were fasted for 4h after the onset of the light phase. Blood was sampled and measured via tail nick. Basal blood glucose was sampled at -15 min, and glucose was administered via IP (2g/kg of 25% dextrose) injection or oral (300uL of 25% dextrose) gavage. Blood samples were taken at 15, 30, 45, 60, and 120 minutes after glucose administration.

Real-time Quantitative PCR (qPCR)

To examine systemic cytokine expression, necropsied livers were rapidly removed and frozen for later analysis. After homogenization in Trizol reagent, tissue RNA was extracted using a Pure Link RNA mini kit (Invitrogen, Carlsbad, CA). cDNA was synthesized (iScript cDNA synthesis kit, BioRad, Hercules, CA), and qPCR was performed using a TaqMan 7900 Sequence Detection System with TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA.). Relative mRNA expression of hepatic *Il6 (*Mm00446190_m1*), Tnfa (*Mm00443258_m1*), IL-10* (Mm01288386_m1) and *Ccl2 (*Mm00441242_m1) were calculated relative to *β*-actin (Mm02619580_g1) samples using the ΔΔCT method.

Hormone Assays

Plasma levels of total GLP-1 and IL-6, TNF-alpha, and IL-1β (Mesoscale Discovery, Rockville, MD, USA), insulin (Crystal Chem Inc., Elk Grove Village, IL, USA) and glucagon (Mercodia, Uppsala, Sweden) were measured using ELISA kits.

Tissue Macrophage Accumulation

On experiment day, mice (n=3-4/group) were administered LPS (100ng/g) or vehicle treatment via IP injection. 24 hours after administration, mice were euthanized by CO_2

asphyxiation and pancreas was immediately removed and placed in 10% formalin overnight. Tissues were processed for subsequent paraffin embedding and cut into 4 µm sections for immunohistochemistry staining. The sections were incubated overnight with a F4/80 antibody (1:250; Cell Signaling Technology) as a marker of general macrophage accumulation and counterstained hematoxylin. The slides were scanned using Leica Aperio AT2® scanner and quantified using ImageJ as percent F4/80 area of total, islet, or acinar area.

Macrophage Characterization via Flow Cytometry

Mouse pancreata (Con-Veh n=5, Con-LPS n=5, *Gcg* Null-Veh n=5, *Gcg* Null-LPS n=5; HFD-Con-LPS n=5, HFD-Con-Veh n=5, HFD *Gcg* Null n=4, *Gcg* Null-LPS n=4; wild-type n=1, Glp1r-GFP-LPS n=2) were excised immediately after euthanasia and then mechanically dissociated with scissors in sterile PBS. Pancreata were washed and pelleted under centrifugation, resuspended in 1mg/mL Collagenase V (Sigma), digested for 15 minutes at 37°C, quenched with RPMI + 10% FBS, then filtered through 40µm filters (Corning). The cells underwent RBC lysis, washed in PBS, then blocked and stained with the CD45-PerCP (Biolegend) for total immune cells, CD64-PE (BD Biosciences) for macrophages, and CD11b-APC (Biolegend) for a specific subpopulation of macrophages at 1:100 dilution in 100% FBS. Separately, bone marrow was extracted (Wild-type=1, Glp1r-GFP-LPS n=2), homogenized to a single cell suspension and stained with the following antibodies CD45-eFluor450 (Invitrogen/eBioscience) and CD11b-APC (Invitrogen/eBioscience). The cells were then washed in FACS buffer (1% FBS, HBSS), and run on a ZE4 Analyzer flow cytometer (BioRad). While I had a validated F4/80 IHC antibody, I tested both F4/80 and CD64 to identify macrophage populations with flow cytometry and the CD64 population was more distinct for flow cytometry of the pancreas.

Statistics

The number of animals studied per treatment and genotype are indicated within each experiment and were determined by a power analysis completed using variance from previous data. All data are expressed as mean ± SEM. The statistical procedures were performed as indicated in the figure legends. Normally distributed data were analyzed

via standard parametric statistics including two-way, or three-way ANOVA's and t-tests. If significant interactions were detected between independent variables, Tukey's *post-hoc* analysis was performed to determine where significant differences lie. Statistical analyses were performed using GraphPad Prism v.8.1.1, and statistical significance was accepted when $p<0.05$.

Results

Impact of LPS on plasma GLP-1 in HFD versus chow-fed mice.

LPS is a well-established tool to induce inflammation and important for this study, increases GLP-1 (151,293,294). Additionally, plasma GLP-1 responses to severe inflammation are higher in patients with T2DM (152,295). To determine whether HFD alters physiological responses to LPS compared to chow-fed mice, I analyzed plasma levels of GLP-1 and glucose before and after LPS administration in chow and HFD-fed mice. As expected, HFD for 8 weeks significantly increased body weight (mean ± SEM; Chow-Veh 30.2 grams (g)±1.11, Chow-LPS 29.5g±0.95, HFD-Veh 48.4g±2.16, HFD-LPS 47.9g±2.30, $p<0.05$) compared to chow-fed mice. As expected, HFD animals had greater baseline glucose levels. 2h after LPS, both chow and HFD-fed mice had decreased blood glucose levels compared to baseline but HFD animals were still significantly higher compared to chow-fed mice (**Figure 3.1A**). 4h after LPS, there were no longer statistical differences between the LPS-treated chow and HFD animals indicating a greater relative fall from baseline in HFD-fed mice. Total GLP-1 (**Figure 3.1B**) levels were increased with LPS and were significantly greater in HFD-fed compared to chow-fed mice 2h after LPS. Consistent with previous work, the LPS dose was calculated based on body weight. However, because HFD-fed mice weigh more, their absolute LPS dose was higher, which, in and of itself, could increase plasma GLP-1 levels in the obese animals. To determine if this was the case, I then administered a flat dose (0.32ug) to a second cohort of chow vs. HFD-fed mice. Again, HFD significantly increased body weight (mean ± SEM; Chow-Veh 32.43g±0.66, Chow-LPS 32.07g±0.32, HFD-Veh 45.66g±1.86, HFD-LPS 45.43g±1.90; $p<0.05$). Similar to the body weight relative LPS dose, glucose (**Figure 3.1C**) levels in chow and HFD-fed decreased over 4h after LPS and this greater relative decrease was greater in HFD-fed

mice. Total plasma GLP-1 (**Figure 3.1D**) was significantly increased in the HFD- compared to chow-fed mice 2 hours after LPS. Taken together, these data indicate that HFD alters glucose and the GLP-1 responses to LPS.

HFD-fed mice have a prolonged anorectic effect in response to LPS compared to chow-fed mice

LPS is known to decrease food intake and because of this reduction, conserve energy expenditure and alter fuel utilization (296,297). Typically, this results in a shift away from carbohydrate oxidation (296). To further examine the interaction between energy homeostasis, immune responses, and diet, I utilized metabolic chambers to monitor end-points associated with energy homeostasis after LPS. Baseline body weight was greater in HFD vs. chow-fed mice but within diet the LPS and Veh groups were matched for body weight (**Figure 3.2A**). Consistent with the literature, LPS-treated mice had a significant decrease in body weight (**Figure 3.2B**) over 4 days. In addition, there was a significant decrease in lean but not fat mass 4 days after LPS indicating that the majority of the body weight lost was from muscle mass (**Figure 3.2C-D**). As expected, LPS-treated mice decreased their food intake both within hours of administration (**Figure 3.2E**) and this suppression of feeding lasted for 3 days (**Figure 3.2F**). During Day 1 after LPS, the HFD mice had significantly decreased food intake compared to the Chow-fed LPS group (**Figure 3.2E**). As the mice recovered on Day 2 and 3, the HFD-LPS mice continued to have decreased cumulative food intake compared to chow-fed mice (**Figure 3. 2F**).

As stated above, a typical response to LPS is a decrease in energy expenditure to prioritize immune cell function (296). I found a significantly lower energy expenditure in the Chow-LPS vs. Veh group during Day 1 dark cycle and then no significant difference between these groups during the first light cycle or after (**Figure 3.2G-H**) indicating that the animals were quickly recovering from the LPS injection. However, the HFD-LPS group has similar energy expenditure to their Veh-treated control after LPS (**Figure 3.2G-H**) implying that HFD induces a dysregulation of energy prioritization. Because the HFD-fed mice have greater body weight, I chose to calculate absolute energy expenditure. However, I saw similar results when energy expenditure was expressed

relative to body weight as well (**Supplementary Figure 3.1**). Together these data suggest that HFD-fed mice have an increased sickness-induced anorexia and dysregulated energy expenditure during severe inflammation.

Under immune activation, competing metabolic demands of immune cells vs. physiological functions must be balanced (296). The respiratory exchange ratio (RER) is an indicator of whole-body fuel utilization and was reported for all groups 24h following LPS (**Figure 3.2I**). The average RER (**Figure 3.2J**) for Day 1 was significantly higher in the Chow-Veh group compared to all others, and on Days 2 and 3 both Veh- and LPS-treated chow groups were significantly higher compared to the HFD-fed groups. These RER data show that the chow-fed mice shift away from oxidizing carbohydrates (100% carbohydrate oxidation indicated by a RER=1) as an energy source (**Figure 3.2I**). The HFD-LPS mice are already oxidizing fatty acids (100% fatty acid oxidation indicated by a RER=0.7) as an energy source at baseline so no decrease in RER is detected. By Day 2 after LPS, RER in the chow-fed mice is similar to baseline indicating that they have recovered from the LPS administration. Conversely, the HFD-LPS mice dips below 0.7 on the RER graph, implying the mice are resorting to either fatty acids or ketone utilization. Day 3 after LPS, both LPS-treated mice have similar RER to their respective control groups.

HFD-fed mice show increased inflammatory responses to LPS compared to chow-fed mice

Having observed differences in responses to LPS between chow vs. HFD-fed mice, I wanted to further explore the systemic physiological and inflammatory responses. Mice underwent vascular catherization and LPS (4ug; flat dose) was administered via the jugular vein while blood draws were taken from the carotid artery. This is beneficial compared to tail bleeding as it allows me to take a higher volume and frequency of blood draws because I can maintain blood volume with a constant red blood cell infusion, and it is less stressful to the mice compared to repeated tail bleeding. The LPS-treated mice had decreased blood glucose levels (**Figure 3.3A**) and had increased plasma GLP-1 (**Figure 3.3B**) over time relative compared to Veh-treated mice and these effects were magnified (Diet x Treatment p=0.02) in mice fed HFD. Interestingly,

although insulin was higher in HFD- vs. chow-fed mice, the increase in GLP-1 did not result in increased plasma insulin with LPS (**Figure 3.3C**). The LPS treated mice had significantly increased glucagon (**Figure 3.3D**) levels compared to Veh-treated mice at 120 and 180min after LPS in both HFD- and chow-fed mice. The pro-inflammatory cytokines, IL-6 (**Figure 3.3E**) and TNF-α (**Figure 3.3F**), were greater in the HFD-LPS group compared to the Chow-LPS group while IL-1β (**Figure 3.3G**) increased with LPS similarly between the two dietary groups. Because the liver is an important link between the gut and systemic circulation (298), I evaluated liver cytokine expression in the HFD and chow-fed mice. In LPS-treated mice, there is a significant increase of liver cytokine expression including IL-6 (**Figure 3.3H**), TNF-α (**Figure 3.3I**), IL-10 (**Figure 3.3J**), and MCP-1 (**Figure 3.3K**) compared to Veh-treatment, but there were no significant interactions between diet and LPS. Together these data suggest that HFD-fed mice have increased systemic and liver inflammatory responses to LPS compared to chow-fed mice.

The role of Gcg in the physiological responses to LPS in chow and HFD-fed mice

Next, I wanted to determine the function of the increase in GLP-1 in response to LPS. The traditional function of GLP-1 focuses on its ability to decrease appetite and increase insulin secretion in response to nutrients (37,38,42,43,243,268). However, GLP-1 has also been implicated as an anti-inflammatory agent (285,286). To determine the necessity of GLP-1 in the metabolic and immunologic responses to LPS, I studied both PDX1-*Cre* and Vil-*Cre* litter-mate controls (indicated by Con in figures), *Gcg* Null, pancreatic and intestinally *Gcg* reactivated mice that were fed chow (**Figure 3.4A-M**) or HFD (**Figure 3.5A-L**). There was no significant impact of genotype on the feeding response to LPS (**Figure 3.4B-C**) demonstrating that *Gcg* is not necessary, nor is pancreatic or intestinal *Gcg* sufficient, for the anorectic effect of LPS. There was no significant change in glucose in response to saline (**Figure 3.4D**) over time or between genotypes. While glucose was significantly lower at 240 min in response to LPS, (**Figure 3.4E**) there was no significant difference in this decrease between the genotypes. At 240 minutes after LPS, (**Figure 3.4F**) total GLP-1 was increased in the chow-fed control and GcgRA$^{\Delta Panc}$ mice and, as expected, was undetected in the *Gcg*

Null mice. The GcgRA$^{\Delta Int}$ mice had no significant increase in total GLP-1 levels in response to LPS. This indicates that the pancreas is a more significant contributor to plasma GLP-1 responses during LPS-induced inflammation. There was no significant impact of genotype or LPS on insulin levels (**Figure 3.4G**). Together these data suggest that GLP-1 does not function to increase insulin during severe inflammation and is not necessary for the glucose response to insulin. Glucagon (**Figure 3.4H**) was significantly higher in response to LPS in control and GcgRA$^{\Delta Panc}$ mice and undetectable in both the *Gcg* Null and GcgRA$^{\Delta Int}$ mice. Several cytokines were significantly increased in response to LPS in both the plasma (**Figure 3.4I-K**) and liver (**Figure 3.4L-M**) but there was no additional impact of genotype. Because genotype did not impact hepatic expression of cytokines in the control, *Gcg* Null or GcgRA$^{\Delta Panc}$ mice, this endpoint was not assessed GcgRA$^{\Delta Int}$ groups.

I previously observed difference in total GLP-1 expression in chow versus HFD-fed mice under inflammatory conditions, therefore, I also assessed similar endpoints in a separate cohort of HFD-fed mice. There was no significant impact of genotype on food intake (**Figure 3.5A-B**) demonstrating that the lack of impact of *Gcg* on the anorectic effect of LPS is not impacted by diet. Similar to chow-fed, there was no significant change in glucose in response to saline (**Figure 3.5C**) over time or between genotypes. Glucose was significantly lower at 240 min in response to LPS (**Figure 3.5D**) but there was no significant difference in this decrease between the genotypes. Together these data suggest that *Gcg* does not play a role in regulating glucose, feeding, or systemic cytokine responses to LPS, regardless of diet.

Total GLP-1 was increased in the Con-LPS HFD-fed mice only (**Figure 3.5E**). When I examined the GLP-1 levels after LPS as percent of average saline, the chow and HFD control mice contributed about the same levels of total GLP-1 (mean ± SEM; Chow-Con 289.36±30.02, HFD-Con 238.22±28.31; p=0.25), regardless of diet. The pancreatic contribution trended towards being higher in the chow-fed (mean±SEM; Chow-GcgRA$^{\Delta Panc}$ 364.40±95.40, HFD-GcgRA$^{\Delta Panc}$ 196.71±45.07, p=0.08). The intestinal contribution (mean±SEM; Chow-GcgRA$^{\Delta Int}$ 69.21±21.35, HFD-GcgRA$^{\Delta Int}$ 181.54±40.08, p=0.01) increased in the HFD compared to chow-fed mice. These data imply that in

chow-fed mice the pancreas was the major contributor but in the HFD-fed mice both the pancreas and intestine contribute to circulating GLP-1 levels. In the HFD-fed mice, only the Con-LPS group had a significant increase in glucagon (**Figure 3.5G**).

Gcg Null mice have increased macrophage accumulation in the pancreas after LPS

To test whether *Gcg* had a role in regulating local inflammation, I determined pancreatic macrophage accumulation and characterization via two methods: flow cytometry and immunohistochemistry. Pancreatic immune cells were sorted on a flow cytometer, and the cells were (**Figure 3.6A, Figure 3.7A**) gated on singlets, scatter, and CD45+ cells. The population of interest, CD64+CD11b+, which represents a specific subpopulation of macrophages, was quantified and analyzed (**Figure 3.6A, Figure 3.7**). The *Gcg* Null-LPS treated mice had significantly increased macrophage accumulation compared to all other groups in the chow-fed mice (**Figure 3.6A**). In the HFD-fed mice, LPS significantly increased CD64+CD11b+ (main effect of treatment ($p=0.03$)) and the *Gcg* Null mice had significantly greater CD64+CD11b+ cells (main effect of genotype ($p=0.04$)) but there was no significant interaction between the two variables.

In a separate cohort, LPS was administered and pancreata were collected and processed for immunohistochemistry. Slides were stained for F4/80 (**Figure 3.6B, Figure 3.7B**) as general marker for macrophages using DAB. These data were quantified as percentage of area positive for F4/80 staining compared to the total (**Figure 3.6C, Figure 3.7C**) islet (**Figure 3.6D, Figure 3.7D**), and acinar area (**Figure 3.6E, Figure 3.7E**), respectively. In both chow and HFD-fed mice, there was an increase in F4/80 staining in response to LPS, but this increase was similar in control, *Gcg* Null, and GcgRA$^{\Delta Panc}$ mice. The *Gcg* Null HFD mice had a baseline increase as shown by the *Gcg* Null-Veh group. In the acinar ($p=0.02$) and total ($p=0.04$) area of the HFD pancreata, I found a main effect of LPS treatment. Together, these data imply that under severe inflammation, *Gcg* Null mice have more macrophage accumulation in the pancreas compared to control as shown by the CD64+CD11b+ population in the flow cytometry, and the IHC demonstrates that the macrophages accumulate in both the islet and acinar cells of the pancreas.

Macrophages isolated from the pancreas express GLP-1R

Next, I investigated if GLP-1 could be directly regulating macrophage accumulation. Whether GLP-1R are expressed on macrophages, and other immune cells for that matter, is unknown as GLP-1R antibodies are questionable (299). To address this problem, I crossed an eGFP-L10a reporter mouse to a *Glp1r*$^{\Delta Cre}$ (**Figure 3.8A**) mouse. I administered LPS and pancreatic tissues were stained for immune cells, acquired on the flow cytometer, and gated on singlets, scatter, CD45+, and CD64+ cells (**Figure 3.8B**). Validating that I was able to detect the GFP signal, the wild-type mice showed no GFP signal (**Figure 3.8C**), whereas *Glp1r*-GFP mice showed a strong GFP signal (**Figure 3.8C**). Additionally, this population of GFP+ cells were positive for CD11b+, the subpopulation of macrophages I saw elevated in *Gcg* Null mice. Interestingly, only the pancreas showed this population and not immune cells isolated from the bone marrow (**Figure 3.8C**). This implies that under the stress of inflammation, macrophages in the pancreas express GLP-1R and are likely directly regulated by locally produced GLP-1.

HFD-fed mice have impaired IP glucose tolerance 3 weeks after LPS

To determine whether pancreatic macrophage accumulation after LPS could have a long-term effect on endocrine function, chow- and HFD-fed mice underwent oral and intraperitoneal (IP) glucose tolerance tests (GTTs) 2 and 3 weeks after LPS, respectively. On the day of both the oral GTT (mean±SEM; Chow-Veh 31.91g±0.45; Chow-LPS 30.46g±0.53; HFD-Veh 48.43g±1.19; HFD-LPS 46.59g±1.23) and IP GTT (mean±SEM; Chow-Veh 31.11g±0.29; Chow-LPS 30.09g±0.67; HFD-Veh 49.58g±1.17; HFD-LPS 48.43g±1.06) the body weight of LPS vs. Veh-treated mice were not different. LPS had no significant impact on oral glucose tolerance (**Figure 3.9A**) in chow- or HFD-fed mice. However, while HFD-fed mice had significantly greater glucose responses compared to chow fed mice, both LPS treated groups had impaired IP glucose tolerance, regardless of diet (Time x Diet $p<0.0001$, Time x Treatment $p=0.0049$) (**Figure 3.9B**). Together these data suggest that LPS treatment has a lasting impact on glucose tolerance that can be overcome by incretin control of glucose.

Discussion

Type 2 diabetes mellitus (T2DM) remains a pervasive problem both in the United States and around the world (275). The COVID-19 pandemic was a prime example of how T2DM and the associated immune dysfunction can adversely impact the immune responses (278). I found that high-fat diet (HFD) increases physiological responses to severe inflammation including greater increases in total GLP-1, greater sickness-induced anorexia and dysregulated energy homeostasis, and that GLP-1 directly regulates macrophage accumulation in response to LPS. Thus, GLP-1 plays an immunologic, rather than metabolic, role in response to LPS.

GLP-1 is traditionally thought of as an incretin hormone which increases after a meal and functions to insulin secretion and thus control blood glucose levels (37,38,42,43). Many studies have investigated the GLP-1 response to inflammation (139,143,285,286). Under severe inflammatory conditions, such as sepsis, patients have remarkably increased GLP-1 levels and this increase is associated with higher mortality rates (300).

As stated above, I used LPS as an inflammatory stimulus which results in a strong, systemic inflammatory response including increased systemic cytokine production and decreased food intake (296,297). In my HFD vs. chow-fed experiments I saw that HFD-fed mice had a stronger physiological response to the LPS. I found that HFD mice had increased plasma GLP-1 two hours after LPS administration. This greater increase in GLP-1 is consistent with what is seen in human T2DM data who have had sepsis (152,295,300). In addition, the HFD-fed mice had increased sickness-induced anorexia as seen by the food intake data and increased cytokine production. Together, these data show that HFD increases the inflammatory state after LPS, including plasma GLP-1 levels suggesting that GLP-1 is involved in the immune response. Indeed, researchers have shown that GPL-1 levels predict mortality of patients with critical illness (300).

The function of endogenous GLP-1 under inflammatory conditions remains unknown. Because LPS is a known anorectic agent that alters glucose homeostasis, both functions of GLP-1, I predicted that the role of GLP-1 in response to LPS would be an extension of its postprandial functions. While pancreatic GLP-1 contributed relatively more GLP-1 to the circulation, neither the pancreatic nor intestinal sources of GLP-1

were necessary for the anorectic or glucose responses to LPS. Together these data imply that the function of GLP-1 under inflammatory conditions differs from its function postprandially. This is corroborated by my insulin data which shows no increase in plasma insulin despite increased circulating GLP-1 levels after LPS administration.

Because obesity and T2DM impact GLP-1 levels, in a separate cohort of mice fed HFD, I investigated these same endpoints. In these experiments, only the control mice had a significant increase in total GLP-1. This lack of increase in the GcgRA$^{\Delta Panc}$ mice could be due to baseline increases of GLP-1 in the HFD-fed mice, or that the HFD GcgRA$^{\Delta Panc}$ mice have dysregulated responses to diet and/or LPS due to lack of *Gcg* in either the gut or hindbrain.

GLP-1 agonists have been implicated in anti-inflammatory signaling in multiple tissues (153,287,288,301). Exendin-4 (Ex-4) has been show to decrease circulatory cytokine levels after both LPS and Ex-4 administration (153). Similarly, patients administered liraglutide over six weeks had decreased cytokine levels measured in mononuclear cells (287). Additionally, GLP-1 agonists increased functional recovery and viability of cardiomyocytes with and without GLP-1R (302). Despite well-established anti-inflammatory effects of GLP-1, how GLP-1 agonists contribute to an anti-inflammatory state remains unknown. Furthermore, whether endogenous GLP-1 has the same effect as long-acting GLP-1 agonists in unknown. In the above experiments, I did not observe any difference in systemic cytokine levels between *Gcg* Null, GcgRA$^{\Delta Panc}$, GcgRA$^{\Delta Int}$ vs. control mice, regardless of diet. The discrepancy between the effects of endogenous GLP-1 and exogenous administration of long-lasting GLP-1 agonists could be explained by the rapid degradation of GLP-1 in circulation (155–157). While long-lasting agonists have systemic anti-inflammatory properties, perhaps endogenous GLP-1 has a local effect in GLP-1 producing tissues, the intestine and pancreas. As mentioned above, under LPS induced inflammation there is an increase in pancreatic produced GLP-1 in chow-fed mice. This is different from what is seen postprandially, where the predominant source of GLP-1 is from the intestine (33). Given this more novel source of GLP-1, I wanted to investigate specifically the impact of *Gcg*- derived peptides on pancreatic inflammation. The flow cytometry data comparing *Gcg* Null versus control

mice shows that after LPS, the *Gcg* Null-LPS group had significantly more CD64+CD11b+ cells compared to all other groups. This population was of interest to me because under HFD-induced chronic inflammation, this subpopulation of macrophages are the major contributor to the immune environment of the islet (259). I saw similar results in both the chow fed and HFD-fed mice. However, it is important to note that the HFD mice had a greater number of unhealthy cells leaving the populations of macrophages less distinct in the HFD flow analysis and this effect was greater in *Gcg* Null vs. control mice. This implies that the absence of *Gcg* increases the inflammatory state of the pancreas.

The strength of flow cytometry is that it allows both the characterization and quantification of the macrophages, but not the localization. To look at the anatomical accumulation of the immune cells, I quantified percentage of area positive for F4/80 staining compared to the total, islet, and acinar area. In both chow and HFD-fed mice, there was an increase in F4/80 staining in response to LPS, but this increase was similar in control, *Gcg* Null, and GcgRA$^{\Delta Panc}$ mice. While I had a validated F4/80 antibody and protocol available for the IHC samples, I used both F4/80 and CD64 antibodies for the flow experiment. The CD64 population was more distinct so I used it to distinguish the macrophages along with CD11b rather than F4/80. Both F4/80 and CD64 are common macrophage markers (303,304). However, while flow cytometry revealed a significant increase in macrophages in the pancreas in in the *Gcg* Null mice in response to LPS, I saw no significant genotype effect with IHC. This difference can be explained by the fact that the flow experiment used the entire pancreas is isolate macrophages, whereas the IHC images were taken of the islets and the surrounding tissue. Images taken only around the islet might skew the results for the acinar population. Despite this discrepancy, these data imply that macrophages accumulate in the pancreas after an inflammatory insult, and *Gcg* Null mice have increased CD64+CD11b+ macrophages compared to control mice.

As discussed above, GLP-1 agonists result in anti-inflammatory signals (153,287,288,301,302). However, it is unclear how GLP-1 agonists are contributing to the anti-inflammatory state. Is it through CNS or peripheral signaling, or via direct

regulation of immune cells? To determine whether GLP-1 could directly regulate macrophages I used a *Glp1r*-GFP reporter mouse. It is well established that there are few, perhaps just one, antibody that is sensitive and accurate enough to detect the GLP-1R. To avoid this issue, I used a GLP-1R$^{\Delta Cre}$ mouse crossed to an eGFP-L10 reporter line and used flow cytometry to gate for GFP and immune cells. I found that a population of CD64+ CD11b+ cells were also positive for GFP in the pancreas implying that there are macrophages within the pancreas that express GLP-1R. However, there were no cell population that was positive for both immune cell markers and GFP. Thus, I hypothesize that GLP-1 directly regulates macrophage accumulation in the pancreas. This conflicts with a recent study which looked at the impact of liraglutide on bone marrow macrophages and found GLP-1R expression in both these macrophages and RAW264.7 cells, a macrophage cell line (305). However, single cell transcriptome analysis found GLP-1R expression in both classical monocytes, a macrophage precursor, and natural killer cells, an important cell type in innate immune responses, in the lung (306). This is an entirely new role for GLP-1 and puts forth a new hypothesis of why GLP-1 agonists show anti-inflammatory properties.

One limitation of this study is the specificity of the PDX1-*Cre* expression in our promotor mouse. PDX1-*Cre* is expressed in both the pancreas and duodenum. Previous research has shown that the predominant source of GLP-1 postprandially is the ileum and colon (33), so the PDX1-*Cre* promoter is still useful as a pancreatic promoter for *Gcg*. Additionally, our Cre model targets the entire *Gcg* gene and all the peptides it encodes. Indeed, glucagon is an important counterregulatory hormone that may play a role in the local inflammatory state of the islet and cannot be discounted at this time. Although tools are limited at this time, future studies targeting either GLP-1 and/or glucagon could offer further insight into pancreatic *Gcg* peptides and local inflammatory responses.

This study puts forth an additional role for GLP-1, as a macrophage regulating peptide. LPS-induced inflammation resulted in impaired glucose tolerance 3 weeks later. Previous data has shown that islet macrophage accumulation impacts glucose-stimulated insulin secretion (259). Together, these data show the lasting effect of

inflammation on islets and could be particularly impactful for pre-diabetic patients who have an inflammatory insult as it could exacerbate the progression to T2DM.

Conclusion

These experiments explored the interaction between LPS-induced inflammation and metabolism and found that HFD-fed mice had increased susceptibility to the symptoms of LPS-induced inflammation. It also put forth a novel function of GLP-1 as a direct regulator of macrophage accumulation.

Authorship

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Author Contributions

E.M.Z., M.L., K.S., and D.A.S. conceived and designed research; E.M.Z., C.R.H., B.M., and A.U. performed experiments; E.M.Z. and D.A.S. analyzed data; E.M.Z., C.R.H, K.K, K.S., and D.A.S. interpreted results of experiments; E.M.Z., K.S., and D.A.S. prepared figures; E.M.Z and D.A.S drafted manuscript; E.M.Z., D.A.S., and K.S. edited and revised manuscript; D.A.S. approved final version of manuscript.

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Study Approval

The University of Michigan Institutional Animal Care and Use Committee approved all procedures.

Figure 3.1 Impact of LPS on plasma GLP-1 in HFD versus chow-fed mice. LPS was either dosed per body weight (100ng/g) or as a flat dose (0.32ug) and (A, C) glucose, and (B, D) plasma total GLP-1 were measured, respectively. HFD-fed mice had a greater fall from baseline glucose at 4 hours and a greater increase in total GLP-1 at 2 hours regardless of dosage. Data in this figure were statistically analyzed with a 2-way (or mixed model) (A-B) or 3-way (C-D) ANOVA with Tukey post hoc analysis when appropriate, each animal was tested once, and data are represented as Mean ± SEM. *p<0.05 Chow vs. HFD, **p<0.01 Chow vs. HFD

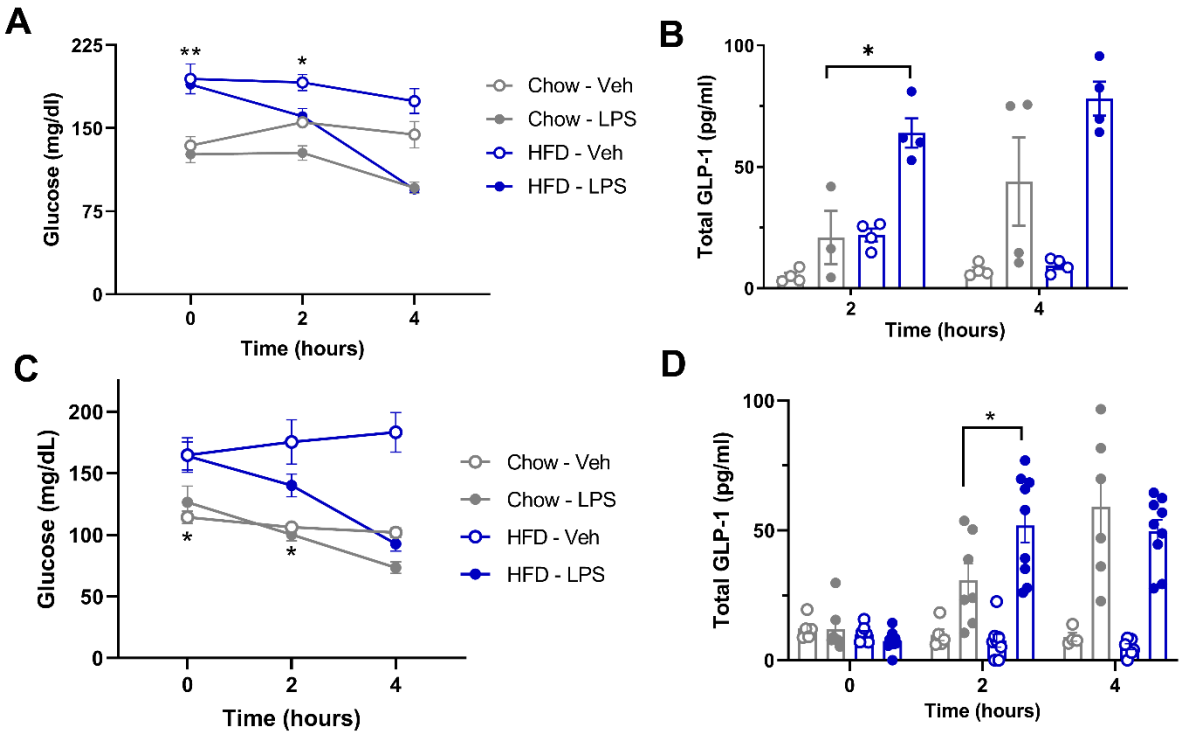


Figure 3.2 HFD-fed mice have a prolonged anorectic effect in response to LPS compared to chow-fed mice. Mice (n=6/group) were acclimated to the metabolic chambers for two days. (A) Mice were matched for body weight. (B) Total body weight, (C) lean mass, (D) and fat mass loss 3 days after LPS (4ug). (E) The HFD-LPS group has decreased food intake compared to the Chow-LPS group. (F) Cumulative food intake during recovery from LPS (Day 2 and 3). (G) Energy expenditure in chow and HFD groups the 3 days following treatment with Veh or LPS. (H) Average energy expenditure separated into dark and light cycles over the three-day experiment; the Chow-LPS mice had significantly lower energy expenditure during the dark cycle of Day 1 compared to the Chow-Veh group. (I) RER for all groups over 3 experiment days in response to Veh vs. LPS. (J) The average RER for Day 1 was significantly higher in the Chow-Veh group compared to all others, and on Days 2 and 3 both chow groups were significantly higher compared to the HFD-fed groups. Data in this figure were statistically analyzed with a 2-way (A-D), or a 3-way (H-J) ANOVA with Tukey post hoc analysis when appropriate, or an unpaired t-test (E-F inset graphs), each animal was tested once, and data are represented as Mean ± SEM. *p<0.05; ***p<0.001; ****p<0.0001 as indicated in each graph.

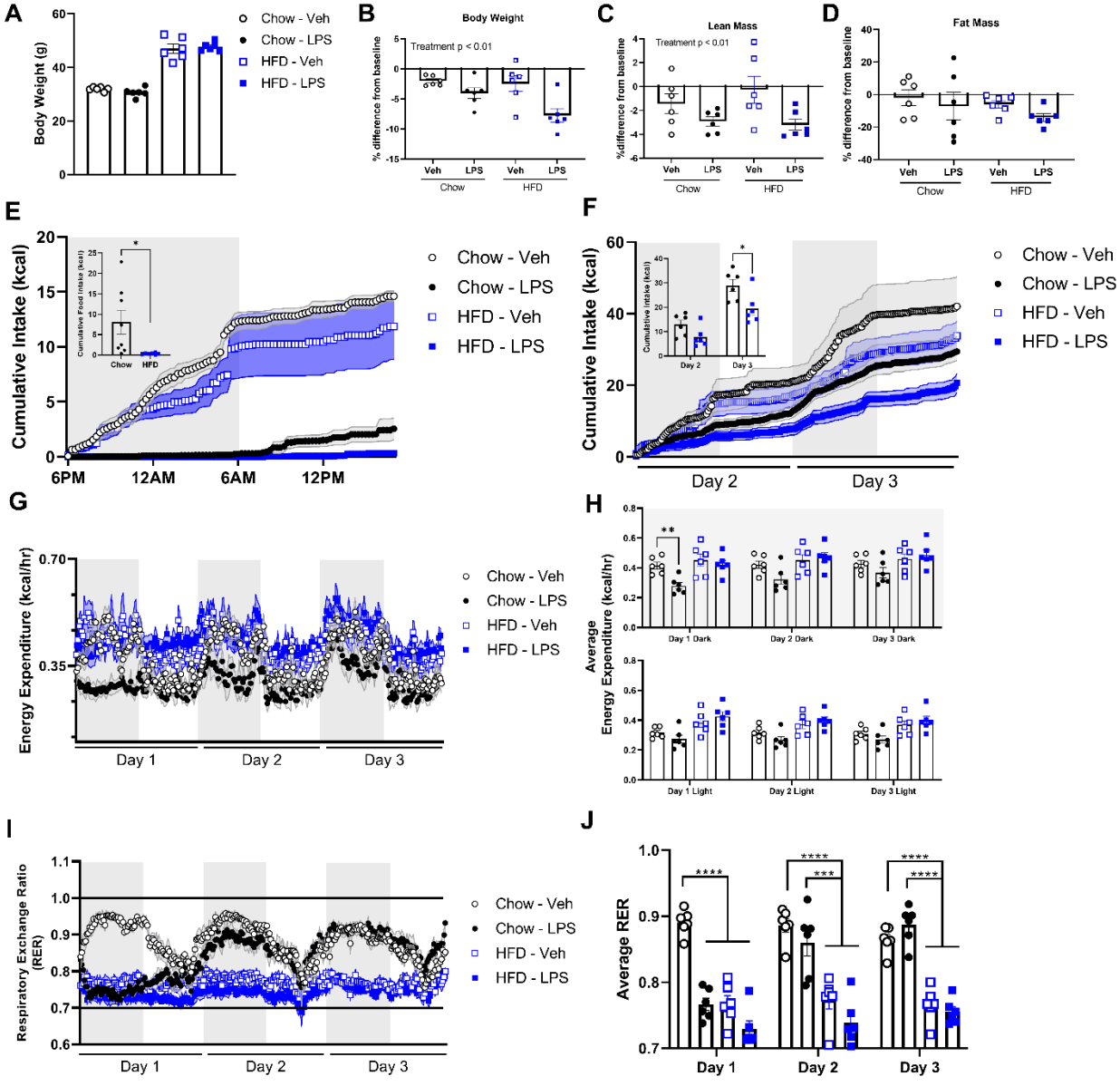


Figure 3.3 HFD-fed mice have increased inflammatory responses to LPS compared to chow-fed mice. Following vascular catheterization, mice (Chow-Veh, n=6; Chow-LPS, n=9; HFD-Veh, n=8; HFD-LPS, n=8) recovered for 3-5 days and were administered LPS (4ug) via the jugular vein and (A) glucose, (B) total GLP-1, (C) insulin, (D) glucagon, (E) IL-6, (F) TNF-α and (G) IL-1β were measured. LPS treated mice had decreased glucose levels over time (Time x Treatment p<0.0001). Total GLP-1 (Time x Treatment p<0.0001) and glucagon (Time x Treatment p<0.0001) were increased over time in LPS-treated groups. Insulin levels were increased by diet but were not significantly impacted by LPS treatment. The iAUC for IL-6 and TNF-α were increased in HFD compared to chow-fed mice. There was no significant difference between dietary groups in the LPS-induced increase in IL-1β. Following the experiment, liver tissue was collected for cytokine and chemokine expression including (H) IL-6, (I) TNF-α, (J) IL-10 and (K) MCP-1. While there was a clear impact of LPS on liver cytokine production, there was no significant additional impact of HFD. Data in this figure were statistically analyzed with a 3-way (A-G) ANOVA with Tukey post hoc analysis when appropriate or 2-way (H-K) ANOVA with Tukey post hoc analysis when appropriate, each animal was tested once, and data are represented as Mean ± SEM. *p<0.05 Chow-LPS vs. HFD-LPS

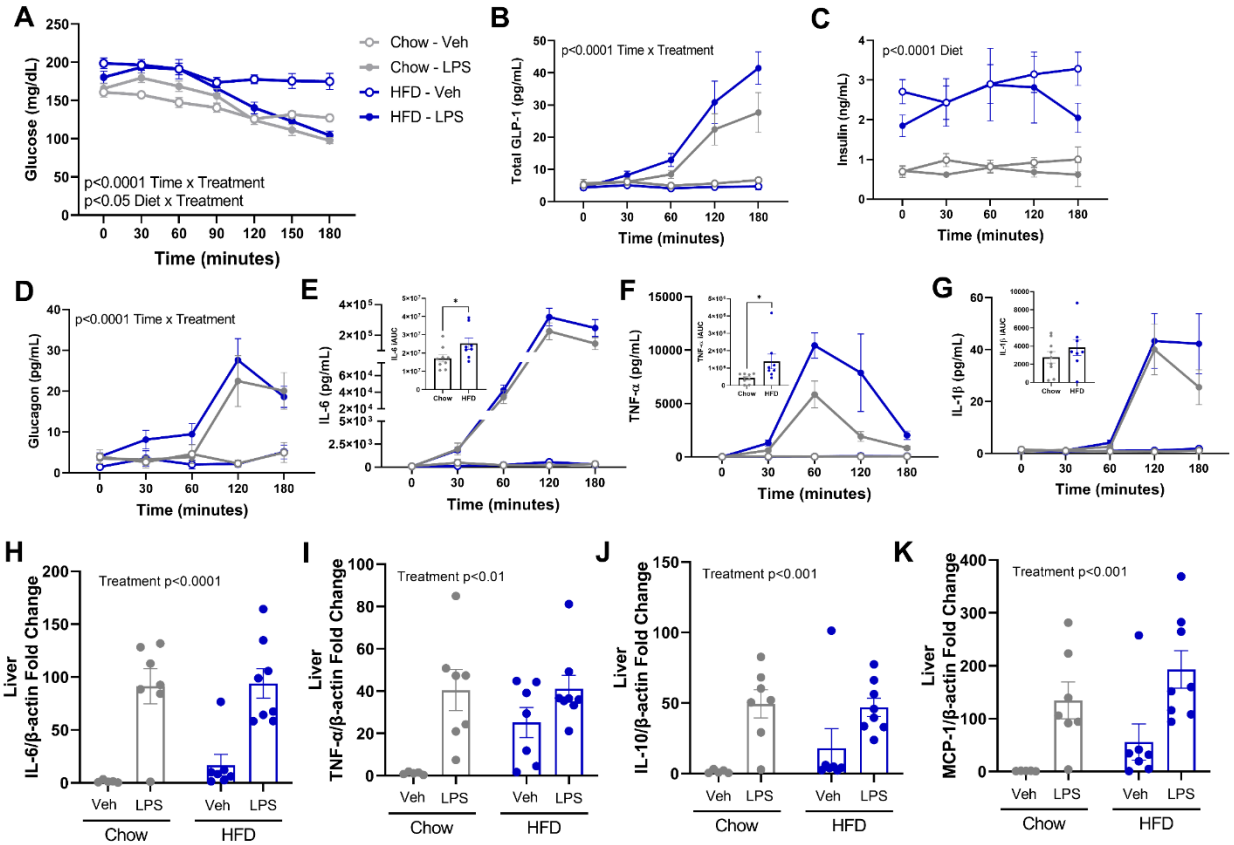


Figure 3.4 The role of *Gcg* in the physiological responses to LPS in chow-fed mice A brief schematic of the (A) *Gcg* genetic models are shown. Following a 4 hour fast, mice (Con-Veh, n=15; Con-LPS, n=13; *Gcg* Null-Veh, n=14; *Gcg* Null-LPS, n=14; GcgRA$^{\Delta Panc}$-Veh, n=6; GcgRA$^{\Delta Panc}$-LPS, n=8; GcgRA$^{\Delta Int}$-Veh, n=7; GcgRA$^{\Delta Int}$-LPS, n=7) were administered LPS (100ng/g) via an IP injection and food intake (B) was measured. (C) Food intake expressed as % difference from saline was similar between genotypes. A separate cohort of mice (Con-Veh, n=13; Con-LPS, n=14; *Gcg* Null-Veh, n=13; *Gcg* Null-LPS, n=14; GcgRA$^{\Delta Panc}$-Veh, n=7; GcgRA$^{\Delta Panc}$-LPS, n=8; GcgRA$^{\Delta Int}$-Veh, n=9; GcgRA$^{\Delta Int}$-LPS, n=10) were administered LPS as in (B) for panels D-K. (D) There was no significant change in glucose in response to saline over time or between genotypes. (E) There was a drop in glucose over time in response to LPS but no significant difference between genotypes. (F) At 240 minutes after LPS, total GLP-1 was increased in the Con and GcgRA$^{\Delta Panc}$ mice. (G) There was no significant increase in insulin detected in response to LPS. (H) Glucagon was significantly higher in response to LPS in Con and GcgRA$^{\Delta Panc}$ mice and undetectable in both the *Gcg* Null and GcgRA$^{\Delta Int}$ mice. (I) Plasma IL-6 response to LPS (Veh levels indicated by dashed line) was significantly higher in the GcgRA$^{\Delta Panc}$ group. (J-K) There were no detectable difference between genotypes for TNF-α and IL-1β plasma levels after LPS (Veh levels indicated by dashed line). Hepatic expression of (Con-Veh, n=5; Con-LPS, n=8; *Gcg* Null-Veh, n=4; *Gcg* Null-LPS, n=7; GcgRA$^{\Delta Panc}$-Veh, n=6; GcgRA$^{\Delta Panc}$-LPS, n=8) both IL-6 (L) and TNF-α (M) were significantly increased in response to LPS but there was no impact of genotype. Data in this figure were statistically analyzed with a 3-way (B, D-E) ANOVA with Tukey post hoc analysis when appropriate or 2-way (C, F-M) ANOVA with Tukey post hoc analysis when appropriate, each animal was tested once, and are represented as Mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001

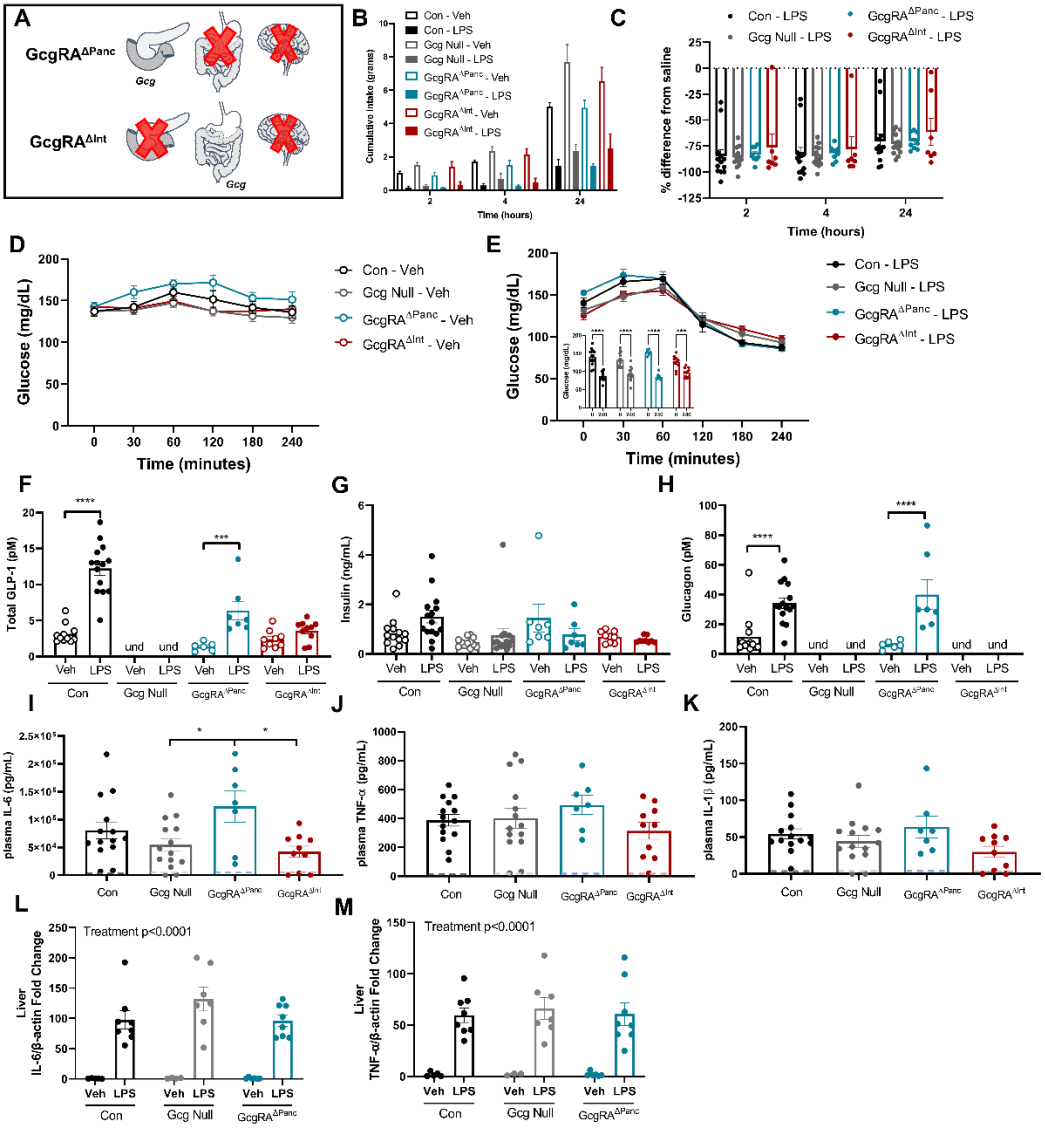


Figure 3.5 The role of *Gcg* in the physiological responses to LPS in HFD-fed mice Following a 4 hour fast, mice (n=7/group) were administered LPS (100ng/g) via an IP injection and food intake (A) was measured. (B) Food intake expressed as % difference from saline was not significantly different between genotypes. In a separate cohort of mice (Con-Veh, n=14; Con-LPS, n=20; *Gcg* Null-Veh, n=14; *Gcg* Null-LPS, n=16; GcgRA$^{\Delta Panc}$-Veh, n=8; GcgRA$^{\Delta Panc}$-LPS, n=9; GcgRA$^{\Delta Int}$-Veh, n=7; GcgRA$^{\Delta Int}$-LPS, n=8) were administered LPS as in (A) for panels C-J. (C) There was no significant change in glucose in response to saline over time or between genotypes. (D) There was a drop in glucose over time in response to LPS but no significant difference between genotypes. Blood was collected at 240 minutes and (F) plasma total GLP-1, (G) plasma Insulin, (H) plasma glucagon, (I) plasma IL-6, (J) plasma TNF-α, (K) plasma IL-1β were measured. All groups, regardless of genotype, responded similarly to LPS (Veh levels indicated by dashed line). Hepatic expression of (L) IL-6 and (M) TNFα were increased in response to LPS, but there was no significant impact of genotype on these responses (Con-Veh, n=7; Con-LPS, n=9; *Gcg* Null-Veh, n=6; *Gcg* Null-LPS, n=6; GcgRA$^{\Delta Panc}$-Veh, n=8; GcgRA$^{\Delta Panc}$-LPS, n=9. Data in this figure were statistically analyzed with a 3-way (A, C-D) ANOVA with Tukey post hoc analysis when appropriate or 2-way (B, H-L) ANOVA with Tukey post hoc analysis when appropriate, each animal was tested once, and are represented as Mean ± SEM. ***p<0.001 ****p<0.0001

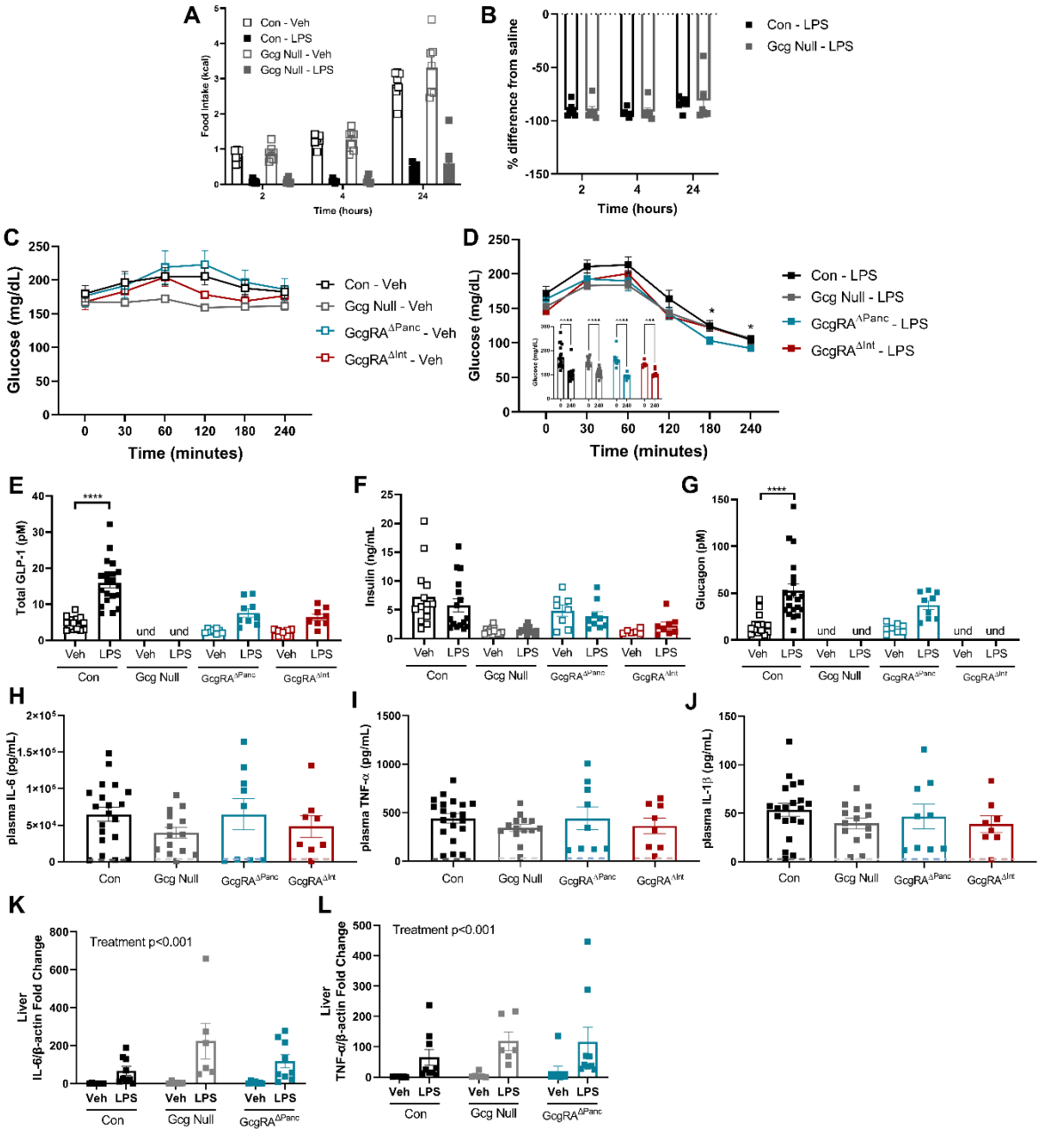


Figure 3.6 *Gcg* Null mice have increased macrophage accumulation in the pancreas after LPS 24h after LPS, mouse (n=4/group) pancreata cells were (A) gated on singlets, scatter, and CD45+ cells. The population of interest, CD64+CD11b+ was quantified and analyzed. *Gcg* Null-LPS mice have increased macrophage accumulation compared to all other groups. In a separate cohort (Con-Veh, n=4; Con-LPS, n=4; *Gcg* Null-Veh, n=3; *Gcg* Null-LPS, n=3; GcgRA$^{\Delta Panc}$-Veh, n=4; GcgRA$^{\Delta Panc}$-LPS, n=4; GcgRA$^{\Delta Int}$-Veh, n=4; GcgRA$^{\Delta Int}$-LPS, n=4) pancreas sections were stained for (B) F4/80 using DAB and (C-E) quantified. LPS-treated mice had increased macrophage accumulation in the (C) total pancreas (p<0.0001), including both the (D) islet (p=0.0117), and (E) acinar cells (p<0.0001) regardless of genotype. Data in this figure were statistically analyzed with a 2-way (A, C-E) ANOVA with Tukey post hoc analysis when appropriate, each animal was tested once, and are represented as Mean ± SEM. **p<0.01

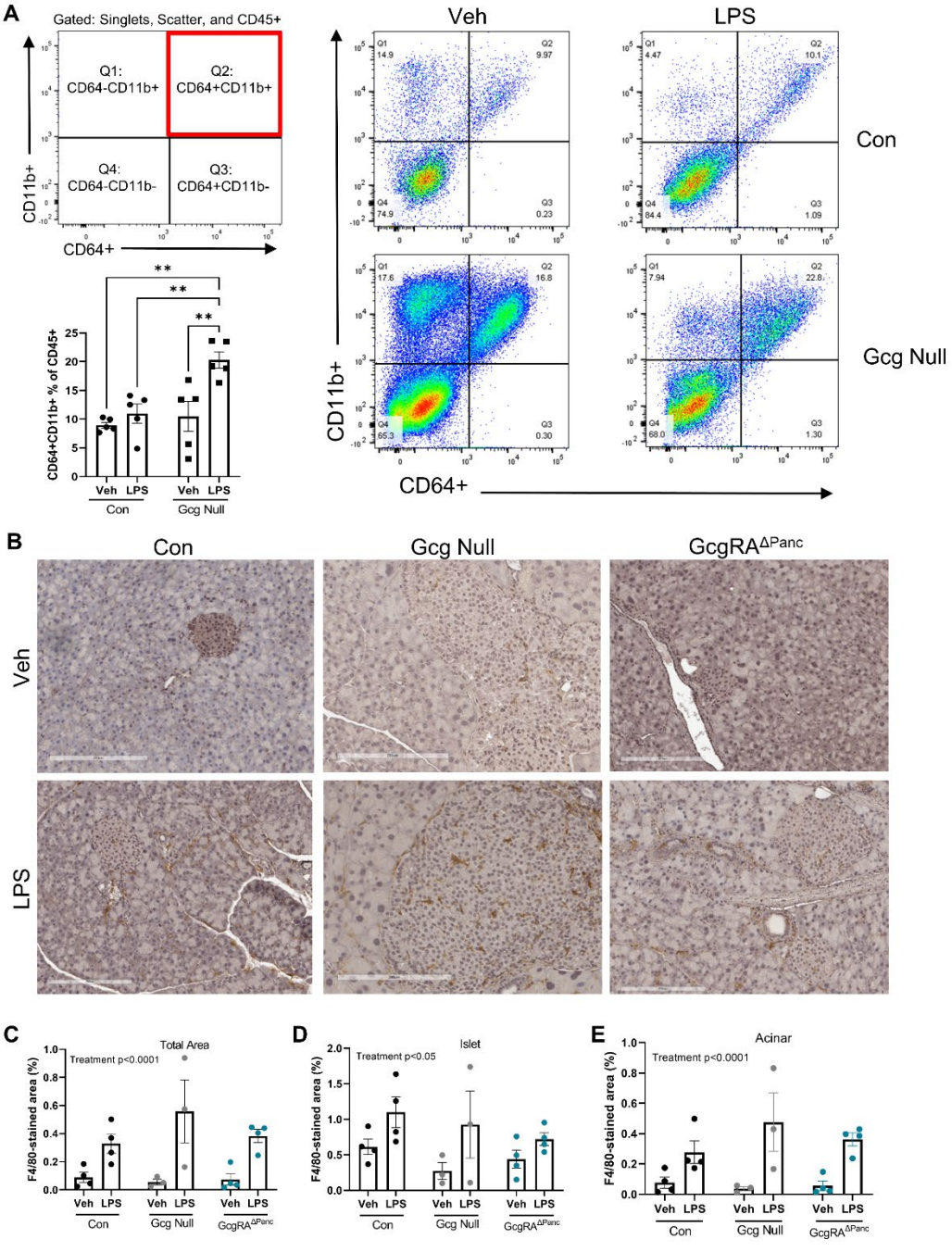


Figure 3.7 *Gcg* Null mice fed HFD also have increased macrophage accumulation in the pancreas after LPS
Mice (Con-Veh, n=4; Cre-LPS, n=4; *Gcg* Null-Veh, n=3; *Gcg* Null-LPS, n=3) pancreata was stained for immune cells and acquired on a flow cytometer. The cells were (A) gated on singlets, scatter and CD45+ cells. LPS mice have increased CD64+CD11b+ accumulation compared to veh groups (Treatment p=0.0297) and macrophages were higher in *Gcg* Null mice (Genotype p=0.0369). In a separate cohort HFD-fed mice pancreata were collected 24 hours after LPS and sections were stained for (B-E) F4/80 using DAB. LPS-treated mice had increased macrophage accumulation in the (C) total pancreas (p=0.0395), but not the (D) islets, implying increase was in the (E) acinar cells (p=0.0245). This was consistent across genotypes. Data in this figure were statistically analyzed with a 2-way (A, C-E) ANOVA with Tukey post hoc analysis when appropriate, each animal was tested once, and are represented as Mean ± SEM.

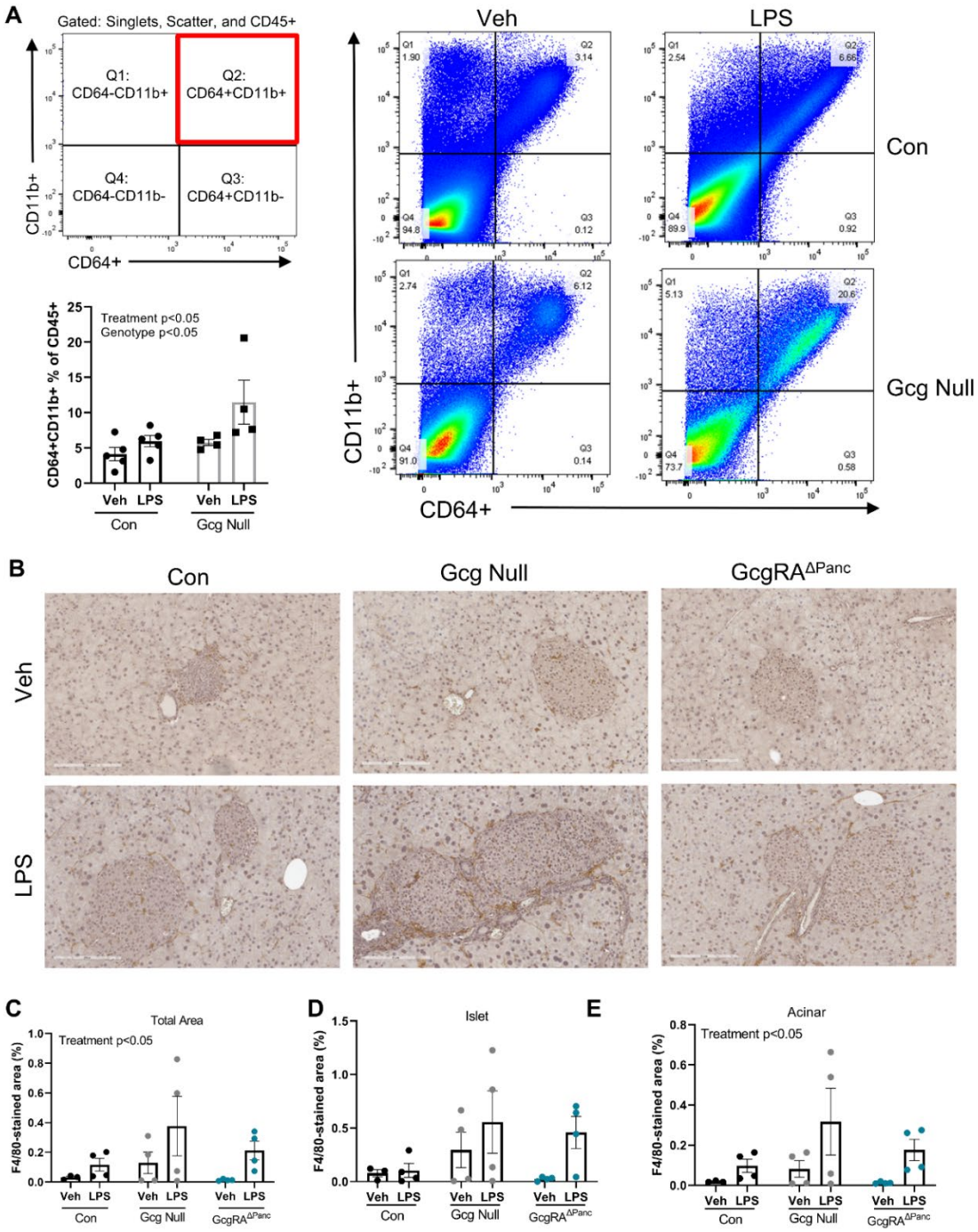


Figure 3.8 Macrophages isolated from the pancreas express GLP-1R. A brief schematic of the (A) *Glp1r*-GFP reporter mouse is shown. 24 hours after LPS (100ng/g) administration, immune cells were isolated from pancreata and bone marrow (Wild-type n=1, *Glp1r*-GFP-LPS n=2), stained, and acquired on the flow cytometer using the gating scheme (B) shown. Wild-type mice showed no GFP signal (C), whereas *Glp1r*-GFP mice showed a strong GFP signal (C). Additionally, a population of cells were positive for both CD11b and GFP.

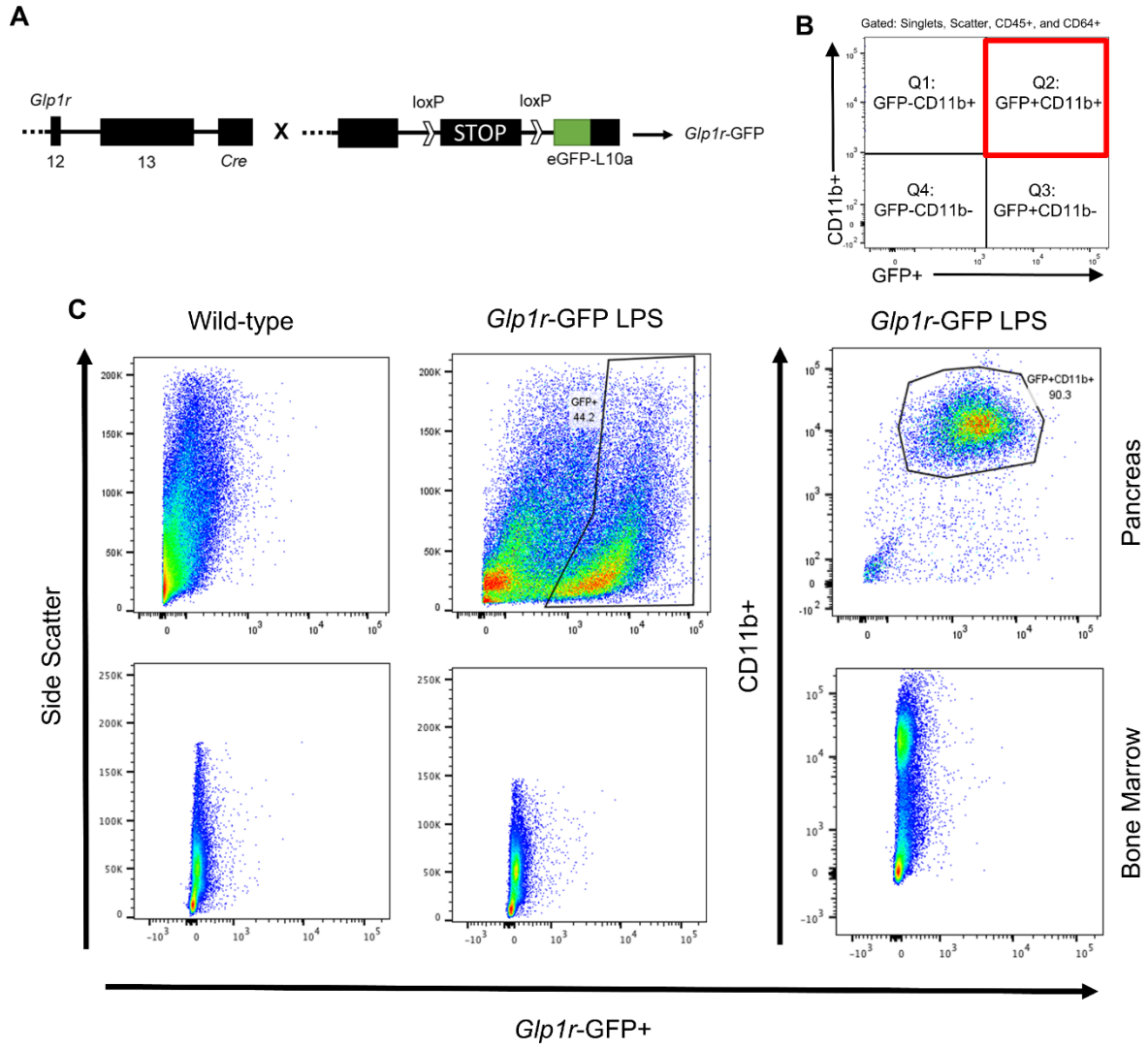
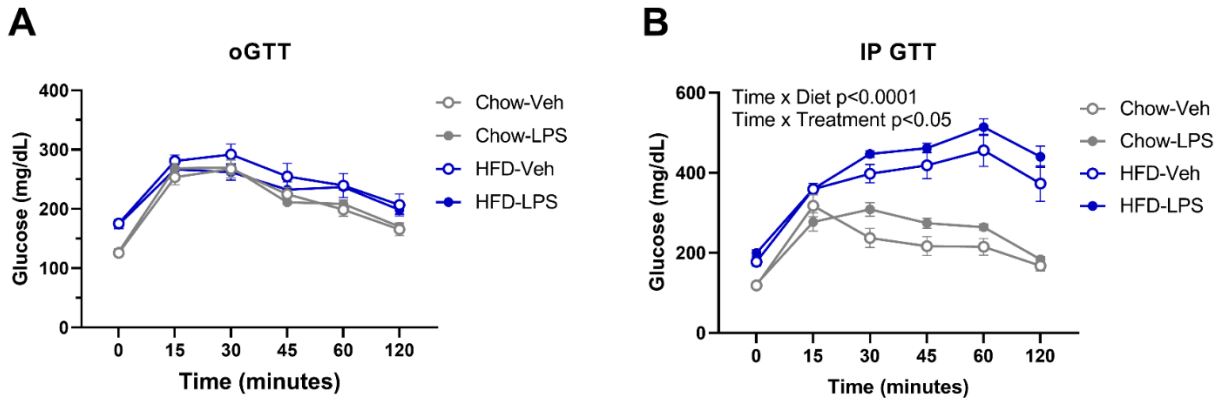
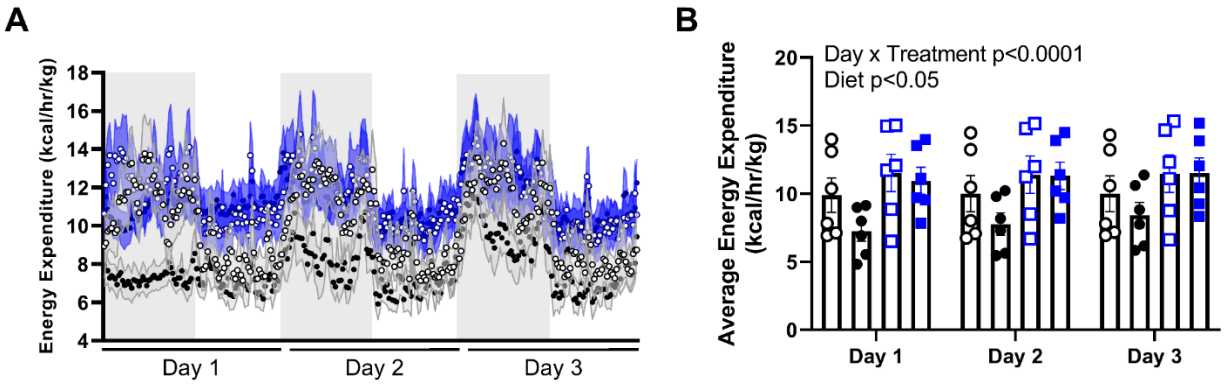


Figure 3.9 HFD-fed mice have impaired IP glucose tolerance 3 weeks after LPS Mice (n=6/group) administered LPS (0.32ug) had no significant difference in (A) oral glucose tolerance (oGTT) compared to saline-treated mice, regardless of diet. (B) Both HFD and LPS treatment impaired IP glucose tolerance but there was no significant interaction of diet and treatment (Time x Diet p<0.0001, Time x Treatment p=0.0049). Data in this figure were statistically analyzed with a 3-way (A-B) ANOVA and are represented as Mean ± SEM.



Supplementary Figure 3.1 Energy Expenditure response to LPS is similar when expressed relative to body weight (A) Energy expenditure expressed relative to body weight was decreased in the Chow-LPS group, but not in the HFD-LPS group. (B) The average energy expenditure did not have a significant 3-way interaction but there was a significant Day x Treatment interaction ($p<0.0001$) and a main effect of diet ($p=0.0441$). Data in this figure were statistically analyzed with a 3-way ANOVA (B) with Tukey post hoc analysis, each animal was tested once, and data are represented as Mean ± SEM.



CHAPTER 4
Conclusion

Significance of Findings

Type 2 diabetes mellitus (T2DM) remains a pervasive problem around the world (276,277). The COVID-19 pandemic was a startling example of the how dysfunction of the adaptive and innate immune systems with T2DM impacts mortality from inflammatory insults (278). It is critical moving forward that we understand how the pathophysiology of T2DM impacts immune responses. This knowledge will help to both decrease mortality rates of T2DM patients under severe inflammatory conditions as well as innovate new therapeutics. This dissertation made important strides towards understanding the local inflammatory state of the pancreas with endotoxemia, the factors that regulate it, as well as the impact of diet-induced obesity on these responses. Recently, researchers have been investigating the impact of GLP-1 and specifically, GLP-1R agonists, on responses to inflammation (139,143,285,286). Under severe inflammatory conditions, such as sepsis, patients have remarkably increased GLP-1 levels and this increase is associated with higher mortality rates (300). However, the function of GLP-1 under inflammatory conditions remains unknown. This dissertation puts forth an additional role for GLP-1, as a macrophage regulating peptide directly through its receptor, GLP-1R.

First, I found that HFD increases total GLP-1 (**Figure 3.1**) responses to severe inflammation, which is consistent with human data in the literature (300). This led me to wonder about symptoms associated with severe inflammation that could be impacted by GLP-1 such as glucose levels and appetite. I found that under HFD conditions, mice had greater sickness-induced anorexia and dysregulated energy homeostasis (**Figure 3.2**) compared to chow-fed mice. Using our innovative *Gcg* tissue-specific reactivation

mouse model, I found that GLP-1 plays an immunologic, rather than a metabolic role (**Figure 3.3-3.7**). Specifically, glucose and insulin levels were not impacted by *Gcg*, and instead, availability of *Gcg* prevents excessive macrophage accumulation in the pancreas. This is significant because it is a new role for GLP-1 which is traditionally described as an incretin (37,38,42,43). Furthermore, using a *Glp1r* reporter mouse, I found evidence that GLP-1R was expressed on pancreatic macrophages (**Figure 3.8**), implying that GLP-1 directly regulates macrophages.

Last, I found that LPS-induced inflammation resulted in impaired glucose tolerance (**Figure 3.9B**) even after the mice had three weeks to recover. Importantly, this was only seen in the intraperitoneal (IP) glucose tolerance test (GTT) whereas the oral GTT (oGTT) was not impacted by LPS. A possible explanation for this is that the incretin activation from the gut after oral administration helps to better control glucose levels after a severe inflammatory insult. This important experiment shows the potential damage to pancreatic and gut function after a severe inflammation. In animal studies, HFD results in accumulation of macrophages in the pancreas and is associated with impaired glucose stimulated insulin secretion (259). The above data suggest that the pancreatic accumulation of macrophages that occurs after LPS leads to long-lasting impairments in glucose tolerance. Together, these data indicate a need for better understanding how inflammation impacts pancreatic function, and whether endogenous GLP-1 or GLP-1R agonists are protective against tissue damage due to inflammation.

Strengths & Limitations

This study utilized several innovative mouse models. First, our *Gcg* reactivation model allowed me to distinguish the impact of tissue-specific production (**Figure 3.4-3.8**) of *Gcg*-derived peptides, rather than using a knockout model. This is especially important for my experiments because I wanted to investigate the impact of *Gcg*-derived peptides on both the systemic and local inflammatory responses; *Gcg* is produced in multiple tissues and each source of *Gcg* has been implicated in energy and glucose regulation. In addition to inflammatory endpoints, this model allowed me to distinguish any potential glucoregulatory differences between intestinal and pancreatic GLP-1, which are reported for post-prandial GLP-1 (37,38,42,43).

However, our Cre model targets the entire *Gcg* gene and all the peptides it encodes. Indeed, glucagon is an important counterregulatory hormone that may play a role in the local inflammatory state of the islet and cannot be discounted. Although tools are limited to create this mouse model now, future studies targeting either GLP-1 and/or glucagon could offer further insight into pancreatic *Gcg* peptides and local macrophage responses. Specifically, GLP-2, another *Gcg*-derived peptide, has also been reported to have anti-inflammatory properties (307,308).

Another potential problem with our PDX1-*Cre* mouse model is the specificity of the PDX1-*Cre* expression in our promotor mouse. PDX1-*Cre* is expressed in both the pancreas and duodenum. Previous research has shown that the predominant source of GLP-1 postprandially is the ileum and colon (33), so the PDX1-*Cre* promoter is still useful as a pancreatic promoter for *Gcg*. Future studies could use an AAV-Cre based virus administered directly through the pancreatic duct to specifically target the pancreas.

Whether GLP-1R are expressed on macrophages, and other immune cells for that matter, is unknown as GLP-1R antibodies are questionable (299). To address this problem, I used a mouse model which crossed an eGFP-L10a reporter mouse to a *Glp1r*$^{\Delta Cre}$ (**Figure 3.8A**) mouse, generously provided by Dr. D. Olson. I was able to detect immune cells that are also positive for GLP-1R without the weaknesses associated with the available GLP-1R antibodies. This finding that GLP-1R are located in pancreatic, but not bone marrow-derived macrophages suggests that, at least this specific population of macrophages that respond to LPS, are resident macrophages. An alternative explanation is that the circulating macrophages may change their genotype once they start accumulating in the pancreas. Macrophages are very plastic cells (309) and thus, this explanation is a distinct possibility. These data provide an increasing amount of rationale for the idea that GLP-1 directly regulates the immune response to inflammatory stress in the pancreas.

I also used an innovative combination of IHC and flow cytometry (**Figure 3.6-3.7**) to look at the accumulation and characterization of macrophages in the mouse pancreas. To look at the accumulation of the immune cells, I quantified percentage of area positive

for F4/80 staining (**Figure 3.6C-E, Figure 3.7C-E**). In both chow and HFD-fed mice, there was an increase in F4/80 staining in response to LPS. While I had a validated F4/80 antibody and protocol available for the IHC samples, I used both F4/80 and CD64 antibodies for pilot flow experiments as I was establishing a panel that could be properly compensated. The CD64 population was more distinct so I used it to distinguish the macrophages along with CD11b rather than F4/80. Both F4/80 and CD64 are common macrophage markers (303,304). However, I saw a statistically different response in the *Gcg* Null-LPS mice in the flow experiment, indicated by CD64, but not with the IHC strategy using F4/80 staining. It is possible that the different antibodies contributed to the lack of ability to detect an impact of genotype with IHC. However, I believe a more likely issue is that the I used the whole pancreas for the flow cytometry experiment, whereas the IHC images were taken of islets and the surrounding cells. Images taken only around the islet might skew the results; future studies could analyze the entire slide rather than focus on the islets. I also used DAB staining, counterstained with hematoxylin, and quantified as area stained rather than by individual nuclei. It is possible that my quantification strategy would be improved with the use of fluorescent antibodies and DAPI instead to quantify by nuclei. A method that I piloted to understand better the islet inflammatory state, was to isolate islets from *Gcg* Null vs. control mice 24 after LPS administration. Unfortunately, the combination of *Gcg* Null and LPS resulted in very unhealthy islets even with an overnight incubation recovery which is standard procedure. In this flow experiment, there was too much debris and too little immune cells to identify populations.

Because my chow-fed vs. HFD mouse metabolic experiments were limited by time points that could be taken via tail nick and blood volume, I designed a study to examine the time course responses of plasma levels of metabolic hormones and cytokines in response to LPS. Mice had catheters placed in the carotid artery and jugular vein in order to minimize handling of the mice. Additionally, this method allowed me to draw larger (about 200 µL), and more frequent blood draws (five blood draws throughout the experiment). I was able to do this with the aid of heparin washed red blood cells from donor mice which were administered via the jugular vein throughout the three-hour

experiment. While this experiment was technically difficult to learn, it was a valuable experience and yielded important findings for my dissertation.

Public Health Relevance

As discussed above, under severe inflammatory conditions, such as sepsis, patients have remarkably increased GLP-1 levels and this increase is associated with higher mortality rates (300). Recent research has shown that macrophages accumulate within the islet during diet-induced obesity leading to elevated levels of pro-inflammatory cytokines (259). Similarly, recent work demonstrates that during obesity, islet-resident macrophages proliferate contributing to a local inflammatory state and this results in impaired β-cell function (259,283). Collectively, these data indicate growing evidence that pancreatic inflammation/macrophage accumulation occurs with obesity, and this contributes to impaired β-cell function and thus could be a critical contributing factor in the progression of T2DM. The impact of COVID-19 on islet function supports the importance of studying pancreatic inflammatory responses (284).

I found that a population of CD64+ CD11b+ macrophage cells that were also positive for the GLP-1R in the pancreas (**Figure 3.8C**) implying that there are macrophages within the pancreas that express GLP-1R. Thus, GLP-1 could directly regulate macrophage signaling in the pancreas. Single cell transcriptome analysis found GLP-1R expression in classical monocytes and natural killer cells in the lung (306) indicating that the pancreas may not be the only organ where GLP-1R are regulating macrophage accumulation. Together, these data provide an increasing amount of rationale for GLP-1 to be directly regulating macrophages. This is an entirely new role for GLP-1 and puts forth a new hypothesis of why GLP-1R agonists show anti-inflammatory properties.

The development of GLP-1-based drugs has been a major advance in T2DM management. The approved pharmaceutical strategies targeting the GLP-1 system either increase endogenous GLP-1 levels with DPP4 inhibitors, or are long-acting GLP-1R agonists resistant to DPP4 cleavage (228,229). DPP4 inhibitors are effective at stimulating insulin and reducing glucagon, attributes that are credited to GLP-1R signaling (230). One of the most used GLP-1R agonists has an extended circulatory half-life is accomplished by the addition of a fatty acid side chain to native GLP-1 which

facilitates albumin binding (Liraglutide/Victoza). Besides being more convenient for the patient, liraglutide treatment also results in a reduction in cardiovascular events (242). The reduction of cardiovascular events is interesting because GLP-1R agonists have been implicated in anti-inflammatory signaling in multiple tissues including cardiomyocytes (153,287,288,301). Exendin-4 (Ex-4), a GLP-1R agonist, has been shown to decrease circulatory cytokine levels after LPS administration (153). Similarly, patients administered liraglutide had decreased cytokine levels measured in mononuclear cells after six weeks of administration (287). A recent paper showed that COVID-19 patients who remained on sitagliptin, a DPP4 inhibitor, while in the ICU had increased survival compared to patients treated with standard of care, stopping sitagliptin and treating with insulin (310). Despite the established anti-inflammatory effects of GLP-1R agonists, how the agonists contribute to an anti-inflammatory state remains unknown. Furthermore, whether endogenous GLP-1 has the same effect as long-acting GLP-1R agonists in unknown. In the above experiments, I did not observe any difference in systemic cytokine levels (**Figure 3.4I-M**, **Figure 3.5H-L**) between *Gcg* Null, GcgRA$^{\Delta Panc}$, GcgRA$^{\Delta Int}$ vs. control mice, regardless of diet. The discrepancy between the effects of endogenous GLP-1 and exogenous administration of long-lasting GLP-1R agonists could be explained by the rapid degradation of GLP-1 in circulation (155–157). While long-lasting agonists have systemic anti-inflammatory properties, perhaps endogenous GLP-1 has a local effect in GLP-1 producing tissues, the intestine, pancreas, and CNS.

As mentioned above, LPS-induced inflammation resulted in impaired glucose tolerance (**Figure 3.9B**) after the mice had three weeks to recover. These data show the lasting effect of inflammation on islets and could be particularly impactful for pre-diabetic patients who have an inflammatory insult as it could exacerbate the progression to T2DM.

Future Studies & Applications

There are many hypotheses that could come from this dissertation regarding GLP-1 expression and receptor signaling, as well as immune cell function. Here I put forth two future studies that are important follow up to the data in this dissertation.

First, in recent years, it has become increasingly evident that too much research is done in male mice only. This dissertation is in fact, guilty of this. While male mice are an important control, it is vital to establish how female physiology is the same or different in the groundbreaking research happening around the world. Specific to this dissertation, sex differences in GLP-1 expression (311) and immune system responses (312) have been detected. An interesting endpoint that I did repeat in female mice was assessing glucose tolerance after LPS. In the female mice, there was no impact of LPS treatment on either oral or IP glucose tolerance (**Figure 4.1**). This indicates that female mice might be protected from the impaired pancreatic function after LPS. Digging into this phenomenon further with female *Gcg* reactivated mice could yield data on sex differences in the immune response of the pancreas.

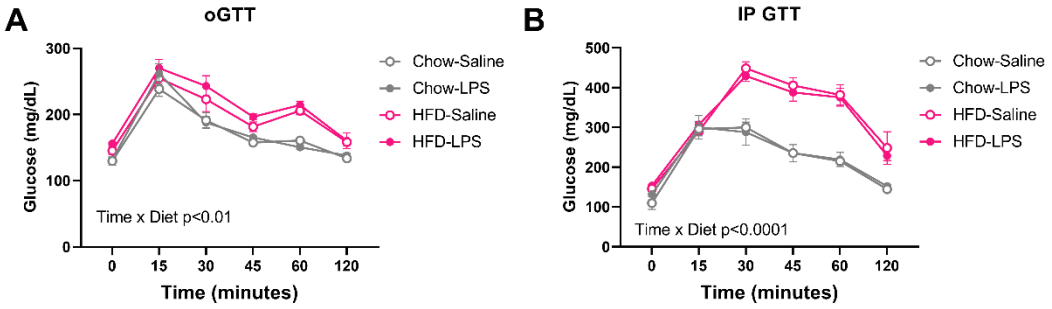
As mentioned above, one of the interesting outcomes from this dissertation was the observation that LPS-treated mice had impaired glucose tolerance, at least in male mice. Future studies could explore pancreatic damage and recovery after severe inflammation. Both endocrine and exocrine function could be impacted.

Finally, the *Gcg* Null, GcgRA$^{\Delta Panc}$ and GcgRA$^{\Delta Int}$ mice used in these experiments are lacking *Gcg* expression in the hindbrain. While it is known that glutamatergic rather than GABAergic neurons (98) are necessary for liraglutide to induce weight loss, whether these neurons are also necessary for or impact any of the physiological responses to LPS is unknown.

Conclusion

The data from this dissertation significantly advances our understanding of the function of the increase in pancreatic GLP-1 production with inflammatory stress. Furthermore, it explores the impact of HFD on immune responses, including *Gcg*. I found that HFD exaggerates physiological responses to LPS including increased GLP-1, decreased blood glucose, and a greater anorectic effect. I also found that *Gcg*-derived peptides play a role in pancreatic macrophage accumulation in response to inflammation. Finally, I found that macrophages isolated from the pancreas express GLP-1R indicating that GLP-1 could be directly signaling macrophages and regulating local inflammation.

Figure 4.1: Female mice do not have impaired glucose tolerance in response to LPS regardless of diet Mice (n=6/group) administered LPS (0.32ug) had no significant difference in (A) oral glucose tolerance (oGTT) compared to saline-treated mice, however there was a significant interaction of diet (Time x Diet p=0.0029). (B) Only HFD impaired IP glucose tolerance but there was no significant interaction of diet and treatment (Time x Diet p<0.0001). Data in this figure were statistically analyzed with a 3-way (A-B) ANOVA and are represented as Mean ± SEM.



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